The Inter-Relation of the Cardio-Autonomic Nervous System and HPA-Axis and its Association with Cardiovascular Precursors in Children

Sivan Rotenberg

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By: Sivan Rotenberg

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Signed by the final examining committee:

Dr. R. Kilgour — Chair

Dr. J. Thayer — External Examiner

Dr. L. Kakinami — External to Program

Dr. J.P. Gouin — Examiner

Dr. S. Miller — Examiner

Dr. J.J. McGrath — Thesis Supervisor

Approved by: Dr. A. Arvanitogiannis, Graduate Program Director

June 23, 2015 Dr. A. Roy, Dean, Faculty of Arts & Science

ABSTRACT

The Inter-Relation of the Cardio-Autonomic Nervous System and HPA-Axis and its Association with Cardiovascular Precursors in Children

Sivan Rotenberg, Ph.D.

Concordia University, 2015

Stress, via the activation of the stress response system, has been linked to the emergence of cardiovascular precursors. The majority of studies relating the autonomic nervous system and the hypothalamic pituitary adrenal (HPA) axis, the two main branches of the stress response system, to stress and cardiovascular precursors have focused on examining each branch of the stress response system in isolation. However, considering only these singular associations disregards the structural and functional inter-connection between the stress response systems. Examining the inter-relation between the stress systems among children and adolescents is particular opportune given the emergence of cardiovascular precursors early in the life course. The objective of the current research program was to examine the patterning of the cardio-autonomic nervous system and HPA axis, and whether the inter-relation between these stress systems was associated with cardiovascular precursors among children.

Three studies were conducted. Study 1 demonstrated the patterning of the stress response system, and found that the inter-relation between stress systems better accounted for the relation between stress and the stress response system than either system alone. Extending these results, Study 2 demonstrated that the inter-relation between stress systems was associated with cardiovascular precursors in a population-based sample of children. Exposure to stress

moderated the association between inter-relation of stress systems and cardiovascular precursors, which was more robust among children with greater stressful life events. Study 3 replicated these results in a sample of children at-risk for obesity, and demonstrated that the inter-relation between stress systems was related to cardiovascular precursors among at-risk children with greater stress exposure.

Overall, this research program found that the inter-relation between the stress systems was related to cardiovascular precursors, and children's environmental exposure to stressful events influenced these associations. Future studies should consider using an inter-relation approach, which may provide greater insight into the association between stress and cardiovascular health than considering the stress systems independently. The patterning of the stress response system has implications for future studies examining how stress "gets under the skin" to promote the development of disease.

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CONTRIBUTIONS OF AUTHOR

The three studies presented as part of my dissertation were based on larger ongoing studies with co-authors contributing differently in each manuscript. For all three studies, I developed the research questions, completed the literature reviews, conducted the statistical analyses, interpreted the results, and drafted and revised the manuscripts. I was also responsible for incorporating the comments and feedback from the co-authors on Study 2 and Study 3, and for the submission of the manuscripts for publication.

In her role as my research supervisor, Dr. Jennifer McGrath co-developed the research questions, supervised the statistical analyses, and carefully edited and revised each manuscript. Dr. McGrath granted approval for the use of the Health Heart Project data (Study 1). The Healthy Heart Project is an ongoing study at the Pediatric Public Health Psychology Laboratory, Concordia University, Montreal. Dr. McGrath obtained grant funding, designed the study, and oversaw all data collection, analyses, and interpretation. Dr. McGrath also secured approval for the use of the QLSCD and QUALITY data used in Study 2 and Study 3.

For Study 2, data from the QLSCD was used. Drs. Richard E. Tremblay led the launch of this original birth-cohort study through the Québec Institute of Statistics. Drs. Gilles Paradis, Louise Séguin, and Marie Lambert (post-humous) led the cardiovascular assessment at age 10. All co-authors (Drs. Jennifer J. McGrath, Melanie Henderson, Gilles Paradis, Louise Séguin, Angelo Tremblay, and Paul Poirier) read the initial draft, and provided suggestions and feedback on the manuscript.

For Study 3, data from the QUALITY Cohort was used. Drs. Marie Lambert (post-humous), Gilles Paradis, Angelo Tremblay, Jennifer O'Loughlin, Melanie Henderson and The QUALITY Cohort Collaborative Group designed and coordinated this cohort study and collected

measurements at clinic visits. Drs. Jennifer J. McGrath and Paul Poirier planned the study design and analysis of the ECG recordings, read the initial draft of the manuscript, and provided suggestions and feedback.

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LIST OF ABBREVIATIONS

AUC_{AG}……Area under the Awakening Response Curve Relative to Ground

AUC_I ……. Area under the Awakening Response Curve Relative to Increase

AUC_{TG}…….Area under the Curve Relative to Ground

BMI……Body Mass Index

DBP……Diastolic Blood Pressure

ECG……..Electrocardiogram

HDL……..High Density Lipoprotein

HF……...…..…High Frequency

HPA…….....Hypothalamic-Pituitary Adrenal

HRV…….. Heart-Rate Variability

LDL…….…..Low Density Lipoprotein

LF……..…Low Frequency

pNN50……..Percent differences in Successive Beat-To-Beat Intervals Greater than 50ms

RMSSD….....Square Root Mean Differences of Successive Beat-To-Beat Intervals

SBP……..Systolic Blood Pressure

SDNN…… Standard Deviation of Beat-To-Beat Interval

GENERAL INTRODUCTION

The current programme of research focuses on the role of the stress response system in the etiology of early cardiovascular precursors during childhood and adolescence. In the following section, the vast literature linking psychological stress and cardiovascular disease is briefly reviewed. The prevalence of cardiovascular precursors during childhood is highlighted. Next, background literature on the stress response system and its non-invasive measurement is summarized. Then, the physiological evidence and theoretical rationale for considering the inter-connection and patterning of the stress response systems is outlined. Through the lens of this interaction of the stress response systems, evidence for an *inter-relation*, or patterning of the stress systems, is presented relevant to cardiovascular precursors. Together, the current programme of research suggests that the inter-relation between the stress systems may be associated with stress, and an enhanced way to predict cardiovascular precursors

Psychological Stress and Cardiovascular Disease

Exposure to stressful experiences, such as the death of a loved one, job strain, or daily hassles, has been implicated in the etiology of cardiovascular disease (Cohen, Janicki-Deverts, Miller, 2007; Hemingway & Marmot, 1999; Rozanski, Blumenthal, Kaplan, 1999; Steptoe & Kivimaki, 2012). Chronic stress, measured prospectively over 6 to 7 years, has been associated with a 40 to 50% increased risk for cardiovascular disease among adults (Rozanski et al., 1999; Hamer, Molloy, Stamatakis, 2008; Steptoe & Kivimaki, 2012). Similarly, a recent meta-analysis of prospective studies with adults found that higher perceived stress – one's perception that their demands exceed their resources and ability to cope - was associated with a 27% increased risk in cardiovascular disease over an average of 13 years (Richardson et al., 2012). Stress is linked to cardiovascular health both indirectly and directly (Brotman, Golden, & Wittstein, 2007; Hamer

et al., 2008; Rozanski, Blumenthal, Davidson, Saab, & Kubzansky, 2005; Steptoe & Kivimaki, 2012). Indirectly, stress is linked to cardiovascular disease by increasing health-risk behaviours, such as greater smoking, alcohol consumption, and sedentary activity (Hamer et al., 2008). Directly, stress leads to physiological changes (e.g., elevated blood pressure, central adiposity), which over time promote the development of precursors to cardiovascular disease (e.g., hypertension, obesity; Bao, Threefoot, Srinivasan, & Berenson, 1995; Steptoe & Kivimaki, 2012). Although the exact mechanisms by which stress "gets under the skin" to promote the development of cardiovascular disease are less clear, dysregulation of the stress response system has been convincingly implicated in the pathophysiology of cardiovascular disease.

Cardiovascular Precursors Begin During Childhood

Examining the association between stress and cardiovascular disease among children and adolescents is paramount given the evidence that cardiovascular precursors evolve early during the lifecourse. In the Bogalusa Heart Study, autopsies of children and adolescents aged 2 to 15 years who died due to unexpected causes (unintentional injuries and homicide) revealed 50% already had fatty streaks in their coronary arteries and 20% had fibrous plaques in their aortas (Berenson et al., 1998; 1992). The classic Barker Hypothesis suggests that cardiovascular precursors have fetal origins (Barker, 1995). Numerous population-based studies have demonstrated the emergence of cardiovascular precursors among children and adolescents (Lambert et al., 2008; Mahoney et al., 1996; May, Kuklina, & Yoon, 2012; Sirnivasan, Frerichs, Webber, & Berenson, 1976; Strong et al., 1999). The presence of cardiovascular precursors in children is concerning, given that precursors track into adulthood and increase individuals' risk for cardiovascular disease (Danese & McEwen, 2012; Hertzman, 1999; Raitakari et al., 2003; Shokoff, Boyce, & McEwen, 2009). As example, elevated low density lipoprotein (LDL)

cholesterol, systolic and diastolic blood pressure, and body mass index in childhood prospectively predicted carotid artery narrowing in adulthood, even after accounting for current adult risk (Raitakari et al., 2003). Altogether, there is compelling cross-sectional and prospective evidence that the emergence of cardiovascular precursors has its early origins during childhood.

Background Information

The Stress Response System. The primary systems involved in the activation of the stress response system are the autonomic nervous system and the hypothalamus-pituitary-adrenal (HPA) axis. The autonomic nervous system is comprised of the parasympathetic and sympathetic nervous system. Upon exposure to a stressor, synaptic signals from cortical brain regions (e.g., prefrontal cortex, amygdala) are sent to the main activation sites of the sympathetic nervous system and the HPA axis: the locus coeruleus-norepinephrine system in the brainstem and the paraventricular nucleus of the hypothalamus, respectively. The sympathetic nervous system has a tonic level of activity that is adjusted by different afferent signals, including those from the parasympathetic nervous system (Malpas, 2010; Porges, 1995). Following an acute stressor, the parasympathetic and sympathetic nervous system respond in a temporal sequence. Mere milliseconds (0.5s) following exposure, the parasympathetic system withdraws its inhibitory effects and facilitates the sympathetic response (Berntson et al., 1997; Porges, 1995; 2007b). The sympathetic response, also known as the "fight or flight response", peaks approximately four seconds later and produces physiological changes throughout the body by releasing noradrenaline from the locus coeruleus (Berntson et al., 1997; Curtis, Lechner, Pavcovich, & Valentino, 1997; Jedema & Grace, 2004) and by stimulating sympathetic preganglionic neurons which project to organs, such as the heart, kidneys, and vasculature (Bengel & Schwaiger, 2004; Engelnad & Arnhold, 2005; Shahar & Palkovits, 2007).

Unlike the rapid response of the sympathetic/parasympathetic system, the activation of the HPA axis is slower in comparison (peak response observed 20-30min post acute stress). Activation of the HPA axis is triggered by the release of the corticotrophin-releasing hormone from the paraventricular nucleus of the hypothalamus. This promotes a series of endocrine events that culminates with the production and release of cortisol from the adrenal cortex (Egliston, McMahon, & Austin, 2007). Cortisol has been implicated in catabolic processes in nearly every system in the human body (e.g., arousal, sleep, metabolism, maintenance of cardiovascular tone, immune and inflammatory responses; Chrousos & Kino, 2007). Cortisol promotes functions related to activation (e.g., arousal) and augments the activity of other physiological systems, including the sympathetic nervous system, where it enhances the sympathetically mediated elevation in heart rate (Saplosky, Romero, & Munck, 2000). Ultimately, cortisol terminates the stress response via a negative feedback loop, wherein cortisol returns to the brain and inhibits the release of the corticotrophin-releasing hormone (Herman, Ostrander, Mueller, & Figueiredo, 2005).

In addition to an acute stressor response, the activity of the autonomic nervous system and HPA axis also follows a circadian or diurnal rhythm (Guo & Stein, 2003; Herman, et al., 2005). The diurnal profile of the autonomic nervous system is characterized by sympathetic activity that increases during the day and decreases at night; mirroring this, parasympathetic activity decreases during the day and increases at night. The diurnal profile of the HPA axis is characterized by increasing cortisol levels that peak within the first hour post-awakening and gradually decline throughout the day (Fries, Dettenborn, & Kirschbaum, 2009). An extensive body of research demonstrates that chronic stressors and daily hassles are related to alterations in the diurnal profile of the stress response system (e.g., caregiving, job strain; Adam, 2006;

Pruessner, Hellhammer, Kirschbaum, 1999; Schubert et al., 2009; Vrijkotte, van Doornen, de Geus, 2000).

Non-Invasive Measurement of Autonomic Nervous System. The autonomic nervous system and HPA axis can be measured in response to distinct stressors (i.e., stress reactivity), or in one's daily natural environment (i.e., ambulatory; Adam & Kumari, 2009; Sinnreich, Kark, Friedlander, Sapoznikov, & Luria, 1998; Task Force, 1996). Autonomic influences can be measured non-invasively using various techniques, including heart rate variability (Thayer, Hansen, & Johnsen, 2010). Heart rate variability (HRV) is an index of cardio-autonomic control, or the amount of variation in beat-to-beat heart rate intervals due to autonomic influences (Pumprla, Howorka, Groves, Chester, & Nolan, 2002). The sinoatrial node, also known as the heart's pacemaker, is dually innervated by the autonomic nervous system; sympathetic modulation increases heart rate, while parasympathetic modulation decreases heart rate (Berntson et al., 1997; Task Force, 1996). Pharmacological blockade studies reveal that the heart is under inhibitory control by the parasympathetic nervous system (Berntson et al., 1994; Cacioppo et al., 1994). Thus, beat-to-beat variations in heart rate activity are predominantly mediated by parasympathetic influences, and HRV is primarily considered an indicator of parasympathetic modulation. (Thayer et al., 2010; the physiological correlates of HRV measures are discussed further below.) As noted earlier, sympathetic modulation is modified by various afferent signals, including inhibitory signals from the parasympathetic nervous system (Malpas, 2010). Lower HRV is thought to represent the loss of parasympathetic inhibition, and a shift towards greater sympathetic activation and catabolic processes (Porges, 2007a).

Heart rate variability is derived from the beat-to-beat heart rate intervals based on continuous electrocardiogram (ECG) recordings. Technological advancements in ECG

monitoring permit ease of recording in ambulatory settings (Crawford et al., 1999; Task Force, 1996). HRV is typically quantified by time domain and frequency domain measures. Time domain measures are based on timing of beat-to-beat intervals or the millisecond differences between successive beat-to-beat intervals. Common time domain measures include SDNN (standard deviation of beat-to-beat interval), RMSSD (square root of the mean squared differences of successive beat-to-beat intervals), and pNN50 (proportion of interval differences of successive beat-to-beat intervals greater than 50ms; Task Force, 1996). Frequency domain measures separate the variance of the beat-to-beat intervals (i.e., power) into different frequency bands (Berntson et al., 1997; Task Force, 1996). Two frequencies are predominantly considered: low frequency and high frequency. Pharmacological blockade studies demonstrate that RMSSD, pNN50, and HF are strongly associated with parasympathetic modulation, while LF is associated with both sympathetic and parasympathetic modulation (Cacioppo et al., 1994; Polanczyk et al., 1998; Saul, 1990). The ratio of LF to HF (LF/HF ratio) reflects the relative relation between sympathetic and parasympathetic influences, and is an indicator of sympathovagal modulation (Lahiri, Kannankeril, & Goldberger, 2008; Sztajzel, 2004; Task Force, 1996; Thayer et al., 2010). It is important to note that the interpretation of LF and LF/HF ratio are actively debated among psychophysiology researchers. Some suggest that LF is a measure of sympathetic modulation (Malliani, 1999; Montano et al., 2009), while others contend it is a measure of both sympathetic and parasympathetic modulation (Cacioppo et al., 1994; Reyes del Paso, Langewitz, Mulder, van Roon, & Duschek, 2013). Yet, despite this debate, the heuristic value of LF and LF/HF ratio as indicators of cardio-autonomic control has been established (Heathers, 2014; Malliani, 2005).

Non-Invasive Measurement of HPA Axis. HPA axis activity can be assessed using salivary cortisol sampling. While some researchers measure cortisol levels in a response to a

laboratory stressor, cortisol levels are also commonly measured in naturalistic settings to characterize the diurnal cortisol profile (Adam & Kumari, 2009). As previously described, the diurnal cortisol profile is characterized by a rapid rise in cortisol levels (50 to 75% increase in the cortisol volume) in the first hour post-awakening, followed by a slow decline throughout the day (Clow, Hucklebridge, Stalder, Evans, & Thorn, 2010; Hanrahan, McCarthy, Kleiber, Lutgendorf, & Tsalikian, 2006). This rapid increase in cortisol following awakening represents a distinct aspect of the diurnal cortisol profile referred to as the cortisol awakening response. The cortisol awakening response is a consequence of the awakening process, and its regulation is unique from cortisol secretion over the rest of the day (Clow et al., 2010; Wilhelm, Born, Kudielka, Schlotz, & Wüst, 2007). Specifically, in addition to the superchiasmatic nucleus that regulates the diurnal cortisol profile, the cortisol awakening response is also regulated by direct sympathetic innervation via the splanchnic nerve (Ulrich-Lai, Arnhold, & Engeland, 2006).

The diurnal cortisol profile can be described using measures of the awakening response, diurnal slope, and the total concentration of cortisol over the day (Rotenberg, McGrath, Roy-Gagnon, & Tu, 2012). The awakening response is measured by the total amount of cortisol released during the awakening response (AUC_{AG}; area under the awakening response relative to ground or zero) and the dynamic increase in the amount of cortisol secreted following awakening (AUC_I; area under the curve relative to increase; Clow, Thorn, Evans, & Hucklebridge, 2004; Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). AUC_I is considered to represent a "response" to awakening and the sensitivity of the HPA axis to stress (Clow, Hucklebridge, & Thorn, 2010; Pruessner et al., 2003). The diurnal slope is characterized by the decline in cortisol from the peak morning cortisol value to the last measured point. Normal declining slopes have negative values, while flattened or blunted slopes are closer to zero. The total cortisol

concentration (AUC_{TG}) represents the overall secretory activity of the HPA axis throughout the day. Single cortisol values, such as the highest cortisol value over the day, are also used to describe the diurnal cortisol profile.

Interconnection of Stress Response System: Physiological Evidence

Given that the autonomic nervous system and HPA axis work in tandem to both activate and then inhibit the stress response, it is not surprising that animal and human studies indicate that these systems are highly coordinated and physically interconnected. For instance, neural signals from the paraventricular nucleus project to the locus coeruleus (Reyes, Valentino, Xu, & Van Bockstaele, 2005), and noradrenergic neurons from the locus coeruleus project to the paraventricular nucleus (Itoi, Jiang, Iwasaki, & Watson, 2004; Ma & Morilak, 2005). Additionally, there is a feed forward mechanism between these two systems. Corticotrophin-releasing hormone stimulates the release of noradrenaline (Jedema & Grace, 2004; Reyes, Valentino, & Van Bockstaele, 2008; Valentino, & Van Bockstaele, 2008), and in turn, noradrenaline promotes corticotrophin-releasing hormone mRNA expression in the paraventricular nucleus (Itoi et al., 2004; Ma & Morilak, 2005). Moreover, pharmacological blockade studies indicate that suppressing sympathetic activity elevates the HPA axis response to a laboratory stressor; while conversely, suppressing HPA activity elevates sympathetic activity (Andrews, D'Aguiar, & Pruessner, 2012; Andrews & Pruessner, 2013). Together, the autonomic nervous system and HPA axis interact in a synchronized response to stress and promote coordinated physiological changes, which overtime lead to adverse cardiovascular changes.

Interconnection of Stress Response System: Theoretical Underpinnings

In addition to the physiological findings that support the inter-relation and patterning of the stress response system, existing theories from the fields of developmental psychobiology and

stress physiology discuss the conceptual underpinning of stress response patterning. Three prominent theories are considered: Bauer's Additive/Interactive Models (Bauer, Quas & Boyce, 2002), Polyvagal Theory (Porges, 1995; 2007a), and Neurovisceral Integration Model (Thayer & Lane, 2000; Thayer & Sternberg, 2006).

Bauer and colleagues (2002) proposed that considering the inter-relation between stress systems may provide additional insight into how stress is related to physical and mental health outcomes, compared to considering just one system independently. Bauer proposed two competing inter-relation models: Additive or Interactive. The Additive Model is based on the premise that the autonomic nervous system and HPA axis are synonymous (i.e., cortisol primarily functions to augment sympathetic effects). As such, individuals are deemed at heightened risk of adverse outcomes (e.g., depression, high blood pressure) if the autonomic nervous system and HPA axis are jointly activated or deactivated (i.e., hyperarousal, hypoarousal, respectively). As increased sympathetic activity is characterized by low cardio-autonomic control, the Additive Model would designate elevated risk patterns as the inter-relation of low cardio-autonomic control with high HPA axis activity, or alternatively, high cardio-autonomic control with low HPA axis activity. The Interactive Model is based on the premise that the autonomic nervous system and HPA axis are complementary, wherein individuals are at heightened risk when the autonomic nervous system acts in opposition to the HPA axis. As such, the Interactive Model would designate elevated risk patterns as the inter-relation of low cardio-autonomic control with low HPA axis activity, or high cardio-autonomic control with high HPA axis activity.

Bauer's Additive/Interactive Models conceptualize elevated health risk based on the patterning of *both* stress response systems. Yet, neither the Additive nor Interactive Models

address the physiology underlying *how* the stress response systems may be inter-related. Theories in the field of stress physiology, including the Polyvagal Theory (Porges, 1995; 2007a) and Neurovisceral Integration Model (Thayer & Lane, 2000; Thayer & Sternberg, 2006), address this conceptual gap. Specifically, both theories suggest that the autonomic nervous system and HPA axis are inter-related via the activity of the parasympathetic nervous system. Although the Polyvagal Theory and Neurovisceral Integration Model evolved from two different theoretical frameworks (evolutionary biology vs. dynamic systems), both theories suggests that decreased parasympathetic activity up-regulates the sympathetic and HPA axis stress response, while increased parasympathetic activity down-regulates the sympathetic and HPA axis stress response (Porges 2007a; Thayer & Sternberg, 2006). These theories have been partly supported by findings from adult laboratory-based stress studies (Cacioppo et al., 1995; Sgoutas-Emch et al., 1994; Uchino, Cacioppo, Malarkey, & Glaser, 1995; Weber et al., 2010); similar studies with children or adolescents could not be identified.

Bauer's Additive/Interactive Models, the Polyvagal Theory, and the Neurovisceral Intergation Model provide conceptual support for the patterning of both stress response systems. Although there are nuanced differences across the models, one commonality is that they all suggest that in the presence of low parasympathetic modulation, the sympathetic nervous system and HPA axis are jointly activated. Bauer's Additive Model most parsimoniously articulates the theoretical underpinnings of the inter-relation of cardio-autonomic and HPA axis activity. Given these theoretical models, it is plausible that considering this inter-relation may facilitate understanding of cardiovascular precursors.

Role of the Stress Response System in Cardiovascular Disease Precursors

The stress response system has been linked to several cardiovascular precursors. The current programme of research focuses on three: elevated blood pressure, central adiposity, and high cholesterol levels. These three precursors were targeted by the American Heart Association, which identified hypertension, central obesity, and hypercholesterolemia as important cardiovascular risk factors to monitor and control in children and adolescents (Kavey et al., 2003). In this section, select experimental and ambulatory studies are presented to highlight key findings for the cardio-autonomic nervous system and HPA axis related to each of these cardiovascular precursors.

Blood Pressure. Blood pressure is regulated by the autonomic nervous system and HPA axis (Björntorp & Rosmond, 2000; Guyenet, 2006). In experimental studies, ablation of sympathetic neurons in rats reduced blood pressure (Madden & Sved, 2003), while cortisol administration in adults increased blood pressure (Mangos et al., 2000; Pirpiris, Sudhir, Yeung, Jennings, & Whitworth, 1992). These experimental findings indicate both increased sympathetic and HPA axis activity is associated with elevated blood pressure. Ambulatory studies with children and adolescents largely support this pattern of results. Specifically, lower cardio-autonomic control (lower RMSSD, HF; higher LF/HF ratio; Farah, Barros, Balagopal, & Ritti-Dias, 2014; Zhou et al., 2012) and higher cortisol awakening (Guzzetti et al., 2014; Soriano-Rodriguez et al., 2010) have been associated with higher systolic and diastolic blood pressure; however, others have observed no association between cortisol awakening and blood pressure (DuBose & McKune, 2013; Hill, Eisenmann, Holmes, & Heelan, 2010).

Central Adiposity. Both the cardio-autonomic nervous system and HPA axis have been linked to visceral fat accumulation (Björntorp, 2001). Two independent reviews of experimental

findings concluded that sympathetic and parasympathetic neural inputs stimulate the growth of adipose tissue (Fliers et al., 2003; Kreier et al., 2002). Cortisol also has been found to direct excess body fat to visceral depots (Bjorntorp, Holm, Rosmond, & Folkow, 2000; Kyrou & Tsigos, 2009). Ambulatory studies with children and adolescents indicate lower cardio-autonomic control (lower HF; Farah et al., 2014; Zhou et al., 2012) and higher morning cortisol have been associated with a larger waist circumference (Barat et al., 2007; Hill, Eisenmann, Gentile, Holmes, & Walsh, 2011); however, one adolescent study observed lower morning cortisol was associated with a larger waist circumference (Ruttle et al., 2013).

Cholesterol. Both cardio-autonomic and HPA axis activity have been implicated in the regulation of cholesterol levels (Berenson et al., 1992; 1998; Björntorp, 2001; Björntorp & Rosmond, 2000; Chrousos, 2000; Porges, 1995; 2007a). In experimental studies, sympathetic and parasympathetic neural signals were found to reciprocally influence total cholesterol and LDL levels: sympathetic efferents increased cholesterol levels, while parasympathetic efferents decreased cholesterol levels (Puschel, 2004; Shanygina, Fomina, Parfenova, & Kalashnikova, 1981). In vitro studies with human tissue demonstrated that cortisol administration increases cholesterol levels (Ottosson, Lonnroth, Bjorntorp, & Eden, 1999). Ambulatory studies with adults have reported lower cardio-autonomic control is associated with higher Total and LDL cholesterol; however, an ambulatory study with children reported no association between cardio-autonomic control and cholesterol levels (Christensen, Toft, Christensen, & Schmidt, 1999; Thayer & Fischer, 2013). Although, higher morning cortisol in children has been related to greater Total and LDL cholesterol levels (DuBose & McKune, 2013; Guzzetti et al., 2014; Prodam et al., 2013; Weigensberg et al., 2008).

Taken together, the findings across experimental and ambulatory studies largely converge to suggest that cardiovascular precursors are related with *both* lower cardio-autonomic control and higher HPA axis activity. However, the existing literature predominantly measured *either* the autonomic nervous system *or* the HPA axis. Researchers have largely disregarded the apparent *inter-relation* between the stress systems. Based on the earlier findings regarding the structural and functional inter-connection between the stress systems, it is plausible that the inter-relation and patterning of the stress response system may better account for cardiovascular precursors than considering either system independently.

Current Program of Research

The aim of the current research program was to extend the existing literature by considering how the inter-relation between the cardio-autonomic nervous system and HPA axis is implicated in the association between stress and cardiovascular precursors. Previous research has established separately that both the autonomic nervous system and the HPA axis are feasible pathways linking stress to cardiovascular precursors. The role of the inter-relation between these two systems has yet to be considered.

For this dissertation, a programmatic line of inquiry was developed to investigate the plausibility of this inter-relation between the stress systems and its association to stress and cardiovascular precursors among children and adolescents. Three complementary studies were conducted. Study 1 examined whether the inter-relation better accounted for the relation between stress and the stress response system than either system alone. This study was also conducted to identify the patterning of the stress response system. Extending these findings, Study 2 tested whether the inter-relation was associated with cardiovascular precursors in a population-based sample of children. Given that stressful life experiences are known to exacerbate the functioning

of the stress response system, Study 2 also tested whether exposure to stressful life experiences moderated these associations. Finally, Study 3 considered whether the association between the inter-relation and cardiovascular precursors was robust in a sample of children at-risk for obesity. The overarching goal of the current programme of research was to investigate the patterning the stress response system and whether its inter-relation was associated with cardiovascular precursors during childhood.

STUDY 1

Inter-relation between autonomic and HPA axis activity in children and adolescents

Sivan Rotenberg and Jennifer J. McGrath

(Manuscript under review)

Abstract

Stress research in youth typically considers *either* the autonomic nervous system or HPA axis. However, these systems are highly coordinated and physically interconnected. We examined whether the inter-relation between cardio-autonomic and HPA axis measures was better associated with perceived stress than their singular associations. Children and adolescents (*N*=201) collected saliva samples to measure cortisol (AUC_{AG}, AUC_{I},, maximum), wore an electrocardiogram monitor to derive heart rate variability (HRV; LF, HF, LF/HF ratio), and completed the Perceived Stress Scale. The interaction between sympathovagal modulation (LF, LF/HF ratio) and cortisol awakening response (AUC_{AG}, AUC_{I},, maximum) explained significantly greater variance in perceived stress than either stress system alone. Higher sympathovagal modulation combined with higher cortisol awakening response was associated with greater perceived stress. Findings suggest that the inter-relation between cardio-autonomic and HPA axis activity may advance our understanding of how stress affects health.

Study 1 Introduction

The stress response system is comprised of the autonomic nervous system and the hypothalamic-pituitary adrenal (HPA) axis. Stress and the repeated activation of the stress response system have been associated with adverse health outcomes in children and adolescents. Specifically, using prospective (Adam et al., 2010; Fuligni et al., 2009; Slopen, Kubzansky, McLaughlin, & Koenen, 2013) and cross-sectional designs (Dreger, Kozyrskyi, HayGlass, Becker, MacNeil, 2010; Kazuma, Otsuka, Matsuoka, & Murata, 1997; Nagai, Matsumoto, Kita, & Moritani, 2003; Sen, Aygun, Yilmaz, & Ayar, 2008; Van den Bergh & Van Calster, 2009), dysregulation of the stress response system has been associated with obesity, asthma, inflammation, and depression among children and adolescents. Studies examining the physiological mechanism by which stress "gets under the skin" to affect health outcomes typically consider *either* the autonomic nervous system or the HPA axis (Lovell, Moss, & Wetherell, 2011; Lucini, Di Fede, Parati, & Pagani, 2005; Sloan et al., 1994). Yet, the autonomic nervous system and HPA axis are highly coordinated and physically interconnected. Investigating the inter-relation between the autonomic nervous system and HPA axis may provide a more thorough understanding of the association between stress and health. Among children and adolescents, few studies have considered how stress is related to the inter-relation between the autonomic nervous system and HPA axis.

Activation of the autonomic nervous system and HPA axis in response to a stressor follows a coordinated, temporal sequence. The autonomic nervous system quickly promotes physiological changes through synaptic transmissions by its two branches: the sympathetic and parasympathetic nervous system. The parasympathetic system facilitates the sympathetic response to stress, commonly referred to as the "fight or flight" response, by withdrawing its

inhibitory effects (Porges, 1995; 2007b). This, in turn, promotes physiological changes including the release of noradrenaline from the locus coeruleus (Curtis, Lechner, Pavcovich, & Valentino, 1997; Jedema & Grace, 2004) and the stimulation of sympathetic preganglionic neurons to increase heart rate (Bengel & Schwaiger, 2004; Engelnad & Arnhold, 2005; Shahar & Palkovits, 2007). Conversely, the HPA axis is a hormonal system; thus, physiological changes associated with its activation occurs minutes after activation. The HPA axis is initiated by the release of the corticotrophin-releasing hormone from the paraventricular nucleus of the hypothalamus, which results in a series of endocrine events that culminates with the release of cortisol from the adrenal cortex (Egliston, McMahon, & Austin, 2007). Cortisol impacts many different physiological systems (e.g., immunity, metabolism) and plays a role in augmenting the activity of the autonomic nervous system, such as enhancing the sympathetically mediated cardiovascular response to stress (e.g., increased heart rate; Saplosky, Romero, & Munck, 2000). Together, the autonomic nervous system and HPA axis work in concert to produce a state of biological and behavioral preparedness.

Interaction Between Stress Systems: Animal Studies

Animal studies examining the physiological link between the autonomic nervous system and HPA axis provide multiple lines of evidence to support their inter-relation. First, the autonomic nervous system and the HPA axis are reciprocally innervated. Corticotrophin-releasing hormone neuronal afferents from the paraventricular nucleus project to the locus coeruleus (Reyes, Valentino, Xu, & Van Bockstaele, 2005) and noradrenergic neurons from the locus coeruleus project to the paraventricular nucleus (Itoi, Jiang, Iwasaki, & Watson, 2004; Ma & Morilak, 2005). Second, there is a feed forward mechanism between the autonomic nervous system and the HPA axis. Corticotrophin-releasing hormone increases the firing rate of locus

coeruleus neurons and stimulates the release of noradrenaline (Jedema & Grace, 2004; Reyes, Valentino, & Van Bockstaele, 2008; Valentino, & Van Bockstaele, 2008). In turn, noradrenaline promotes corticotrophin-releasing hormone mRNA expression in the paraventricular nucleus (Itoi et al., 2004; Ma & Morilak, 2005). Moreover, lesions to the locus coeruleus attenuate the HPA axis response to a stressor (Zeigler, Cass, & Herman, 1999). Third, animal studies suggest that the autonomic nervous system and the HPA axis are both under tonic inhibitory control by the Central Autonomic Network, which includes the prefrontal cortex and limbic structures (Benarroch, 1993; Ulrich-Lai & Herman, 2009); these findings have also been observed in adult human studies (Gianaros, Van der Veen, & Jennings, 2004; Herman, Ostrander, Mueller, & Figueriredo, 2005; Radley, Arais, & Sawchenko, 2006). Thus, studies of the structural and functional connectivity between the autonomic nervous system and the HPA axis highlight their interconnection.

Stress Systems In Humans: Measurement of Autonomic Nervous System and HPA Axis

Autonomic nervous system activity can be measured in humans using heart rate variability (HRV), an indicator of cardio-autonomic control (Pumprla, Howorka, Grove, Chester, & Nolan, 2002). Both the parasympathetic and sympathetic nervous systems innervate the sinoatrial node, the pacemaker of the heart (Berntson et al., 1997; Task Force, 1996), and modulate heart rate. Noradrenaline from sympathetic neurons increases heart rate, while acetylcholine from parasympathetic neurons decreases heart rate (Berntson et al., 1997). HRV is commonly quantified by frequency domain measures, which describe how power is distributed as a function of frequency. Two frequencies are predominantly considered: low frequency (LF, 0.04-0.15 Hz) and high frequency (HF, 0.15-0.40 Hz; Task Force, 1996). Pharmacological blockade studies indicate that HF HRV is strongly associated with parasympathetic modulation,

and LF HRV is associated with both sympathetic and parasympathetic modulation (Cacioppo et al., 1994; Polanczyk et al., 1998). The LF/HF ratio reflects the balance of sympathetic and parasympathetic nervous systems, and is an indicator of sympathovagal modulation (Lahiri, Kannankeril, & Goldberger, 2008; Sztajzel, 2004; Task Force, 1996). Notedly, while the heuristic value of LF and LF/HF ratio has been established (Heathers, 2014; Malliani, 2005), their interpretation remains debated in the literature (de Geus, Montano, Sloan, & Thayer, 2014).

HPA axis activity can be assessed using salivary cortisol sampling. Cortisol is released in a circadian fashion characterized by cortisol levels that peak within the first hour post-awakening and gradually decline throughout the day (Fries, Dettenborn, & Kirschbaum, 2009). The collection of multiple samples across the day are used to derive aggregate measures that describe the diurnal cortisol profile, including the awakening response and diurnal slope (Rotenberg, McGrath, Roy-Gagnon, & Thanh Tu, 2012). The awakening response refers to the rise in cortisol by 50 to 75% (approximately 4-15 nmol/L) during the first hour post-awakening, and is measured by total amount of cortisol released during the awakening response (AUC_{AG}; area under the awakening response relative to ground or zero) and dynamic increase in the amount of cortisol secreted following awakening (AUC_I; area under the curve relative to increase; Clow, Thorn, Evans, & Hucklebridge, 2004; Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003). Diurnal slope is characterized as the decline in cortisol over the day. Single sample cortisol measures are commonly reported as well, including the maximum or specific time of day (e.g., morning, afternoon, bedtime; cf., Blair, Peters, & Granger, 2004; Cohen et al., 2006; El-Sheikh, Erath, Buckhalt, Granger, & Mize, 2008; Lupien, King, Meaney, & McEwen, 2001).

Interaction Between Stress Systems: Human Studies

Human studies examining the concurrent functioning of cardio-autonomic and HPA axis activity provide further evidence in support of their inter-relation. Specifically, higher morning and afternoon cortisol levels have been associated with low HF among adolescents in ambulatory settings (El-Sheikh, Arsiwalla, Hinnant, & Erath, 2011); although, no association has been reported as well (Oldehinkel et al., 2010). Additionally, elevated cortisol awakening response (AUC_I) has been associated with reduced LF and HF among young adults (Stadler, Evans, Hucklebridge, & Clow, 2011). These findings, taken together with those from the animal literature, converge to suggest that autonomic and HPA axis activity are coordinated and work together to respond to stress across the lifespan.

Theoretical Rationale for Considering the Inter-Relation

Extant psychophysiology theories highlight the importance of considering the role of the inter-relation between the autonomic nervous system and HPA axis. Bauer, Quas, & Boyce (2002) hypothesized that the coordination of the autonomic and HPA axis response to stress is related to an individual's risk for adverse outcomes. Bauer and colleagues' proposed two competing inter-relation models: Additive or Interactive. The Additive Model contends that symmetrical activation of both systems (hyper-arousal: high autonomic and HPA axis activity; or hypo-arousal: low autonomic and HPA axis activity) increases risk. In contrast, the Interactive Model contends that asymmetric activation increases risk, as the most adaptive physiological response may be when there is a balance between autonomic and HPA axis activity. Del Giudice and colleagues (2010) extended Bauer and colleagues' model by suggesting that distinct response patterns between the autonomic nervous system and HPA axis emerge due to early life

experiences (e.g., exposure to chronic stress) and that the match or mismatch between environmental context and stress response patterns is vital for determining risk.

The Polyvagal Theory (Porges, 1995; 2007a) and Neurovisceral Integration Model (Thayer & Lane, 2000; Thayer & Sternberg, 2006) also support examining the inter-relation and provide greater insight into how the autonomic nervous system and HPA axis may be inter-related. Grounded in Porges' work on emotion regulation, both theories suggest that the parasympathetic nervous system plays an integral role in regulating the stress response system. Specifically, the parasympathetic nervous system is thought to moderate an individual's stress response by either inhibiting or disinhibiting the sympathetic nervous system and HPA axis. Parasympathetic inhibition promotes a calm or restorative state, while parasympathetic disinhibition promotes the mobilization of energy and the "fight or flight" response (Porges, 2007a). Adult findings from laboratory-based stress studies partially support these theories as both sympathetic and parasympathetic activity have been found to moderate the HPA axis response to stress (Cacioppo et al., 1995; Sgoutas-Emch et al., 1994; Uchino, Cacioppo, Malarkey, & Glaser, 1995; Weber et al., 2010). Similar studies with children and adolescents could not be identified; thus, it remains to be determined whether measures of parasympathetic modulation (e.g., HF) moderate HPA axis activity in youth. Examining the association between stress and the inter-relation of cardio-autonomic control and HPA axis activity in children is particularly opportune given that stressful experiences in childhood shape disease trajectories later in life (Hertzman, 1999).

Perceived Stress and Stress Systems in Children and Adolescents

Studies examining the association between perceived stress with *either* cardio-autonomic or HPA axis activity in children and adolescents yield inconsistent results. Levels of perceived

stress over the past year have been positively (Bevans, Cerbone, & Overstreet, 2008), negatively (Maldonado et al., 2008), and unassociated (Maldonado et al., 2008; Williamson, Birmaher, Dahl, & Ryan, 2005) with cortisol levels in children and adolescents. Adolescents' momentary analysis of perceived stress has also been positively associated with cortisol levels in a single study (Adam, 2006). Previous studies relating perceived stress to HRV in children and adolescents could not be identified; however, in the adult literature, findings have been mixed. Among adults, chronic perceived stress has been positively associated with LF and LF/HF ratio (Lucini et al., 2005); momentary levels of perceived stress have been negatively associated with HF (Looser et al., 2010); and, perceived stress levels over the past month have been unassociated with HRV (Stadler et al., 2011). Thus, the association between perceived stress and the stress response system remains unclear.

Two possible explanations may account for these inconsistencies. First, measurement limitations may contribute to the mixed findings, as the majority of studies with HPA axis activity (Bevans et al., 2008; Maldonado et al., 2008; Williamson et al., 2005) included only two cortisol samples collected on one day, which has been shown to yield poor reliability (Rotenberg, McGrath, Roy-Gagnon, & Tu, 2012). Timing of perceived stress measurement varied across studies, ranging from momentary assessment to the past year. The specific measures of cardio-autonomic control and HPA axis activity have also varied across studies, making synthesis difficult due to measurement differences. Second, the analytical approach may account for the mixed findings. Specifically, previous studies did not test the interaction between the autonomic nervous system and HPA axis, and instead only analyzed cardio-autonomic control *or* HPA axis activity alone. In the context of Bauer's Additive Model (2002), individuals with greater perceived stress may have asymmetrical stress response systems, yet this finding would be

masked if only HRV or cortisol measures are considered singularly. Thus, it is plausible that these measurement limitations and constrained analytical methods may account for the observed inconsistences in the literature. Comprehensive and concurrent assessment of both cardio-autonomic and HPA axis activity is required to tease apart and address these questions.

The overarching aim of the current study was to examine whether the inter-relation between cardio-autonomic and HPA axis activity was a better indicator of the association between perceived stress and the stress response system in children and adolescents, than *either* system alone. Informed by previous theoretical models, we first hypothesized that inter-relation models, which include the interaction between HRV and cortisol, would better explain perceived stress compared to models with HRV *or* cortisol singularly. The secondary aim was to further examine the patterning of the cardio-autonomic and HPA axis inter-relations. We hypothesized that altered cardio-autonomic activity (low parasympathetic, high sympathovagal modulation), combined with elevated cortisol awakening response, would be associated with higher perceived stress. Given the inconsistencies reported in the literature, we explored the patterning of the inter-relations across measures of cardio-autonomic and HPA axis activity.

Study 1 Method

Participants

Children and adolescents aged 9 to 18 years were recruited to take part in the larger Healthy Heart Project, a longitudinal study examining early cardiovascular risk factors, at Concordia University, Montreal, QC. Flyers, postcards, and bookmarks were distributed throughout the community and in schools approved by the Montreal English School Board. Children and adolescents with serious psychopathology or medication use known to interfere

with cardiovascular or endocrine functioning were excluded. This study was approved by the Concordia University Ethics Review Committee (UH2005-077).

Participants (N = 201) included children and adolescents (M_{age} = 12.69, SD = 2.05, see Table 1). About half of the sample was female (n = 90, 45%) and in the early to mid stages of pubertal adrenarche (Stages I-III, n = 91, 45.2%). The majority of participants were of normal body mass (5-85th BMI percentile: n = 136; 67.7%). On average, parents were university educated ($M_{education}$ = 16.37 yrs., SD = 3.37) and had a yearly household income of $78.9K Canadian (SD = $52.4K). Youth had a mean wake-time of 7:31 am (SD = 1.29 h) and typically slept for 8.95 h (SD = 1.22). The majority of the physiological measures were collected while school was in session (78.1%), and most participants (85.1%) indicated that the collection of measures easily fit into their typical routine.

Measures

Cortisol. Saliva samples were collected six times per day for two consecutive weekdays. Samples were collected at awakening ($awake_0$), +30 minutes post-awakening ($awake_{30}$), +45 minutes post-awakening ($awake_{45}$), before lunch, before dinner, and at bedtime.

Saliva samples were collected using the Salivette sampling device. Participants were instructed to place the cotton swab under their tongue for at least 30 seconds. When saturated, it was placed back in the Salivette tube and refrigerated until returned at the second visit. Participants were instructed not to eat, drink, or brush their teeth 10 minutes before taking a sample. Participants recorded the date and time each sample was taken in a daily log, which was initialed by parents or teachers as a marker of compliance (97% of log entries initialed by parent/teacher). Compliance was also verified for the awakening ($awake_0$) sample using accelerometry data (supine to sitting; Rotenberg & McGrath, 2014). Consistent with previous

adult findings, most participants (88.1%) collected the $awake_0$ sample within 15 minutes of the accelerometer-based wake-time (Dockray et al., 2008; DeStantis et al., 2010) and 91% of the participants collected the $awake_{0,}$ $awake_{30}$, and $awake_{45}$ samples within one hour post awakening; further details on compliance can be found in Rotenberg & McGrath (2014).

Upon receipt at the laboratory, saliva samples were stored in a sub-zero freezer until they were packaged in dry ice and couriered to the University of Trier, Germany for cortisol assaying. Cortisol levels are robust to environmental conditions associated with the shipping process (Clements & Parker, 1998). Cortisol levels were determined in duplicate using a competitive solid phase time-resolved fluorescence immunoassay with fluorometric end point detection (DELFIA; Dressendörfer, Kirschbaum, Rohde, Stahl, & Strasburger, 1992). The intra-assay coefficients of variation were less than 11%.

Untransformed cortisol values were used to derive four cortisol measures per day: area under the awakening response relative to ground (AUC_{AG}), dynamic increase of the awakening response (AUC_I), and diurnal slope. The diurnal slope was determined by standard linear regression and was anchored to the maximum sample ($Slope_{Max}$; for formulae see Rotenberg et al., 2012). Maximum was identified as the highest morning cortisol value identified each day. The two-day average of each cortisol measure was used in the analyses. All cortisol measures were normally distributed.

Heart Rate Variability. Continuous electrocardiogram (ECG) recordings were collected with ambulatory monitors (Vivometrics, San Diego, California, USA) worn at home and school. ECG data acquisition began during the first laboratory visit when participants were fitted with the ambulatory monitor and continued for 24 hours. Using self-report measures and accelerometry, ECG recordings corresponding to waking hours were selected; thus, only daytime

ECG data were analyzed.

The ECG data were recorded at a sampling rate of 200 Hz, and processed according to Task Force Guidelines (1996). The ECG signal was imported, converted, and formatted into MindWare files using Biolab 3.0 acquisition software (MindWare Technologies Ltd., Columbus, Ohio, USA). The MindWare file was then converted into an ASCII text file which was processed in Kubios® HRV v.2.0 (University of Eastern Finland, Kuopio, Finland; Niskanen, Tarvainen, Ranta-aho, & Karjalainen, 2004). Artifact correction was conducted manually in 5 min epochs based on Task Force Guidelines (1996), and by visual inspection of the software graphical interface. Using a window width of 256 s, data were tapered using a Hanning window.

Frequency-domain measures of HRV were derived for each 5 min epoch and averaged across the entire daytime recording on each day. The two-day average of each HRV measure was used in analyses. Frequency domain measures were based on power spectral analysis (ms^2) and were derived using the Fast Fourier Transformation. Frequency domain measures included: low frequency (LF; 0.04 - 0.15 Hz), high frequency (HF; 0.15 – 0.40 Hz), and the LF/HF ratio. All frequency measures were normally distributed.

Perceived Stress. Perceived stress was assessed using the 10-item Perceived Stress Scale, which assesses how unpredictable, uncontrollable, and overwhelming an individual considers their life to be over the past month (Cohen, Kamarck, & Mermelstein, 1983; Cohen & Williamson, 1988). The 10-item Perceived Stress Scale has been shown to be valid and reliable in children and adolescents aged 11 to 20 (= .72 - .76; Finkelstein, Kubzansky, Capitman, & Goodman, 2007; Seller, Copeland-Linder, Martin, & L'Heureux Lewis, 2006; = .81 current study).

Pubertal Stage. Pubertal stage was based on adrenarche (pubic hair growth) and was assessed using sex-specific illustrations corresponding to Tanner stages I to V (Growing and Changing Questionnaire; Golding, Pembray, & Jones, 2001). While visual examination performed by physician to assess sexual maturation status is the gold standard, it is often not conducted due to concerns about privacy and the sensitivity of the physical examination. Pubertal illustrations have demonstrated good reliability and validity (r = .77-.91; Morris & Udry, 1980; Netherton, Goodyer, Tamplin, & Herbert, 2004).

Sleep Duration. The quantity of sleep on the night preceding saliva sampling was recorded in the child's daily logs. Sleep duration was calculated as the time elapsed between self-reported bedtime and wake-time. Wake-time was defined as the time children and adolescents woke-up and collected the awake$_0$ saliva sample. Sleep duration is an established covariate of both HRV and cortisol (Jarrin et al., 2015; Rotenberg et al., 2012).

Procedure

Participants and their parents were scheduled for two visits to the laboratory. During the first visit, participants and their parents completed demographic and health questionnaires. Participants also completed the Perceived Stress Scale during the first visit. Participants were instructed how to use the Salivette sampling device (Salimetrics, Inc.) and provided saliva collection kits for home and school. They were also fitted with an ambulatory heart rate monitor. Salivary cortisol samples and the ECG recording began the day after the first visit. During the second visit, participants returned their saliva samples and the heart rate monitor. Informed consent and participant assent were obtained prior to the study. Participants received monetary compensation for their time.

Sample Exclusion Criteria

Of the initial 241 participants who were included in the larger Healthy Heart Project, participants who did not return any saliva samples (n = 15), collected saliva samples only on weekend days (n = 10), or did not have ECG recording due to equipment malfunction (n = 15) were excluded from the sample. Thus, the final sample included 201 participants. Excluded participants did not differ from the final sample on sex, age, pubertal stage, parental education, household income, cortisol measures, HRV measures, or perceived stress (results not shown for parsimony).

Statistical Analyses

Variables were inspected for normality and outliers to ensure the assumptions of the analytic methods were met. Missing data were imputed using multiple imputation (McKnight, McKnight, Sidani, Figueredo, 2007). Imputation of missing cortisol values (single samples not returned, 10%; insufficient saliva for assaying, 0.01%) was informed by data from the larger Healthy Heart Project (e.g., subsequent cortisol samples, day of sampling, puberty); missing values were imputed 20 times with re-sampling techniques. Box plots and scatterplots were visually inspected to check the distributions and linearity. Descriptive data (means, standard deviations, minimum, maximum, skewness, kurtosis) were reviewed for all variables. All analyses were conducted using IBM SPSS Statistics software (Version 20). Analyses were performed with both the original and imputed datasets. Findings did not vary; therefore, results based on the imputed dataset are presented for parsimony.

To test the first hypothesis that the inter-relation models, which include interactions between HRV and cortisol, would better explain perceived stress compared to HRV or cortisol singularly, we conducted General Linear Model regression analyses. Singular models tested the

association between each stress system measure (HRV: HF, LF, LF/HR ratio; Cortisol: AUC_I, AUC_{AG}, $Slope_{MAX}$, Maximum) with perceived stress. Inter-relation models included the main-effects and two-way interaction of HRV and cortisol measures. Both singular and inter-relation models adjusted for developmentally relevant covariates of HRV and cortisol that have been previously established in the literature (Jarrin et al., 2015; Rotenberg et al., 2012), including age, sex, pubertal stage, BMI *Z*-score, household income, parental education, sleep duration, and wake time. (Inclusion of compliance with cortisol sampling as a covariate did not alter results; data not shown for parsimony). Test statistics (ΔF and ΔR^2) and the Generalized F test (F^*) were used to compare the singular and inter-relation models to determine whether the inter-relation models accounted for significantly greater variance in perceived stress.

To test the second hypothesis that altered cardio-autonomic activity (low parasympathetic, high sympathovagal modulation) combined with elevated cortisol awakening response would be associated with higher perceived stress, we examined the interactions of the inter-relation regression models. Simple slopes analyses were used to interpret significant interactions (Aiken & West, 1991). We anticipated the interactions of low HF and high LF/HF ratio with high AUC_{AG} or AUC_I would be associated with the higher perceived stress. Exploratory analyses were conducted for the interactions with LF HRV and Maximum cortisol.

Study 1 Results

Descriptive statistics are presented in Table 1. Correlations among HRV, cortisol, and covariate measures are presented in Table 2.

Singular versus Inter-Relation Models

The first hypothesis that inter-relation models would better explain perceived stress compared to the singular HRV or cortisol models was partially supported (see Table 3). Inter-

relation models with the interactions of LF*AUC_{AG}, LF*Maximum, as well as LF/HF ratio*AUC_I, and LF/HF ratio*Maximum accounted for significantly greater variance in perceived stress than either the singular HRV or cortisol models. No interactions with HF were significant. The inter-relation of HRV and cortisol accounted for an additional 2-4% of the variance in perceived stress. Interestingly, AUC_I was unassociated with perceived stress in the singular cortisol model but its interaction with LF/HF ratio accounted for 4% of the variance in perceived stress.

Patterning of Inter-Relations Across Cardio-Autonomic and HPA Axis Activity

The second hypothesis that altered cardio-autonomic activity (low parasympathetic, high sympathovagal modulation), combined with elevated cortisol awakening response, would be associated with higher perceived stress was partly supported. Simple slopes analyses indicated that LF and LF/HF ratio moderated the association between the cortisol awakening response and perceived stress (Figure 1A & C). Specifically, higher LF combined with greater AUC_{AG} was associated with higher perceived stress; this inter-relation was not significant for AUC_I. Higher LF/HF ratio combined with greater AUC_I, but not AUC_{AG}, was associated with higher perceived stress. Contrary to expectations, no inter-relations of HF, the most robust measure of parasympathetic activity, with the cortisol awakening measures were significant.

Exploratory analyses were conducted to consider the patterning of the remaining inter-relations across measures of cardio-autonomic and HPA axis activity. Higher LF combined with higher maximum cortisol was associated with higher perceived stress (Figure 1B). Similarly, higher LF/HF ratio combined with higher maximum cortisol was associated with higher perceived stress (Figure 1D). No patterns emerged between HF with any cortisol measures. Finally, no patterns emerged between Slope with any HRV measures; however, the inter-relation

model of LF*Slope accounted for significantly greater variance in perceived stress despite the interaction term not being significant.

Study 1 Discussion

Previous studies that have examined the association between perceived stress and the stress response system have typically investigated the autonomic nervous system *or* the HPA axis (Lovell et al., 2011; Lucini et al., 2005; Sloan et al., 1994). Yet, the autonomic nervous system and HPA axis are highly coordinated and physically interconnected (Ma & Morilak, 2005; Reyes et al., 2008; Ulrich-Lai & Herman, 2009). The current study is one of the first to test whether the inter-relation between cardio-autonomic control and HPA axis activity was better associated with perceived stress than the singular effect of either cardio-autonomic or HPA axis activity. We first hypothesized that the inter-relation models would better explain perceived stress compared to the singular HRV or cortisol models. The patterning of the inter-relations was more closely examined as a secondary aim. We further hypothesized that altered cardio-autonomic activity (low parasympathetic, high sympathovagal modulation), combined with elevated cortisol awakening response, would be associated with higher perceived stress. The results partially supported the first hypothesis as the inter-relation between measures of sympathovagal modulation (LF, LF/HF ratio) and the cortisol awakening response was significantly more associated with perceived stress than the singular association of either stress response system alone. The second hypothesis was also partly supported as cardio-autonomic activity moderated the association between the cortisol awakening response and perceived stress. Namely, greater sympathovagal modulation (high LF, high LF/HF ratio) combined with higher cortisol awakening response (AUC_{AG}, AUC_{I}) was associated with greater perceived stress. Exploratory analyses revealed these patterns were largely similar to the maximum cortisol

measure, but no inter-relations with low parasympathetic activity (low HF) or diurnal cortisol slope were significant.

The current results were largely consistent with the few previous studies that considered the interaction between the autonomic nervous system and HPA axis among adults (Cacioppo et al., 1995; Uchino et al., 1995) and children and adolescents (El-Sheikh et al., 2008; Gordis, Granger, Susman, & Trickett, 2006). Similar to our findings, adult studies using laboratory stressors reported the HPA axis stress response was only evident among individuals with greater sympathetic activity, indexed by pre-ejection period; yet, HF was not associated with the HPA axis stress response (Cacioppo et al, 1995; Uchino et al., 1995). Ambulatory or laboratory-based stress studies with children and adolescents that examined the interaction between the autonomic nervous system and HPA axis could not be identified. However, sympathetic activity measured by alpha-amylase has been reported to influence the association between cortisol and aggressive behavior (Gordis et al., 2006), and internalizing and externalizing symptoms (El-Sheikh et al., 2008). Contrary to the current study, one study found that children with asymmetric cardio-autonomic and HPA axis activity (e.g., low HF, high cortisol) had the highest levels of anxiety and depression symptoms (El-Sheikh et al., 2011); however, three methodological differences likely account for these discrepancies: outcome variable, age of participants, and HPA axis measure. First, while the current study examined perceived stress as the outcome variable, El-Sheikh and colleagues measured symptoms of anxiety and depression. Second, participants were younger than those in the current sample (M_{age} = 9.06 vs. M_{age} = 12.69, respectively). Third, only a single afternoon sample was used, which has been previously shown to have poor reliability (Rotenberg et al., 2012), compared to aggregate measures used in the current study. The current findings combined with previous results suggest that cardio-autonomic activity may

moderate HPA axis activity. Prospective studies and experimental research are needed to further examine the patterning of cardio-autonomic and HPA axis activity.

In the present study, significant interactions between cardio-autonomic control and the HPA axis explained a unique portion of the variance (2-4%) in perceived stress. The magnitude of this effect is congruent with previous child studies that considered the inter-relation between autonomic and HPA axis activity. Gordis et al. (2006) found that the interaction of alpha-amylase and cortisol accounted for 7% of the variance in children's aggressive behavior. El-Sheikh et al. (2008) reported that the interaction of HF and cortisol accounted for between 2-14% of the variance in anxiety and depressive symptoms. Overall, the current findings support emerging theories in the field of stress physiology that emphasize the importance of considering the inter-relation among physiological components of the stress response system (Bauer et al., 2002; Del Giudice et al., 2010). The interactions also suggest that existing analytical inconsistencies in the child literature regarding the relationship between stress and the stress response system (Bevans et al., 2008; Maldonado et al., 2008; Williamson et al., 2005) may be accounted for by the interaction between the stress systems. The significant contribution of the inter-relation in this study highlights the need for future research to use multi-dimensional models when considering how the stress response system is related to adverse outcomes.

Interpretations of the significant interactions in the current study indicate that children and adolescents with greater sympathovagal modulation and higher cortisol awakening response had greater perceived stress. This symmetrical activation supports Bauer et al.'s Additive Model of risk, which suggests that children and adolescents are at a greater risk for adverse outcomes when there is simultaneous hyper-arousal of cardio-autonomic and HPA axis activity. Hyper-arousal of the stress response system is thought to exacerbate the development of disease (e.g.,

metabolic syndrome, cardiovascular disease) by promoting physiological changes, such as increased blood pressure and visceral fat accumulation (Bjorntorp et al., 2000).

Our observation that sympathovagal modulation interacts with cortisol awakening response coincides with anatomical studies regarding the regulation of the cortisol awakening response. In addition to the HPA axis, the cortisol awakening response is also regulated by direct sympathetic innervation via the splanchnic nerve (Clow, Hucklebridge, Stalder, Evans, & Thorn, 2010; Engeland & Arnhold, 2005). Ulrich-Lai and colleagues (2006) conducted splanchnic nerve transection in rats, which have a diurnal cortisol profile opposite that of humans (cortisol levels peak in evening and reach nadir in morning). The splanchnic nerve transection markedly reduced cortisol concentrations during the evening, but only modestly increased cortisol levels in the morning, evidencing that the sympathetic nervous system plays a prominent role in regulating the rise in cortisol post-awakening. We anticipated that parasympathetic modulation would have significantly interacted with the cortisol awakening response based on these findings and informed by the Polyvagal Theory and Neurovisceral model. However, contrary to expectations, no inter-relations with HF emerged in our findings. Further research examining the relationship between perceived stress and the inter-relation of cardio-autonomic control and HPA axis activity is warranted.

It is important to note that the physiological underpinning and interpretation of the LF and LF/HF ratio HRV measures remains actively debated in the field of psychophysiology (de Geus et al., 2014). Some suggest that LF is a measure of sympathetic modulation (Malliani, 1999; Montano et al., 2009); others argue that it is a measure of both sympathetic and parasympathetic modulation (Cacioppo et al., 1994; Reyes del Paso et al., 2013); and still others suggest that it represents baroreflex activity (Heathers, 2014). Friedman (2007) suggests that in

some contexts LF can serve as an index of sympathetic modulation, whereas in other contexts there can be extensive parasympathetic influence. Given that the current findings related to LF paralleled LF/HF ratio, a measure of sympathovagal modulation, rather than HF, a known measure of parasympathetic modulation, the current study supports the presence of sympathetic modulation in LF (Friedman, 2007). This is consistent with several experimental and ambulatory studies, which contend that stress is associated with increased sympathetic control, as evidenced by increased LF (cf., Berntson & Cacioppo, 2004). Despite the lack of consensus on the interpretation of LF and LF/HF ratio, the results of the present study support the heuristic value of LF and LF/HF ratio, and their role as a moderator of the association between stress and cortisol.

Investigating the association between the inter-relation of cardio-autonomic control and HPA axis activity with perceived stress during childhood and adolescence is particularly important given that this early timing in lifecourse development is considered a critical period when the effects of stress become biologically embedded (Danese & McEwen, 2012; Goodman, McEwen, Huang, Dolan, & Adler, 2005; Shokoff et al., 2009). Children and adolescents experience numerous physiological changes (e.g., puberty, shift in sleep-wake cycle) and begin a vital stage of brain development (i.e., frontal cortex development; Goodman et al, 2005; Lupien, McEwen, Gunnar, & Heim, 2009; Shokoff et al., 2009). The importance of considering the impact of stress during childhood was recently highlighted by an animal study, which found that exposure to an acute stressor during childhood resulted in greater anxiety-like behavior and the highest basal cortisol levels later in life, compared to exposure to stress during the prenatal period, infancy, or adulthood (Cymberblit-Sabba et al., 2015). Identifying different patterns in stress responsivity, and relating these patterns to adverse outcomes, is important to advance the

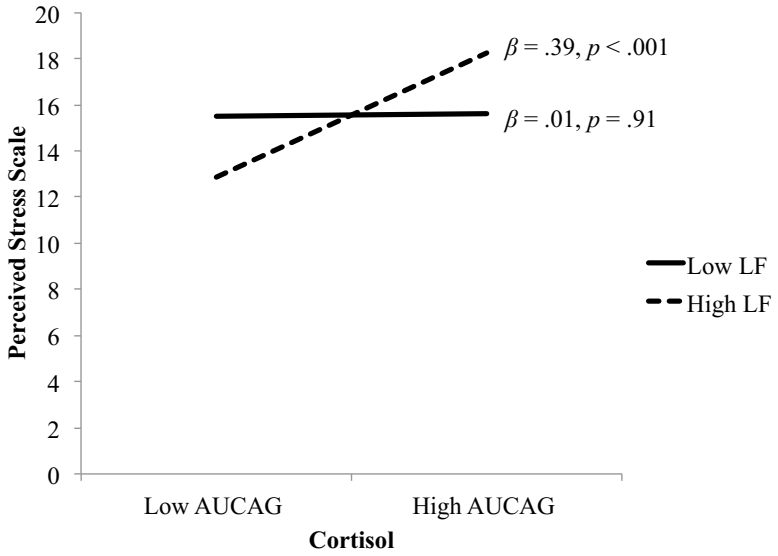
current understanding of how stress impacts children's health. Examining the role of the inter-relation across different developmental periods is recommended for future research.

Three study limitations merit discussion. First, we were unable to make inferences about causality because we employed a cross-sectional design. Future research should examine longitudinal, developmental trajectories of the inter-relation between autonomic and HPA axis functioning and its link with stress. Second, we used the Perceived Stress Scale to measure the construct of daily hassles and stress over the past month (Cohen et al., 1983; Cohen & Williamson, 1988). In addition to perceived stress, chronic stress and stressful life events have also been associated with adverse health outcomes in children and adolescents (Slopen et al., 2013). Future research should consider measuring stress from a multidimensional perspective (i.e., longer duration, early life adversity). Third, we measured cardio-autonomic activity using HRV, which is the gold standard for measuring parasympathetic activity, but only an indirect measure of sympathetic activity (Lahiri et al., 2008). Future research should consider other autonomic measures (i.e., salivary alpha amylase, pre-ejection period) to more comprehensively characterize the autonomic nervous system. In summary, future research would benefit from extending the current findings by examining how the inter-relation between the autonomic nervous system and the HPA axis is associated with other constructs of stress (e.g., stressful life events), over a longer period of time (e.g., past year, childhood), in clinical populations (e.g., children and adolescents with obesity, depression), and using additional measures of autonomic activity (e.g., alpha amylase). Despite these limitations, our methodological protocol to comprehensively measure cardio-autonomic control and HPA axis activity was an important strength of this study. Previous studies relating the autonomic nervous system or HPA axis to perceived stress have yielded inconsistent results in part, possibly, due to measurement

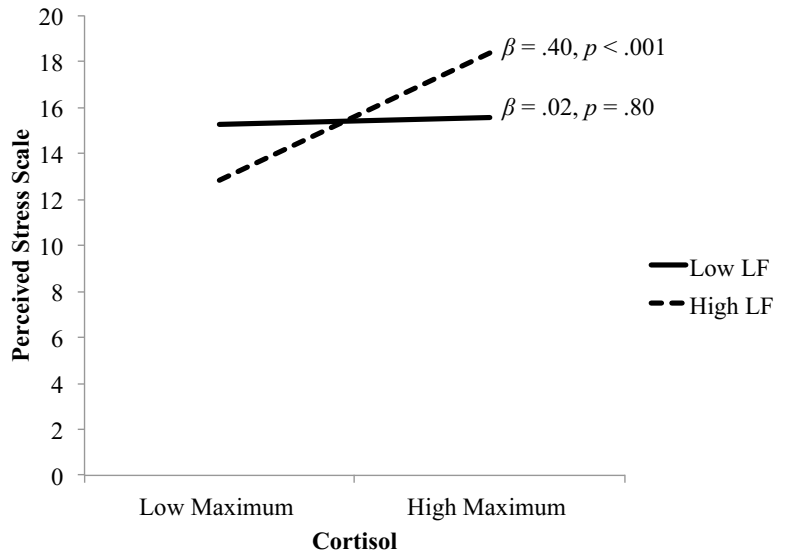
limitations (e.g., collecting a single cortisol sample). In the current study, we collected measures over multiple days using continuous ambulatory ECG monitoring and repeated salivary cortisol sampling to derive stable and reliable indices of cardio-autonomic and HPA axis activity, in accordance with existing methodological guidelines (Adam & Kumari, 2009; Rotenberg et al., 2012; Task Force, 1996).

Taken together, our results are among the first to provide evidence for the importance of considering the inter-relation between the autonomic nervous system and the HPA axis when investigating the association between stress and the stress response system. We found that the inter-relation between cardio-autonomic control and the HPA axis was more associated with perceived stress than the singular association of either stress response system alone. Children with higher sympathovagal modulation in combination with a higher cortisol awakening response had greater perceived stress. These results contribute to the existing literature by providing empirical evidence suggesting that the interaction between the autonomic nervous system and HPA axis is uniquely associated with perceived stress. Future studies examining the association between stress and health should consider the inter-relation between the autonomic nervous system and the HPA axis to gain a more thorough understanding of the mechanism by which psychological stress effects health.

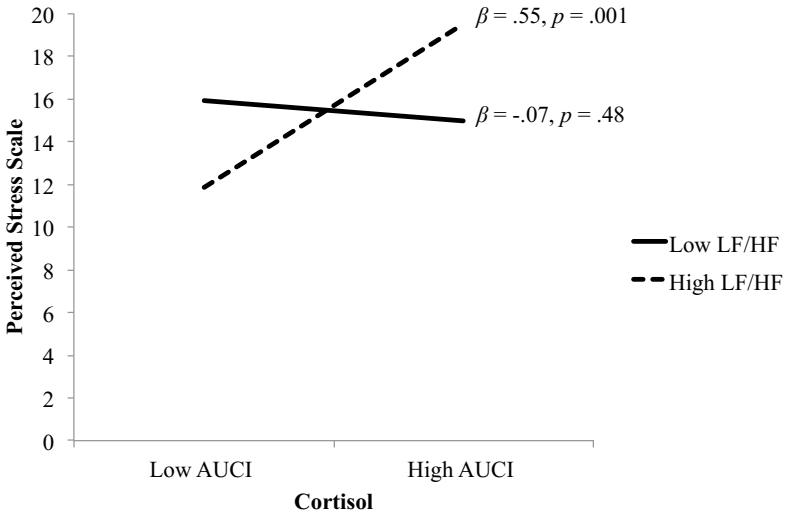
A)


20
18
16
14
12
10
8
6
4
2
0
Perceived Stress Scale
β = .39, p < .001
β = .01, p = .91
Low LF
High LF
Low AUCAG
High AUCAG
Cortisol

B)


20
18
16
14
12
10
8
6
4
2
0
Perceived Stress Scale
β = .40, p < .001
β = .02, p = .80
Low LF
High LF
Low Maximum
High Maximum
Cortisol

C)


20
18
16
14
12
10
8
6
4
2
0
Perceived Stress Scale
β = .55, p = .001
β = -.07, p = .48
Low LF/HF
High LF/HF
Low AUCI
High AUCI
Cortisol

D)

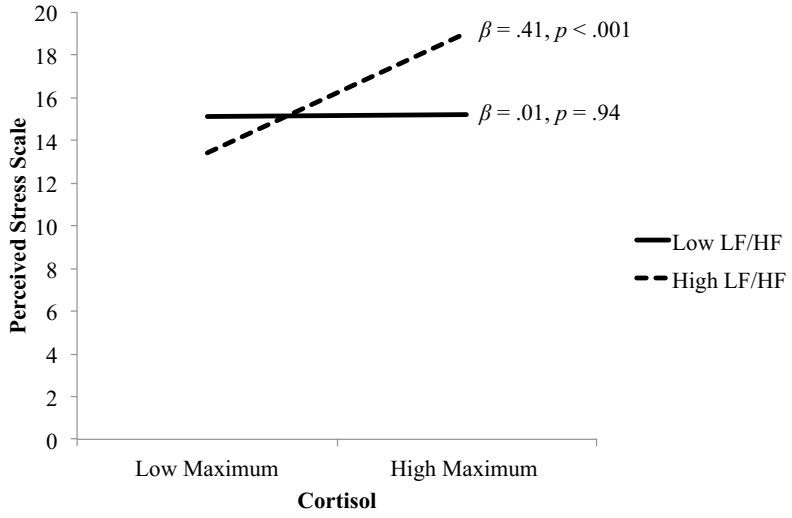

20
18
16
14
12
10
8
6
4
2
0
Perceived Stress Scale
β = .41, p < .001
β = .01, p = .94
Low LF/HF
High LF/HF
Low Maximum
High Maximum
Cortisol

Figure 1. Simple slopes interpretation of significant inter-relations. Higher sympathovagal modulation and higher cortisol awakening response associated with greater perceived stress. In the panels, A) higher LF and higher AUC_{AG}; B) higher LF and higher maximum cortisol; C) higher LF/HF ratio and higher AUC_I; and, D) higher LF/HF ratio and higher maximum cortisol, associated with higher perceived stress. Data were dichotomized (mean ± 1 SD) for interpretation purposes only; continuous data were retained in analyses.

Table 1

Descriptive Statistics: Demographics, Cortisol, and HRV Measures

	M (n)	*SD (%)*
Demographics		
Age (8-18)	12.69	2.05
Female	(90)	(44.8%)
Adrenarche		
Stage 1	(33)	(16.4%)
Stage 2	(23)	(11.4%)
Stage 3	(35)	(17.4%)
Stage 4	(49)	(24.4%)
Stage 5	(45)	(22.4%)
BMI *Z*-score (-3.00- 2.60)	0.49	0.97
Normal weight	(136)	(67.7%)
Overweight	(39)	(19.4%)
Obese	(22)	(10.9%)
Parental Education (0 - 22 years)	16.37	3.37
Household Income ($5K - 21K CAN)	78.9K	52.4K
Perceived Stress Score (0 - 40)	15.56	6.87
Cortisol Measures		
AUC_{AG}	11.25	5.40
AUC_I	2.31	5.38
$Slope_{Max.}$	-1.05	0.51
Maximum	18.38	8.04
HRV Measures		
LF (ms^2)	2080.70	1086.32
HF (ms^2)	1699.33	1138.75
LF/HF Ratio	1.57	0.66

Note. $N = 201$. AUC_{AG} = area under curve relative to ground. AUC_I = area under curve relative to increase. $Slope_{max}$ = diurnal slope anchored to max sample using regression. LF = low frequency. HF = high frequency.

Table 2

Correlations among HRV, Cortisol, and Covariate Measures

	1	2	3	4	5	6	7
HRV Measures							
1. LF	-						
2. HF	**.81****	-					
3. LF/HF Ratio	**-.29****	**-.62****	-				
Cortisol Measures							
4. AUC_{AG}	.05	.05	-.07	-			
5. AUC_{I}	.01	.03	.01	.02	-		
6. $Slope_{Max}$	-.11	-.09	-.08	**-.73****	**-.38****	-	
7. Maximum	.08	.07	-.06	**.81****	**.39****	**-.92****	-
Covariates							
Age	.05	-.07	**.16***	-.02	**.14***	.01	.03
Sex	**-.30****	**-.24****	.08	.10	.08	-.09	**.14***
Adrenarche	.09	.01	.03	-.06	**.16***	.01	.01
BMI *Z*-score	-.01	-.02	-.03	-.05	-.06	.10	-.04
Parent Education	-.02	.01	-.03	-.00	.11	.06	-.04
Household Income	.05	.06	-.02	.03	.12	-.08	.08
Sleep Duration	.08	.13	-.05	-.08	**-.14***	.03	-.12
Wake-time	.13	.12	-.04	**-.25****	-.04	**.18***	**-.23****
Compliance	.07	.07	-.04	.01	-.02	-.04	.00

Note. Zero-order correlations are presented. AUC_{AG} = area under curve relative to ground. AUC_{I} = area under curve relative to increase. $Slope_{max}$ = diurnal slope anchored to max sample using regression. LF = low frequency. HF = high frequency. Bold values indicate significance: $^{\pm}p<.07$ $^{*}p<.05$ $^{**}p<.01$.

Table 3

Singular vs. Inter-relation Model Comparisons

		Singular Models HRV			Singular Models Cortisol			Inter-relation Models					Model Comparison		
HRV	Cortisol	β_{hrv}	F	R^2	β_{cort}	F	R^2	β_{cort}	β_{hrv}	β_{int}	F	R^2	ΔF[b]	ΔR^2	F[*c]
LF		.05	4.21	.17	-	-	-	-	**-**	**-**	**-**	**-**	**-**	**-**	
	AUC_{AG}	-	-	-	**.17***	4.74	.19	-.15	**-.40***	**.56****	4.86	**.23**	**4.47***	**.04**	**3.42***
	AUC_I	-	-	-	.08	4.16	.17	.01	.02	.13	3.87	.19	0.80	.00	2.26
	$Slope_{Max}$[a]	**-**	-	-	.06	4.65	.20	.22	**-.48****	-.29	4.85	.25	**4.01***	**.04**	**3.57****
	Maximum	-	-	-	**.21****	5.18	.20	-.13	**-.42***	**.59***	5.16	**.24**	**3.40***	**.03**	**3.27***
HF		.00	4.15	.17	-	-	-	-	-	-	-	-	-	-	
	AUC_{AG}	-	-	-	-	-	-	.06	-.20	.22	4.01	.20	0.67	.01	1.48
	AUC_I	-	-	-	-	-	-	.12	-.01	.00	3.70	.18	0.00	.00	1.84
	$Slope_{Max}$[a]	**-**	-	-	-	-	-	.13	-.12	-.09	3.73	.21	0.22	.00	1.66
	Maximum	-	-	-	-	-	-	.18	-.11	.10	4.43	.21	0.22	.00	1.63
LF/HF Ratio		.01	4.15	.17	-	-	-	-	-	**-**	**-**	**-**	**-**	**-**	
	AUC_{AG}	-	-	-	-	-	-	-.15	-.27	**.42**$^{\pm}$	4.27	**.21**	1.82	**.**02	2.06
	AUC_I	**-**	-	-	-	-	-	**-.39***	-.12	**.58****	4.61	**.22**	**4.10***	**.04**	**3.98****
	$Slope_{Max}$[a]	**-**	-	-	-	-	-	-.00	-.10	-.25	4.34	.24	1.13	.04	1.39
	Maximum	**-**	-	-	-	-	-	-.19	**-.38**$^{\pm}$	**.58***	5.00	**.23**	**2.67**$^{\pm}$	**.02**	**2.90***

Note. All analyses controlled for age, sex, BMI *Z*-score, adrenarche, parental education, household income, sleep duration, & wake-time. β_{cort} = standardized beta coefficient for cortisol measure. β_{hrv} = standardized beta coefficient for HRV measure. β_{int} = standardized beta coefficient for interaction term. AUC_{AG} = area under curve relative to ground. AUC_I = area under curve relative to increase. $Slope_{max}$ = diurnal slope anchored to max sample using regression. LF = low frequency. HF = high frequency. Bold values indicate significance: $^{\pm}p<.07$ $^{*}p<.05$ $^{**}p<.01$.

[a]Analyses also controlled for maximum cortisol sample, anchor point for calculation of $Slope_{Max}$.

[b]Change in F test-statistic conservatively based on higher *F* test-statistic of either singular model.

[c]Generalized F test compares the higher *F* test-statistic of either singular model to the inter-relation model.

TRANSITION TO STUDY 2

The goal of Study 1 was to examine whether the *inter-relation* between the autonomic nervous system and HPA axis was a better measure of the relation between stress and the stress response system than either system singularly, among children and adolescents. Extant conceptual theories had previously posited that the inter-relation between the stress systems should be associated with adverse health outcomes; however, no empirical studies testing the interaction of these systems could be found. The aim of Study 1 was to test whether the inter-relation was associated with perceived stress, over and above either system alone. Demonstrating this association was an important first step in this line of inquiry.

In the process of planning Study 1, different approaches to modeling the inter-relation between the stress systems were considered. First, I examined whether the measures of HRV and cortisol mathematically covaried in a meaningful and interpretable way to characterize the underlying systems. Namely, factor analytic methods were conducted with all HRV and cortisol measures; however, this approach was futile given that only two factors emerged: one with the HRV measures and the other with the cortisol measures. In hindsight, only two factors emerged due to the obvious greater covariance within the HRV and cortisol measures. Second, to address this covariance issue, I wondered if factor analytic methods on the interactions between the HRV and cortisol measures would better characterize the pattern of associations across the two systems. An interesting pattern of results emerged (e.g., interactions with AUC_I loaded as a separate factor, interactions with LF and LF/HF ratio loaded as another factor); however, meaningful interpretation of these factors was obfuscated and unclear. Third, another angle using principles from the field of epidemiology was considered by creating quartiles for each HRV and cortisol measure. Chi-square analyses then were used to determine if there were

"groups" based on being in the highest or lowest quartile. These findings were inefficient and analyses drastically decreased sample size ($N = 201$ to $N = 50$). Finally, interaction models were used to represent the inter-relation between the stress systems, which resulted in clear, parsimonious, and interpretable findings.

Overall in Study 1, the inter-relation between the stress systems (i.e., interaction of HRV and cortisol measures) was associated with perceived stress in a community sample of children and adolescents. More specifically, the inter-relation of higher sympathovagal modulation and higher cortisol awakening response was related to higher perceived stress. These significant interactions better predicted perceived stress than the singular association of either stress system alone, albeit the effect sizes were small ($\Delta R^2_{avg} = .03$). Moreover, the significant interactions better predicted stress than simultaneously including both the HRV and cortisol measure in the model (Paired Models, Appendix 1). Yet, there were some unexpected findings. Based on the Polyvagal Theory (Porges, 1995) and Neurovisceral Integration Model (Thayer & Lane, 2000), it was expected that parasympathetic modulation would have also interacted with the HPA axis; however, no significant interactions with HF (i.e., parasympathetic modulation) were found. Also, only the interactions with measures of the cortisol awakening response (AUC_{AG}, AUC_I, maximum sample) were significantly associated with perceived stress. Additional measures of the diurnal cortisol profile (AUC_{TG}, Diurnal Slope, bedtime sample; see Appendix 1) did not significantly interact with HRV measures to predict perceived stress. Despite these unexpected findings, Study 1 provided initial support for considering the patterning and inter-relation between the stress systems.

Considering Study 1 results and the existing literature linking stress to cardiovascular health, I decided to extend the concept of stress response patterning to cardiovascular precursors

during childhood. Cardio-autonomic control and HPA axis activity had been linked independently to cardiovascular precursors in children and adolescents, but prior researchers largely neglected to simultaneously consider these systems. Thus, I became increasingly interested in examining whether the inter-relation would be an improved predictor of cardiovascular precursors.

While Study 1 considered the link with perceived stress, I was also curious about how exposure to stressful life experiences may influence the inter-relation. There is also convincing evidence that the match, or mismatch, between environmental context (e.g., exposure to stressful life experiences) and physiological functioning influences a person's risk for physical health outcomes (Del Giudice et al., 2010; Ellis & Del Giudice, 2014; Schwartz et al., 2003). In other words, the interaction between stressful life experiences with cardio-autonomic and HPA axis activity may contribute to the development of cardiovascular precursors. Previous studies with children and adolescents that considered the influence of stressful life experiences on the relation between the stress response system and cardiovascular precursors could not be identified. Thus, Study 2 also evaluated the moderating role of stressful life experiences on the relation between the stress response system and cardiovascular precursors (referred to as cardiovascular risk factors in Study 2). Of note, the measurement and conceptualization of stress differed from Study 1 to Study 2. Stress in Study 1 was based on a self-report measure of perceived daily hassles over the past 30 days; Study 2 was based on a self-report measure of stressful life experiences over the past 3 months. This change in study design was largely attributable to the data available in the large population-based sample used in Study 2; yet, there were conceptual advantages to this change as well. Given that stressful life experiences were measured over a

longer period of time, Study 2 may better characterize cumulative stress exposure, which is hypothesized to promote the wear and tear of physiological systems (McEwen & Seeman, 1999).

Altogether, Study 2 was designed to address two gaps observed in the literature. First, Study 2 examined whether the inter-relation between cardio-autonomic and HPA axis activity was associated with cardiovascular precursors in children. Second, Study 2 considered whether greater exposure to stressful life experiences moderated this association.

STUDY 2

Stress moderates the association between cardiovascular risk factors and the inter-relation of autonomic and HPA axis activity among youth

Sivan Rotenberg and Jennifer J. McGrath

(Manuscript under review)

Abstract

Objective. Dysregulated autonomic and HPA axis activity have been singularly associated with cardiovascular risk factors in youth. Yet, these associations do not characterize their complex interconnections, and are incongruent with current models emphasizing the multi-system perspective. Current study examined whether the inter-relation between autonomic and HPA axis activity was associated with cardiovascular risk factors, and whether stressful life events moderated this association. **Method.** Youth (N = 575; M_{age} = 10.14 yrs.) participated in the 10th wave of the Quebec Longitudinal Study of Child Development, a population-based birth cohort in Canada. Youth answered questionnaires about stressful life events. Resting blood pressure (SBP, DBP), cholesterol levels (total, LDL, HDL), and waist circumference were measured. Youth wore ambulatory Holter monitors to measure heart rate variability (HRV; SDNN, LF, HF) and collected salivary samples to measure cortisol (AUC_I). **Results.** Interaction of HRV and cortisol was significantly associated with LDL ($\beta_{SDNN*AUCI}$ = -.32; $\beta_{LF*AUCI}$ = -.19; $\beta_{HF*AUCI}$= -.15) and Total cholesterol ($\beta_{LF*AUCI}$= -.16). Associations were amplified among children with greater stressful life events. Singularly, HRV was associated with blood pressure (SBP β_{SDNN}= -.15, β_{LF}= -.17, β_{HF}= -.14; DBP β_{SDNN}= -.20, β_{LF}= -.19, β_{HF}= -.17) and HDL (β_{SDNN}= .08, β_{LF}= .12); cortisol was associated with waist circumference (β_{AUCI}= -.09). **Discussion.** Among children with greater reported stressful life events, inter-relation of autonomic and HPA-axis activity may better explain certain cardiovascular precursors. Findings partly support hypothesis that children are at greatest risk for adverse outcomes when both autonomic and HPA axis systems are activated.

Study 2 Introduction

Stress is a known risk factor for cardiovascular disease (Chrousos, 2009; Richardson et al., 2012). Although the mechanism by which stress "gets under the skin" is not fully elucidated, cardiovascular risk in youth has been associated with the stress response system, including the autonomic nervous system and the hypothalamic-pituitary adrenal (HPA) axis (Prodam et al., 2013; Soriano-Rodríguez et al., 2010; Zhou, Xie, Wang, & Yang, 2012). Studies examining the association between stress and cardiovascular risk factors in youth (children and adolescents, ages 6-17) typically measure *either* the autonomic nervous system or the HPA axis, despite research findings demonstrating that the autonomic nervous system and HPA axis are highly coordinated and physically connected (Reyes, Valentino, Xu, & Van Bockstaele, 2005; Thayer & Sternberg, 2006). To better characterize the link between stress and cardiovascular disease in youth, the present study investigates the association between cardiovascular risk factors and the *inter-relation* of the autonomic nervous system and HPA axis.

When exposed to stress, the autonomic nervous system and the HPA axis respond in a coordinated manner to promote physiological adaptations commonly referred to as the "fight or flight" response. The autonomic nervous system responds within seconds of a stressor through the neural networks of the sympathetic and parasympathetic nervous system. The sympathetic nervous system initiates the "fight or flight" response by releasing adrenaline, increasing heart rate, and influencing other physiological systems (e.g., metabolism; Chrousos, 2009). This response is enabled by the parasympathetic nervous system withdrawing its inhibitory effects on the sympathetic system (Porges, 1995; 2007b). The HPA axis responds minutes following stressor onset, as its activation requires a series of cascading hormonal events that culminates in the release of cortisol. Upon initial release, cortisol augments the activity of the autonomic

nervous system, but ultimately terminates the "fight or flight" response (McEwen & Seeman, 1999; Sapolsky, Romero, & Munck, 2000). In tandem, the autonomic nervous system and HPA axis protect the body from the impact of stress and promote adaptation.

Animal and human studies examining the inter-relation between the autonomic nervous system and HPA axis highlight their physiological inter-connection, and further support their coordination. Lesions to the sympathetic nervous system have been found to attenuate the HPA axis response to stress (Ziegler, Cass, & Herman, 1999), and impair the cortisol awakening response, one phase of the diurnal cortisol profile (Ulrich-Lai, Arnhold, & Engeland, 2006). In a series of studies using animal models, Reyes and colleagues (2005) found that the autonomic nervous system and the HPA axis were reciprocally innervated and part of a feed forward mechanism. Human studies examining autonomic and HPA axis activity support these animal findings. Specifically, studies of adults and youth with measures of heart rate variability (HRV; indices of sympathetic and parasympathetic activity) and salivary cortisol (afternoon levels, cortisol awakening response) indicate there is a moderate inverse relation between the autonomic nervous system and HPA axis (El-Sheikh, Arsiwalla, Hinnant, & Erath, 2011; Stalder, Evans, Hucklebridge, & Clow, 2011; c.f., Oldehinkel et al., 2010). Using cross-correlation analyses, Engert and colleagues (2011) showed that autonomic activity (measured using salivary alpha amylase) precedes cortisol release by 13.5 min. The convergence of animal and human findings provides evidence for the inter-relation between the autonomic nervous system and HPA axis, and suggests that an investigation of this inter-relation may further elucidate the mechanisms underlying the well-known association between stress and cardiovascular disease.

The autonomic nervous system and HPA axis play an important role in regulating a wide range of cardiovascular risk factors, including blood pressure, cholesterol and lipoprotein levels,

and indices of adiposity/obesity (Girod & Brotman, 2004; Guyenet, 2006; Thayer, Yamamoto, & Brosschot, 2010; Ullian, 1999). For instance, lesions to the sympathetic nervous system have been associated with reduced blood pressure (Madden & Sved, 2003); although others have observed no association (Julien et al., 1990). Cortisol administration has been found to augment sympathetic activity resulting in increased blood pressure (Mangos et al., 2000; Pirpiris, Sudhir, Yeung, Jennings, & Whitworth, 1992; Sapolsky et al., 2000). Cortisol administration has also been shown to decrease the rate of lipid metabolism, resulting in greater blood cholesterol and lipoproteins concentrations (Ottosson, Lonnroth, Bjorntorp, & Eden, 2000). Finally, both parasympathetic and sympathetic branches of the autonomic nervous system, as well as cortisol, have been associated with visceral fat accumulation (Kyrou & Tsigos, 2009; Rosmond, 2005; Tchernof & Despres, 2013).

To date, several studies have examined the association between stress markers (i.e., autonomic and HPA axis activity) and cardiovascular risk factors in healthy adults, including cholesterol (Christensen, Toft, Christensen, & Schmidt, 1999; Colhoun, Francis, Rubens, Underwood, & Fuller, 2001; DeSantis, Adam, Mendelsohn, & Doane, 2011; Jaiswal et al., 2012; Lampert et al., 2008; Park, Blumenthal, Lee, & Georgiades, 2011; Thayer & Fischer, 2013; Walker, Soderberg, Lindahl, & Olsson, 2000), central adiposity (Champaneri et al., 2013; Hemingway, 2005; Soares-Miranda et al., 2012), and blood pressure (Schroeder et al., 2003; Hemingway, 2005; Soares-Miranda et al., 2012). Together, these findings suggest that certain cardiovascular risk factors are associated with *both* the autonomic nervous system and HPA axis, while others are associated with *either* autonomic or HPA axis activity. For instance, total cholesterol and low-density lipoprotein (LDL) have been negatively associated with HRV (SDNN, RMSSD, pNN50; Christensen et al., 1999; Jaiswal et al., 2012; Thayer & Fischer, 2013;

c.f. Colhoun et al., 2001), and positively associated with morning cortisol levels (sample collected before noon; Park et al., 2011; c.f. Walker et al., 2000); whereas, high density lipoprotein (HDL) has been positively associated with HRV (SDNN, LF, HF; Hemingway, 2005; Lampert et al., 2008; c.f. Jaiswal et al., 2012), but unassociated with cortisol (AUC_I, morning; DeSantis et al., 2011; Park et al., 2011; Walker et al., 2000). Similar patterns to HDL have been found for resting systolic (SBP) and diastolic pressure (DBP; Hemingway, 2005; Schroeder et al., 2003; Soares-Miranda et al., 2012; Walker et al., 2000; c.f. Jaiswal et al., 2012; Park et al., 2011). Studies examining the association between central adiposity and HRV or cortisol are inconsistent, as HRV (SDNN) and cortisol measures ($awake_0$, AUC_I) have been negatively (Champaneri et al., 2013; Hemingway, 2005) and unassociated (Soares-Miranda et al., 2012) with waist circumference. The role of the inter-relation between autonomic and HPA axis activity has yet to be considered; thus, it remains to be determined whether this inter-relation may better account for the relation between cardiovascular risk factors and stress than their singular associations.

To date, the few studies that have examined the association between cardiovascular risk factors with autonomic and HPA axis activity in healthy youth have yielded inconsistent findings. Soriano-Rodriguez and colleagues (2010) observed that morning serum cortisol was positively related to resting SBP, but was not related to resting DBP, HDL levels, or waist circumference. In contrast, others have found that cortisol measures ($awake_{30}$, AUC_{AG}, morning) were negatively associated with HDL, and unassociated with resting SBP (DuBose & McKune, 2013; Hill, Eisenmann, Holmes, & Heelan, 2010). Only two studies have examined the relation between cardiovascular risk factors and autonomic activity in healthy youth. Both studies found that HRV (SDNN, RMSSD, pNN50, HF) was negatively associated with resting SBP and waist circumference (Farah, Barros, Balagopal, & Ritti-Dias, 2014; Zhou et al., 2012); however, Farah

and colleagues (2014) found that certain indices of HRV (LF, LF/HF) were positively associated with resting SBP. With regards to DBP, measures of HRV have been negatively (SDNN, RMSSD, pNN50, HF), positively (LF, LF/HF), and unassociated with resting DBP (Farah et al., 2014; Zhou et al., 2012). No association between HRV and total cholesterol, LDL, or HDL levels has been found (Zhou et al., 2012). To our knowledge, no studies have investigated the relation between cortisol with total cholesterol or LDL levels in healthy youth. These early findings suggest there is a link between cardiovascular risk factors with autonomic and HPA axis activity early in the life course, yet further research is needed.

Two important gaps in the existing literature remain to be addressed. First, the association between cardiovascular risk factors and the *inter-relation* of the autonomic nervous system and the HPA axis has yet to be examined. Existing theories, such as Bauer et al.'s (2002) Additive Model suggests that individuals with concurrent excessive or inadequate autonomic and HPA axis activity are at the greatest risk for adverse outcomes. Moreover, models such as the Polyvagal Theory (Porges, 1995; 2007a) and the Neurovisceral Integration Model (Thayer & Sternberg, 2006), describe how the autonomic nervous system and the HPA axis may be inter-related, by hypothesizing that parasympathetic activity regulates both the sympathetic and the HPA axis response to stress. Adult findings from studies measuring laboratory stress (Cacioppo et al., 1995; Weber et al., 2010) support these models, as autonomic activity was found to moderate the relationship between stress and the HPA axis.

The second gap in the literature is that the role of stressful life experiences as a moderator of the association between cardiovascular risk factors and the stress response system (diathesis-stress model) has not been examined in youth. The diathesis-stress model proposes that a physiological predisposition (e.g., susceptibility for altered autonomic and HPA axis activity)

will increase the risk of disease if the predisposed individual also experiences high stress (Everson et al., 1997; Kamarck et al., 2005; Vrijkotte, van Doornen, & de Geus, 2000). Adult findings from laboratory-based stress studies support this model (Everson et al., 1997; Kamarck et al., 2005); however, similar studies with youth and/or diurnal measures of autonomic and HPA axis activity have not been conducted. Thus, the question remains whether the autonomic nervous system and the HPA axis are *concomitantly* associated with cardiovascular risk factors in healthy youth, and whether this association is moderated by stressful life experiences.

To address the current gaps in the literature, the aims of the current study are two-fold. First, we examine whether the inter-relation of the autonomic nervous system and the HPA axis better predicts cardiovascular risk factors in youth, compared to their singular associations. Second, given that greater exposure to stressful life experiences exacerbates the functioning of the stress response system, we consider whether stressful life experiences moderate the association between cardiovascular risk factors with autonomic and HPA axis activity. We hypothesize that the interaction between the autonomic nervous system and the HPA axis will be independently associated with cardiovascular risk factors (e.g., total cholesterol, LDL, blood pressure) over and above their singular associations, and that the association between cardiovascular risk factors and the stress response system will be strongest in youth with more stressful experiences. Based on the Polyvagal Theory and previous studies, we hypothesize that low HRV in conjunction with high cortisol will be associated with greater cardiovascular risk.

Study 2 Method

Participants

Participants were from the population-based Quebec Longitudinal Study of Child Development (QLSCD), a birth cohort study conducted by the Québec Institute of Statistics

(Jetté, 2002). The initial cohort was selected from the master birth registry of the Ministère de la Santé et des Services Sociaux using a multistage cluster (by region and municipality), random sampling strategy that was representative of singleton births from 1996 to1998. Families living in Aboriginal territories or remotes areas of Quebec (2.1%), infants born before 24 or after 42 weeks gestation (0.1%), as well as infants whose gestational age was unknown (1.3%) were excluded from the initial cohort. The present study is based on the cross-sectional cardiovascular health screening that was conducted when the children were 10 years of age (2006 to 2008). The ethics review boards of the Institut de la Statistique du Québec, the Centre Hospitalier Universitaire Sainte-Justine, the Louis-Hippolyte Lafontaine Hospital, and the Faculty of Medicine of Université de Montréal, approved original data collection.

Procedure

Children and their parents were invited to participate in the cardiovascular health screening, and provided their informed assent and consent, respectively. A registered nurse arrived at the child's home in the morning and took blood samples as well as anthropometric measures. During the standardized morning visit, children and their parents completed questionnaires and were instructed on how to collect saliva samples at home using the passive-drool technique. Additionally, throughout the standardized visit children wore a continuous ECG ambulatory monitor (8500 Marquette MARS Holter).

Measures

Cortisol. Saliva samples were collected at awakening ($awake_0$) and +30 minutes post-awakening ($awake_{30}$) on two non-consecutive days. Saliva samples were collected using the passive-drool technique. Participants were instructed not to eat, drink, or brush their teeth before samples, and to record the date and time of each sample in a daily log. To improve compliance,

telephone reminders were provided the day before sampling, and saliva collection was completed under parental supervision. Ninety-two percent of participants collected the $awake_0$ sample within 15 minutes of awakening, and 98.5% of participants collected the $awake_{30}$ sample within 45 minutes of the $awake_0$ sample. These ranges are within methodological guidelines (Adam & Kumari, 2009; Dockray, Bhattacharyya, Molly & Steptoe, 2008). When collection was complete, all saliva samples were placed in a pre-paid envelope and mailed back to the laboratory. Cortisol levels are robust to environmental conditions associated with the shipping process (Clements & Parker, 1998). Cortisol levels were determined in duplicate using a sensitive enzyme immunoassay kit (Salimetrics, State College, PA, USA). The intra-assay coefficient of variation was 2.14%. Untransformed cortisol values were used to derive: 1) area under the awakening response relative to ground (AUC_{AG}), 2) dynamic increase of the awakening response (AUC_I), and, 3) maximum sample (i.e., highest morning cortisol value; for formula see Rotenberg, McGrath, Roy-Gagnon, & Tu, 2012). The two-day average of each cortisol measure was used in the analyses. All cortisol measures were normally distributed except for the maximum sample, which was square root transformed to address non-normality.

Heart Rate Variability. Continuous electrocardiogram (ECG) recordings were collected with an 8500 Marquette MARS Holter monitor (GE Marquette Medical Systems, Milwaukee, Wisconsin, USA) while the children were seated quietly for the 2.5 hour standardized visit. ECG data were sampled at a rate of 128 Hz, combined with QRS template matching based on 1024 samples/300 sec. The ECG signal was imported into the MARS® Holter Analysis Workstation (GE Marquette Medical Systems, Milwaukee, Wisconsin, USA) where data were formatted for editing and ECG interpretation and analysis. ECG data were processed according to Task Force Guidelines (1996). Artifact correction was conducted based on a 20% change from the previous

signal as a criterion (Kleiger, Miller, Bigger, & Moss, 1987), and by visual inspection by a qualified trained technician. Using 10 min epochs, ECG data were linearly detrended, mean-centered, and tapered using a Hanning window.

Time- and frequency-domain measures of HRV were derived for each epoch and averaged across the recording period. The time domain measure was the standard deviation of the beat-to-beat interval (SDNN). Frequency domain measures were based on power spectral analysis and were derived using the Fast Fourier Transformation. Frequency domain measures were low frequency (LF; 0.04 - 0.15 Hz), high frequency (HF; 0.15 – 0.40 Hz), and the LF/HF ratio. Results related to RMSSD and pNN50 were similar to HF, and were not shown due to parsimony.

Metabolic Factors. After fasting overnight, children's blood samples were collected by a registered nurse for measurement of Total cholesterol, LDL, and HDL. Immediately after collection, blood samples were placed on ice, centrifuged for 30 minutes, aliquoted, and frozen on dry ice. Lipid concentrations were determined with a Synchron LX20 with Beckman Instruments reagents. LDL was calculated based on the Friedewald equation (Friedewald, Levy, & Fredrickson, 1972).

Blood Pressure. After a 30 min acclimatization period, resting SBP and DBP was measured on the right arm with the appropriate sized cuff (Webber et al., 1995) using an oscillometric instrument (BpTRU, model BPM-100, VSM MedTech Ltd, Vancouver, Canada). While seated, five blood pressure readings were taken at 1 min intervals. The mean of all readings was used in analyses.

Stressful Life Experiences. Stressful life experiences was measured using a list of events typically experienced by children (e.g., school problems, parental divorce; Deschesnes,

1998). Children rated the extent to which they were worried or stressed about 14 items over the past 3 months on a 4-point scale (*not at all* to *a whole lot*). This scale has good internal consistency (α = .83–.89, Deschesnes, 1998; α = .67, present study). Children were separated into high vs. low stress groups based on a median split (n = 319 vs. 256, respectively). Children in the high stress group endorsed significantly greater problems at school (t (555) = -12.65, p < .001), feeling more isolated (t (475) = -8.62, p < .001), and greater interpersonal difficulties with their parents (mother, t (554) = -8.74, p < .001; father, t (550) = -8.92, p < .001) compared to children in the low stress group.

Demographic Factors. Income sufficiency was determined by the low-income cut-off established by Statistics Canada (2008). Statistic Canada's low-income cut-off represents a threshold below which a family will likely spend 20% more of their income than the average family on necessities (food, shelter, clothing), after adjusting for household income, size, and geographic region (e.g., cut-point for family of 4 in rural area $22, 206 vs. $33,946 for similar family in large city). Families were classified as having 'sufficient income' if household income was above the low-income cut-off; 'insufficient income' if household income was between 60% and 90% of the low-income cut-off; and 'very insufficient income' if household income was <60% of the low-income cut-off (Statistics Canada, 2008). Parental education was based on the highest level of education of both parents.

Pubertal Stage. Pubertal stage was based on adrenarche (pubic hair growth) and was assessed using sex-specific illustrations corresponding to Tanner stages I to V (Growing and Changing Questionnaire; Golding, Pembray, & Jones, 2001). Pubertal illustrations have demonstrated good reliability and validity (r = .77-.91; Morris & Udry, 1980; Netherton, 2004).

Sleep Duration. The quantity of sleep on the night preceding saliva sampling and wake-time was self-reported on the child's daily logs. The two-day average of sleep duration and wake-time was used in the analyses.

Statistical Analyses

Prior to conducting the analyses, missing cortisol values due to single saliva samples that were not returned (1.2%), and missing blood samples (32%) were imputed using multiple imputation that was informed by data from the larger QLSCD study (e.g., age, sex, day of sampling, puberty, HRV measures, etc.; McKnight, McKnight, Sidani & Figueredo, 2007). Of the 582 participants, one participant was excluded since cortisol data was an extreme outlier (>6 SD). Analyses were performed with both the original and imputed datasets; findings did not vary. Therefore, results based on the imputed dataset are presented for parsimony.

To examine the first hypothesis and determine whether the inter-relation of HRV and cortisol measures was uniquely associated with cardiovascular risk factors (total cholesterol, LDL, HDL, SBP, DBP, waist circumference), we compared the associations between cardiovascular risk factors and the singular models (HRV *or* cortisol measures) to the associations between cardiovascular risk factors and the interaction models (main effects and the interaction of HRV and cortisol). We hypothesized that the interaction between HRV and cortisol would be independently associated with cardiovascular risk factors, such that HRV would moderate the association between cortisol and cardiovascular risk factors. Simple slopes analyses (Aiken & West, 1991) were conducted to examine the moderating role of HRV in all significant interactions. To examine the second hypothesis and determine whether the relation between HRV, cortisol, and cardiovascular risk factors was moderated by stressful life experiences, stratified analyses were conducted. All models adjusted for age, sex, pubertal stage,

BMI *Z* score, income level, parental education, sleep duration, and wake time. All analyses were conducted using IBM SPSS Statistics software (Version 20).

Study 2 Results

Participant demographics ($N = 581$) are presented in Table 4. Overall the majority of the children were female (55.1%), in early stages of adrenarche (88.2%), and of normal body weight (5-85th BMI percentile: 72.5%). On average, children's blood pressure (M_{SBP} = 95.57 mmHg, SD = 9.22; M_{DBP} = 61.43 mmHg, SD = 8.81), cholesterol level (M_{TC} = 4.30 mmol/L, SD = 0.65), and lipoprotein levels (M_{LDL} = 2.51 mmol/L, SD = 0.59; M_{HDL} = 1.45 mmol/L, SD = 0.26) were within normal limits. Parents were predominately university educated ($M_{education}$ = 15.95 yrs., SD = 4.11) and had sufficient income (92.1%) based on the low-income threshold cut-point determined by Statistics Canada (2008). Correlations between HRV and cortisol measures are presented in Table 5.

Cardiovascular Risk Factors and Inter-Relation of Autonomic and HPA Axis Activity

To examine whether the interaction between HRV and cortisol measures was independently associated with cardiovascular risk factors, we compared the test statistics (ΔF and ΔR^2) and beta coefficients of the singular and interaction models to identify the best fitting regression model. Singular models are presented in Table 6. Higher Total cholesterol and LDL as well as smaller waist circumference were associated with greater AUC_I, but unassociated with AUC_{AG}, maximum cortisol, and HRV measures. Lower HDL was related to lower SDNN and LF, but unrelated to the remaining HRV indices or cortisol measures. Elevated SBP was related to low HRV (SDNN, LF, HF), but unassociated with LF/HF and cortisol measures. Elevated DBP

was associated with low HRV (SDNN, LF, HF; except for LF/HF). DBP was also inversely related to AUC_I.

Relative to the singular models, Total cholesterol was best associated with the interaction of AUC_I with LF (ΔF (1,569) = 4.11, p = .04; Table 7, top panel). LDL cholesterol was best associated with the interaction of AUC_I with SDNN (ΔF (1,568) = 4.67, p = .03), LF (ΔF (1,569) = 5.65, p = .02), and HF (ΔF (1,569) = 4.43, p = .04). No other interactions were statistically significant. Simple slope analyses revealed that HRV measures moderated the association between cortisol with total cholesterol and LDL levels. Among children with low LF, greater AUC_I was associated with higher total cholesterol (β = .18, p = .001), and higher LDL levels (β = .22, p < .001); among children with high LF there was no relation between AUC_I and total cholesterol or LDL levels (β = .01, p = .85 and β = .01, p = .81, respectively). Relatedly, greater AUC_I was associated with higher LDL levels only for participants with low SDNN or HF (β = .20, p < .001 and β = .20, p < .001, respectively). There was no association between AUC_I and LDL among participants with a high SDNN or HF (β = .02, p = .74 and β = .02, p = .73, respectively). Total cholesterol was best associated with the interaction of AUC_I with LF and LDL was best associated with the interaction of AUC_I with SDNN, LF or HF. Diastolic blood pressure was best associated with the main effects of HRV and AUC_I; HDL and SBP were best associated with HRV measures singularly; and waist circumference was best associated with AUC_I.

Stress as Moderator of the Relation Between Cardiovascular Risk Factors with Autonomic and HPA Axis Activity

Stress moderated the association between Total cholesterol, LDL, HDL, and waist circumference, with HRV and cortisol measures (Table 7, middle and bottom panel). Among

children in the high stress group with low LF, greater AUC_I was associated with higher total cholesterol ($\beta = .29$, $p < .001$) and higher LDL (Figure 2). Additionally, lower AUC_I was singularly associated with a larger waist circumference among children with high stress. For children in the low stress group, high HDL was related to high HRV. There was no association between total cholesterol, LDL and waist circumference, with HRV and cortisol measures in the low stress group; or between HDL and HRV in the high stress group. Stress did not moderate the association between SBP and DBP, and HRV measures. Among children in the high stress group, the interaction of LF with AUC_I was associated with total cholesterol and LDL; AUC_I was singularly associated with waist circumference. Among children in the low stress group, HRV was singularly associated HDL.

Study 2 Discussion

The autonomic nervous system and HPA axis have been singularly associated with cardiovascular disease risk factors among youth (Soriano-Rodríguez et al., 2010; Zhou et al., 2012). Yet, studies investigating the relation between the autonomic nervous system and HPA axis indicate that these systems are physiologically and physically inter-connected (Reyes et al., 2005; Thayer, Hall, Sollers, & Fischer, 2006). Additionally, exposure to stressful life experiences promotes dysregulation of autonomic and HPA axis activity (McEwen & Seeman, 1999). Thus, the association between cardio-autonomic and HPA axis activity with cardiovascular risk factors may be moderated by stress.

The results of this study provide limited support for our first hypothesis that the interaction of the autonomic nervous system and HPA axis would be associated with cardiovascular risk factors above and beyond the singular associations. The interaction of the autonomic nervous system and HPA axis was better associated with Total cholesterol and LDL

than their singular associations; however, the singular association of either HRV or cortisol was best associated with HDL, blood pressure, and central adiposity. Blood pressure and HDL were associated with autonomic activity, whereas central adiposity was associated with HPA axis activity. Consistent with these findings, adult's cholesterol (Total and LDL) has been previously associated with both autonomic and HPA axis activity (Jaiswal et al., 2012; Park et al., 2011; Thayer & Fischer, 2013; c.f., Colhoun et al., 2001; Walker et al., 2000); while HDL cholesterol and blood pressure have been associated with autonomic activity (Hemingway, 2005; Lampert et al., 2008; Schroeder et al., 2003; Soares-Miranda et al., 2012, c.f., Jaiswal et al., 2012), but not HPA axis activity (DeSantis et al., 2011; Park et al., 2011; Walker et al., 2000). Together, these data suggest that the relation between stress and cardiovascular risk is evident during childhood, and that certain cardiovascular risk factors are better associated with the inter-relation of the autonomic nervous system and HPA axis, while others are not. The latter observation may be accounted for by the differing roles of the autonomic nervous system and HPA axis. Specifically, blood pressure and abdominal fat accumulation are predominately regulated by autonomic and HPA axis activity, respectively (Bjorntorp, Holm, Rosmond, & Folkow, 2000; Guyenet, 2006), whereas cholesterol and lipid metabolism is influenced by both autonomic *and* HPA axis inputs (Kalsbeek, Bruinstroop, Klieverik, La Fleur, & Fliers, 2010; Ottosson et al., 2000). Adult studies that concurrently measured autonomic and HPA axis activity could not be identified. While the current study highlights the role of the inter-relation between cardio-autonomic and HPA axis activity during childhood, it would also be pertinent for researchers to consider this inter-relation during adulthood.

The current results are partially consistent with previous studies with youth. Aligned with current study findings, youth's autonomic activity has been previously negatively associated with

SBP (Zhou et al., 2012); HPA axis activity has been unassociated with HDL cholesterol (Soriano-Rodríguez et al., 2010) and SBP (Hill et al., 2010). In contrast to our findings, youth's autonomic activity has been unassociated with cholesterol levels (Total, LDL, HDL) and DBP (Zhou et al., 2012). HPA axis activity has been negatively associated with HDL cholesterol (DuBose & McKune, 2013), positively associated with SBP (Soriano-Rodríguez et al., 2010), and unassociated with DBP (Soriano-Rodríguez et al., 2010). Previous research that considered the association between Total cholesterol or LDL and cortisol could not be found. These inconsistencies may be accounted for by restrictive samples (girls only; Dubose & McKune, 2013), the age and pubertal status of participants (prepubescent children 6-8 years of age; Dubose & McKune, 2013; Hill et al., 2010; Soriano-Rodriguez et al., 2010), or methodological differences (single sample of cortisol; Hill et al., 2010; Soriano-Rodriguez et al., 2010). Despite the paucity of studies in youth, overall these findings, together with the previous research, suggest that the relation between cardiovascular risk factors with autonomic and HPA axis activity is present early in the life course.

The second hypothesis that the association between cardiovascular risk factors with the autonomic nervous system and/or the HPA axis would be more robust among youth with greater stressful life experiences was supported. Specifically, the above-mentioned relation between dysregulated autonomic and HPA axis activity with greater cholesterol (total, LDL), and with central adiposity was observed among youth with high stress. Moreover, greater HRV was associated with greater HDL levels among youth with low stressful life experiences, which suggests that youth with high stress and dysregulated autonomic functioning are at risk for dyslipidemia and may not benefit from the protective effects of HDL. These results are consistent with previous adult laboratory stress studies, which found that markers of

atherosclerosis (e.g., carotid artery plaques, intima-media thickness) were associated with the interaction between stress reactivity and individuals' perceptions of how demanding their daily life is (Everson et al., 1997; Kamarck et al., 2005). Thus, the current study supports the Diathesis-Stress Model of disease, and suggests that stressed youth with dysregulated stress response systems are at the greatest risk of developing risk factors for cardiovascular disease.

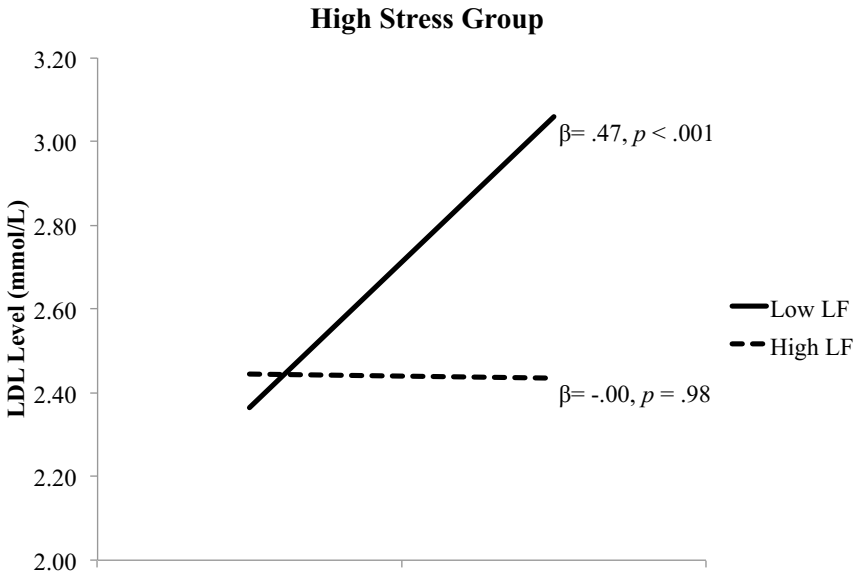
The observation that greater HPA axis activity was associated with higher Total cholesterol and higher LDL levels among youth with dysregulated autonomic activity is consistent with Bauer et al.'s (2000) Additive Model, which posits that children are at the greatest risk for adverse outcomes when both autonomic and HPA axis systems are activated. Additionally the observation that autonomic activity moderates the relation between cardiovascular risk factors and HPA axis activity is consistent with existing models in stress physiology such as the Polyvagal Theory (Porges, 1995; 2007a) and the Neurovisceral Integration Model (Thayer & Sternberg, 2006), which highlight the importance of parasympathetic activity in regulating the sympathetic and the HPA axis stress response. In accordance with these theories, LF HRV emerged as a predominant moderator. However also based on these theories, we anticipated that other parasympathetic measures (i.e., HF HRV) would have emerged as significant moderators as well.

This study is not without limitations. First, examining the interaction between autonomic and HPA axis activity is one approach to characterize the complex physiological interconnections between these two systems. However, other statistical approaches have been proposed (e.g., salivary alpha amylase over cortisol ratio, Ali & Pruessner, 2012). Second, the present study is cross-sectional; therefore, we were unable to address causality. Third, the measures of autonomic and HPA axis activity were derived from short-term assessments during

a standardized clinic visit. Although the methodology used in the current study was ecologically valid and consistent with existent guidelines (Rotenberg et al., 2012; Task Force, 1996), the inclusion of a laboratory stressors would be of interest in order to examine how cardiovascular risk is associated with the stress reactivity. Fourth, the generalizability of the findings is limited as the sample included youth aged 10 years; although, the participants were from a population-based sample of healthy youth. Future research should employ longitudinal designs, consider alternative approaches to characterizing the inter-connection between the autonomic and the HPA axis systems, and include a laboratory stressor to examine the association between cardiovascular risk factors with autonomic and HPA axis activity across childhood and adolescence.

In summary, the current study indicates that the inter-relation between the cardio-autonomic nervous system and the HPA axis is better associated with certain risk factors for cardiovascular disease, and that dysregulation of the stress response system *in combination* with high stress is associated with the greatest cardiovascular risk. These findings suggest that beyond the singular effects of autonomic or HPA axis activity, it is also important to consider the coordination between these systems in the pathophysiology of cardiovascular disease. Future work considering the dynamic nature of both systems will be vital as understanding the patterning of the stress response system may give more insight into how stress "gets under the skin" to promote the development of cardiovascular disease.

A.



B.

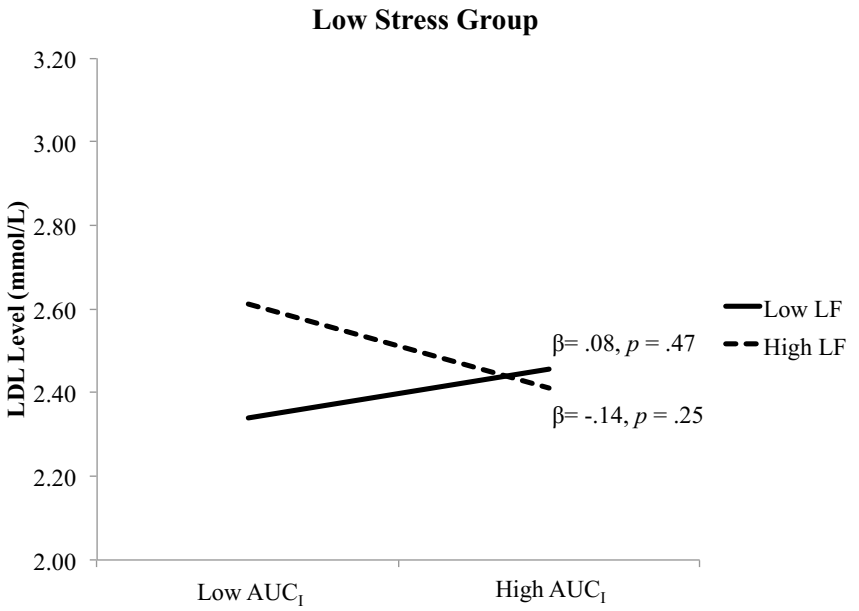


Figure 2. Higher LDL levels associated with greater AUC_I only among youth with low HRV and high stress. A. Effect of low and high LF and AUC_I (± 1SD from the mean) on LDL levels in high stress group. B. Effect of low and high LF and AUC_I (± 1SD from the mean) on LDL levels in low stress group. Data were dichotomized for interpretation purposes; continuous data were retained in analyses.

Table 4

Descriptive Statistics for Demographics, Risk Factors, and Cortisol and HRV Measures

	M (n)	*SD (%)*
	Demographics	
Age	10.14	.25
Female	(320)	(55.1%)
Adrenarche		
Stage 1	(244)	(42.0%)
Stage 2	(268)	(46.2%)
Stage 3	(68)	(11.7%)
Stage 4	(2)	(0.3%)
BMI *z*-score		
Normal weight	(421)	(72.5%)
Overweight	(68)	(11.7%)
Obese	(43)	(7.4%)
Parental Education (0- 20 years)	15.95	4.11
Income[a]		
Sufficient Income	(535)	(92.1%)
Insufficient Income	(30)	(5.2%)
Very Insufficient Income	(16)	(2.8%)
Stress Level (0-25)	4.01	3.97
	Cardiovascular Risk Factors	
Systolic Blood Pressure (mmHg)	95.57	9.22
Diastolic Blood Pressure (mmHg)	61.43	8.81
Total Cholesterol (mmol/L)	4.30	0.65
Low Density Lipoprotein (mmol/L)	2.51	0.59
High Density Lipoprotein (mmol/L)	1.45	0.26
Waist Circumference (cm)	63.59	9.13
	Cortisol Measures	
AUC_{AG} (nmol/L)	4.06	1.83
AUC_I (nmol/L)	0.42	0.92
Maximum[b] (nmol/L)	3.05	0.67
	HRV Measures	
SDNN (ms)	86.99	24.99
LF (ms^2)	1536.51	1032.18
HF (ms^2)	821.65	666.15
LF/HF Ratio	2.20	0.85

Note. AUC_{AG}, area under curve relative to ground for awakening response; AUC_I, area under curve relative to increase; SDNN, standard deviation of the beat-to-beat interval; LF, low frequency; HF, high frequency. (n = 581).

[a]Categories determined by Statistics Canada (2008)

[b]Square-root transformed to address non-normality

Table 5
Correlations between HRV and Cortisol measures

	1	2	3	4	5	6
HRV Measures						
1. LF	-					
2. HF	**.89****	-				
3. LF/HF Ratio	**-.18****	**-.47****	-			
Cortisol Measures						
4. AUC_{AG}	.02	.00	.00	-		
5. AUC_{I}	.08	.06	.01	**.18****	-	
6. Maximum[a]	.02	.02	-.01	**.90****	**.33****	**-**

Note. Zero-order correlations are presented.
[a]Square-root transformed to address non-normality
$^{*}p < .05$, $^{**}p < .01$

Table 6

Univariate analyses predicting cardiovascular risk factors with cortisol and HRV measures

	Total Cholesterol		LDL		HDL		DBP		SBP		Waist Circumference	
Measures	*β*	R^2	*β*	R^2	*β*	R^2	*β*	R^2	*β*	R^2	*β*	R^2
HRV												
SDNN	-.07	.03	**-.10***	.04	**.08***	.17	**-.21****	.09	**-.15****	.14	-.03	.04
LF	-.06	.03	**-.10***	.04	**.12****	.18	**-.20****	.08	**-.17****	.13	-.03	.03
HF	-.05	.03	-.08	.03	.07	.17	**-.18****	.07	**-.14****	.12	.01	.03
LF/HF	-.02	.03	-.05	.03	.05	.17	.04	.04	.04	.10	-.02	.03
Cortisol												
AUC_{AG}	.03	.03	.04	.03	.00	.16	-.03	.04	.01	.10	-.06	.04
AUC_I	**.10***	.04	**.12****	.04	-.01	.16	**-.10***	.05	-.06	.10	**-.09***	.04
Maximum	.04	.03	.06	.03	-.03	.16	-.05	.04	-.00	.10	-.06	.04

Note. All analyses controlled for: age, sex, pubertal stage, parental education, household income, sleep duration and wake time; BMI *Z* score was a covariate in all analyses except for waist circumference; HRV recording duration was included as a covariate in analyses with SDNN.

$^{*}p < .05$, $^{**}p < .01$

Table 7.
Best fitting model for all participants, high stress group, and low stress group

	Total Cholesterol				LDL				HDL		DBP			SBP		Waist Circumference	
Best fit	Interaction Model				Interaction Model				Univariate		Paired Model			Univariate		Univariate	
	β_{AUCi}	β_{hrv}	β_{int}	ΔR^{2a}	β_{AUCi}	β_{hrv}	β_{int}	ΔR^{2a}	β_{hrv}	ΔR^{2a}	β_{AUCi}	β_{hrv}	ΔR^{2a}	β_{hrv}	ΔR^{2a}	β_{AUCi}	ΔR^{2a}
All participants (n = 581)																	
AUC_I																**-.09***	.01
SDNN	**.33***	-.03	-.24	.00	**.42****	-.05	**-.32***	.01	**.08***	.01	**-.09***	**-.20****	.05	**-.15****	.02		
LF	**.23****	-.01	**-.16***	.01	**.27****	-.04	**-.19***	.01	**.12****	.02	**-.09***	**-.19****	.05	**-.17****	.03		
HF	**.18****	-.02	-.11	.00	**.23****	-.03	**-.15***	.01	.07	.00	**-.09***	**-.17****	.04	**-.14****	.02		
LF/HF	.14	-.02	-.04	.00	.13	-.04	-.01	.00	.05	.00	**-.10***	.04	.01	.04	.00		
High stress group (n = 319)																	
AUC_I																**-.17****	.03
SDNN	**.41***	-.12	-.25	.05	**.45***	**-.13**$^{\pm}$	-.27	.07	.03	.00	-.05	**-.17****	.03	**-.11***	.01		
LF	**.35****	-.05	**-.23***	.06	**.38****	-.08	**-.24***	.07	.08	.01	-.05	**-.15****	.03	**-.11***	.01		
HF	**.27****	-.09	-.13	.05	**.32****	-.08	-.17	.06	.02	.00	-.05	**-.15****	.02	**-.10**$^{\pm}$	.01		
LF/HF	.24	.04	-.08	.03	.18	-.01	.01	.03	.09	.01	-.06	.06	.01	**.11***	.01		
Low stress group (n = 256)																	
AUC_I																-.02	.00
SDNN	.17	.06	-.14	.00	.33	.03	-.31	.01	**.15***	.02	**-.14***	**-.24****	.07	**-.21****	.04		
LF	.11	.09	-.11	.00	.15	.04	-.17	.01	**.18****	.03	**-.12***	**-.25****	.09	**-.25****	.06		
HF	.08	.09	-.07	.01	.11	.06	-.12	.01	**.14***	.02	**-.13***	**-.20****	.06	**-.19****	.04		
LF/HF	.11	-.07	-.08	.01	.14	-.07	-.12	.01	-.01	.00	**-.16***	-.01	.02	-.05	.00		

Note. All analyses controlled for: age, sex, pubertal stage, parental education, household income, sleep duration and wake time; BMI *Z* score was a covariate in all analyses except for waist circumference; HRV recording duration was included as a covariate in analyses with SDNN.

[a]Change in R^2 from covariate model

$^{\pm}p < .07$, $^{*}p < .05$, $^{**}p < .01$

TRANSITION TO STUDY 3

The objectives of Study 2 were to examine whether the inter-relation between the stress systems was associated with cardiovascular precursors in a population-based cohort of children, and to consider whether this association was moderated by stress. This study focused on three precursors (blood pressure, cholesterol, central adiposity), which previous research had convincingly demonstrated to be predictive of later development of cardiovascular disease. While Study 1 established that the inter-relation was better associated with stress than either stress system singularly, Study 2 extended this work to consider its association with cardiovascular precursors and the moderating role of stress exposure. It was thought that the inter-relation of the stress systems would be more robustly associated with cardiovascular precursors among children with more stressful life experiences.

Consistent with Study 1, the inter-relation of the stress systems provided greater insight about the relation between stress and children's health, compared to their singular considerations. Specifically, the patterning of lower parasympathetic modulation and higher cortisol awakening response was associated with greater Total and LDL cholesterol levels. Unexpectedly, the inter-relation was not related to blood pressure or central adiposity. Rather, lower parasympathetic modulation was singularly associated with higher systolic and diastolic blood pressure; lower cortisol awakening response was singularly associated with larger waist circumference. Initially, the present results raised the question whether the findings related to Total and LDL cholesterol were spurious. However, given that the results emerged across two distinct measures of cardio-autonomic control (LF, HF), and the overall pattern of results was consistent with Study 1, an alternative explanation may account for the unexpected findings. Compared to the prevalence of cardiovascular precursors reported in other studies, Study 2 sample demographics suggest the

participants may not have accumulated sufficient physiological wear and tear. For example, Study 2 participants had lower resting systolic blood pressure than children in a study where higher morning cortisol was linked to higher systolic blood pressure (SBP: 95.57 vs. 101 mmHg, respectively; Soriano-Rodriquez et al., 2010). As another example, Study 2 participants had a smaller waist circumference compared to children in a previous study linking lower HRV to greater central adiposity (waist circumference: 63.6 vs. 76.6 cm, respectively; Farah et al., 2014). These noted demographic differences across studies raised the question of whether the levels of cardiovascular precursors were too low to observe an association with the inter-relation of cardio-autonomic and HPA axis activity. This led to the decision to examine the relation in a targeted sample of children at greater risk for cardiovascular pathology.

Overall, the patterning of results was consistent across Study 1 and Study 2. Namely, children were at greater risk of adverse outcomes (higher perceived stress, higher Total and LDL cholesterol) when lower cardio-autonomic control interacted with higher cortisol awakening response. However, there were nuanced differences between the specific inter-relations across the two studies. In Study 1, the inter-relation of *higher sympathovagal modulation* with higher cortisol awakening response was related to higher perceived stress. In Study 2, the inter-relation of *lower parasympathetic modulation* with higher cortisol awakening response was related to higher Total and LDL cholesterol. Sympathovagal modulation and parasympathetic modulation are closely related but distinct constructs that independently represent the regulatory capacity of the parasympathetic nervous system on the stress response system. Therefore, the results of Study 1 and Study 2 emphasized the importance of considering parasympathetic modulation when relating HPA axis activity to adverse outcomes.

Finally, the Study 2 hypothesis that the relation between cardiovascular precursors and the stress response system would be more robust among children with greater stressful life experiences was supported. Specifically, the inter-relation between the stress systems was related to Total and LDL cholesterol among children with high stress. Similarly, the singular association between the cortisol awakening response and waist circumference was observed among children with high stress. These findings are consistent with the Diathesis-Stress Model and suggest that the association of the inter-relation and cardiovascular precursors should be considered in the context of exposure to stress. In Study 1, the inter-relation was associated to higher perceived stress, which is a known risk factor for cardiovascular disease (Richardson et al., 2012). Study 2 extended these results to stress exposure over a longer interval, to indicate that children exposed to more stressful life events, who have dysregulated stress response system patterning, have an increased cardiovascular risk. Altogether, Study 1 and Study 2 findings suggested a pattern of lower cardio-autonomic control with higher cortisol awakening response may be related to children's cardiovascular precursors in the context of greater stressful life experiences.

Given the aforementioned demographic differences regarding the low prevalence of cardiovascular precursors in the population-based sample, I decided to examine the inter-relation in a targeted sample of children at greater risk for cardiovascular pathology. The goal of Study 3 was to replicate and extend Study 2 by examining whether the inter-relation of cardio-autonomic and HPA-axis activity was associated with cardiovascular precursors, and whether exposure to stressful life experiences influenced this association, in a sample of children at risk for obesity. In Study 3, children taking part in the larger QUALITY Cohort were recruited based on their parents' weight status, not their own, thereby affording a unique opportunity to examine these associations in a targeted, vulnerable sample.

STUDY 3

Exposure to stress moderates the association between the inter-relation of heart rate variability and cortisol with cardiometabolic markers in children at-risk for obesity

Sivan Rotenberg and Jennifer J. McGrath

(Manuscript under review)

Abstract

Despite current models and physiological findings regarding the functional inter-relation between the autonomic nervous system and HPA axis, the majority of child studies relating cardiometabolic markers to the stress response system consider either system in isolation. Additionally, although it is well established that stressful life events exacerbates the functioning of the stress response system, few studies consider the impact of stress on the association between the stress response system and health. The current study addressed these gaps of knowledge, and examined whether the association between the inter-relation of the autonomic nervous system and HPA axis and cardiometabolic markers was moderated by exposure to stressful life events in children at-risk for obesity. **Method.** Participants included 534 children (M_{age} = 9.12 yrs.) from the QUebec Adipose and Lifestyle InvesTigation in Youth (QUALITY) Cohort, a longitudinal study of children at-risk for the development of obesity. Children wore ambulatory Holter monitors to derive heart rate variability (LF, HF, LF/HF ratio) and collected saliva samples to measure cortisol (AUC_{AG}, AUC_{I}, AUC_{TG}, maximum sample). Children answered questionnaires about stressful life events. Resting blood pressure (SBP, DBP), cholesterol levels (Total, LDL, HDL), and waist circumference were measured. **Results.** Three-way interaction of HRV, cortisol, and stress was significantly associated with Total cholesterol, LDL, HDL, and waist circumference. Among children with high stress, simple slopes (*ss*) indicated that inter-relation of HF and AUC_{I} was associated with higher Total (β_{ss} = .15;), HDL cholesterol (β_{ss} = .17) and larger waist circumference (β_{ss} = -.19). HRV was associated with blood pressure (SBP β_{LF}= -.08, β_{HF}= -.08, $\beta_{LF/HF}$= .09; DBP β_{LF}= -.18, β_{HF}= -.17), irrespective of cortisol levels or stress. **Discussion.** The inter-relation between the autonomic nervous system and HPA axis was associated with higher cardiometabolic markers among children with greater

stressful life events. Findings support hypothesis that considering the inter-relation of the stress systems, and children's exposure to stressful life events, provides greater insight into the relation between stress and health.

Study 3 Introduction

Childhood obesity is a major risk factor for the development of cardiometabolic diseases in adulthood (Nadeau, Maahs, Daniels, & Eckel, 2011). Overweight and obese children have higher prevalence rates of markers for cardiometabolic disease (e.g., high cholesterol, high blood pressure) than normal-weight children (Lambert et al., 2008; Maximova, Kuhle, Davidson, Fung, & Veugelers, 2013). Cardiometabolic markers track into adulthood and increase risk for morbidity and mortality (Juonala et al., 2011; Reilly & Kelly, 2010; c.f. Lloyd, Langley-Evans, & McMullen, 2010). Greater exposure to stressful life events is one factor that has been implicated in the etiology of cardiometabolic markers in children, through its dysregulation of the stress response system (Anagnostis, Athyros, Tziomalos, Karagiannis, & Mikhailidis, 2009; Foss & Dyrstad, 2011; Holmes, Ekkekakis, & Eisenmann, 2009). Specifically, altered functioning of the autonomic nervous system and hypothalamic pituitary adrenal (HPA) axis has been observed among obese compared to normal-weight children (Kaufman, Kaiser, Steinberger, Kelly, & Dengel, 2012; Kjölhede, Gustafsson, Gustafsson, & Nelson, 2013; Nagai, Matsumoto, Kita, & Moritani, 2003; Ruttle et al., 2013), and among obese children with high versus average blood pressure (Gutin, Barbeau, Litaker, Ferguson, & Owens, 2000; Guzzetti, Pilia, Ibba, & Loche, 2014; Weigensberg, Toledo-Corral, & Goran, 2008). One central limitation of these studies is that *either* autonomic *or* HPA axis activity is measured, despite physiological evidence supporting their functional inter-relation (Reyes, Valentino, Xu, & Van Bockstaele, 2005; Reyes, Valentino, & Van Bockstaele, 2008; Ziegler, Cass, & Herman, 1999). Moreover, certain cardiometabolic markers (e.g., total cholesterol, low density lipoprotein) in healthy children seem to be better associated with the inter-relation of the autonomic nervous system and HPA axis (Rotenberg et al., 2015). To better characterize the role of the stress response system in the

pathophysiology of cardiometabolic disease, the inter-relation between the autonomic nervous system and HPA axis must therefore be considered.

Cardiometabolic Markers: Cholesterol, Blood Pressure, and Central Adiposity

Animal and human studies show the importance of *both* the autonomic nervous system and HPA axis in the regulation of cardiometabolic markers, including cholesterol, blood pressure, and central adiposity (Björntorp, 2001; Björntorp & Rosmond, 2000; Chrousos, 2000; Haqq et al., 2012; Thayer, Yamamoto, & Brosschot, 2010).

Cholesterol. The autonomic nervous system and HPA axis play a predominate role in cholesterol homeostasis. Lesions to the sympathetic and parasympathetic branches of the autonomic nervous system decrease and increase total cholesterol and low-density lipoprotein (LDL) levels, respectively (Puschel, 2004; Shanygina, Fomina, Parfenova, & Kalashinikova, 1981). Cortisol, the end product of the HPA axis, is implicated in the regulation of cholesterol levels by altering the rate of lipid metabolism (Ottosson, Lonnroth, Bjorntorp, & Eden, 1999). This pattern of associations is largely supported in studies with children and adults. Heart rate variability (HRV), a measure of cardio-autonomic modulation, has been both negatively (Christensen, Toft, Christensen, Schmidt, 1999; Rotenberg et al., 2015; Thayer & Fischer, 2013) and unassociated (Zhou et al., 2012) with Total and LDL cholesterol levels in child and adult studies. Across several child studies, cortisol has been positively associated with Total cholesterol and LDL, and unassociated with high-density lipoprotein (HDL; DuBose & McKune, 2013; Guzzetti et al., 2014; Prodam et al., 2013; Weigensberg et al., 2008).

Blood Pressure. Physiological evidence suggests that the sympathetic nervous system is the primary regulator of blood pressure, and that sympathetic activity is augmented by cortisol and the HPA axis. (Bjorntorp, Holm, Rosmond, Folkow, 2000; Guyenet, 2006). Denervation of

the sympathetic nervous system is associated with reduced blood pressure (Madden & Sved, 2003; c.f., Julien et al., 1990) and the administration of cortisol increases blood pressure by augmenting the sympathetic effects (Mangos et al., 2000; Pirpiris, Sudhir, Yeung, Jennings, & Whitworth, 1992; Sapolsky, Romero, & Munck, 2000). Among normal-weight and overweight/obese children, low HRV is related with high systolic and diastolic blood pressure (Farah, Barros, Balagopal, & Ritti-Dias, 2014; Rotenberg et al., 2015; Zhou et al., 2012); although, others observed no association between HRV and diastolic blood pressure (Guízar, Ahuatzin, Amador, Sánchez, & Romer, 2005; Gutin et al., 2000). Among overweight/obese children, high cortisol is consistently associated with high systolic and diastolic blood pressure (Guzzetti et al., 2014; Prodam et al., 2013; Weigensberg et al., 2008). In contrast, among normal-weight children, cortisol is unassociated with diastolic blood pressure (Soriano-Rodriguez et al., 2010), while positively (Soriano-Rodriguez et al., 2010) and unassociated with systolic blood pressure (DuBose & McKune, 2013; Hill, Eisenmann, Holmes, & Heelan, 2010).

Central Adiposity. Both the autonomic nervous system and HPA axis are implicated in central adiposity and visceral fat accumulation. Cortisol is mainly responsible for directing excess body fat to visceral depots (Bjorntrop et al., 2000; Kyrou & Tsigos, 2009); although, both sympathetic and parasympathetic neural inputs influence this process and promote the growth of adipose tissue (Fliers et al., 2003; Kreier et al., 2002). In studies with normal-weight, overweight, and obese children, HRV is negatively associated with waist circumference (Farah et al., 2014; Farah, Pardo, Tenorio, & Ritti-Dias, R.M, 2013; Zhou et al., 2012). Studies relating cortisol to waist circumference are less consistent. Cortisol has been positively (Barat et al., 2007; Hill, Eisenmann, Gentile, Holmes, & Walsh, 2011; Steptoe, Kunz-Ebrecht, Brydon, & Wardle, 2004), negatively (Ljung et al., 2000; Ruttle et al., 2013) and unassociated (Guzzetti et al., 2014;

Soriano-Rodriguez et al., 2010; Weigensberg et al., 2008) with waist circumference in child and adult studies. Taken together, findings suggest that cardiometabolic markers are related to *both* low cardio-autonomic control and high HPA axis activity.

One key limitation of the existing literature is that only the singular associations between cardiometabolic markers and *either* HRV *or* cortisol have been examined. Examining only singular associations is counterintuitive given the physiological evidence that supports their functional inter-relation (Itoi, Jiang, Iwasaki, & Watson, 2004; Reyes, Valentino, Xu, Van Bockstaele, 2005; Zeigler, Cass, Herman, 1999). The Polyvagal Theory (Porges, 1995; 2007a) and Neurovisceral Integration Model (Thayer & Sternberg, 2006) provide conceptual support for the functional inter-relation between the stress systems by contending that parasympathetic inhibition enables the sympathetic and HPA axis to respond to stress, and that parasympathetic disinhibition is associated with dysregulated stress response systems. Examining the singular associations is also conceptually problematic as it oversimplifies the complex multi-system dynamic relating stress and health outcomes (Andrews, Ali, & Pruessner, 2013). Thus, despite the anatomical and theoretical rationale to examine the association between cardiometabolic markers and the inter-relation of the autonomic nervous system and HPA axis, one gap in the previous literature is the limited focus on singular associations between cardiometabolic markers and cardio-autonomic *or* HPA axis activity alone.

Another limitation in the extant literature is that only a paucity of studies have considered the impact of stressful life events on the relation between cardiometabolic markers and the stress response system among children and adolescents. Akin to the Diathesis Stress Model (Everson et al., 1997; Kamarck, et al., 2005), Schwartz et al. (2003) posit that it is the *interaction* between stress exposure and physiological predispositions (e.g., altered cardio-autonomic and HPA axis

activity) that contributes to the development of cardiometabolic risk, and, in the absence of stress, individuals with altered stress response systems remain at lower risk of developing cardiometabolic risk. Previous laboratory stress studies with adults that examined the interaction between cardiovascular reactivity (i.e., change in blood pressure) and stressful life events on markers of atherosclerosis (e.g., carotid artery plaques, intima-media thickness) underscore the role of these interactive effects (Everson et al., 1997; Kamarck et al., 2005; Lynch, Everson, Kaplan, Salonen, Salonen, 1998). Specifically, these studies found that adults with higher stress and greater cardiovascular reactivity had greater progression of intima-media thickness four years later (Everson et al., 1997; Lynch et al., 1998). Only one previous study with children could be found (Rotenberg et al., 2015). In our previous cross-sectional study, the interaction of HRV and cortisol was associated with Total and LDL cholesterol among children with greater stressful life events, such that those with low HRV and high cortisol had higher cholesterol levels. Additional studies are needed to examine whether stress exposure moderates the relation between the stress response system and cardiometabolic markers early during the lifecourse. Given that the effects of stressful life experiences are considered to become biologically embedded and shape disease trajectories (Danese & McEwen, 2012; Goodman, McEwen, Huang, Dolan, & Adler, 2005; Hertzman, 1999; Shokoff et al., 2009), another gap in the literature is the notable absence of the examination of the association between stressful life events and the inter-relation of cardio-autonomic control and HPA axis activity in children.

To address these gaps in the literature, the aim of the current study was to examine whether the association between the inter-relation of cardio-autonomic and HPA axis activity with cardiometabolic markers (e.g., Total cholesterol, LDL, HDL, blood pressure, waist circumference) was moderated by exposure to stressful life events among children. Extending

our earlier work, we aimed to examine this association in a sample of children at-risk for obesity, and therefore at higher risk for having these cardiometabolic markers. We hypothesized that the association between the inter-relation with cardiometabolic markers would be heightened in children with greater exposure to stressful life events.

Study 3 Method

Participants

Children aged 8 to 10 years from the QUebec Adipose and Lifestyle InvesTigation in Youth (QUALITY) Cohort were included. The QUALITY Cohort is a prospective study designed to examine the genetic, biological, environmental, and psychosocial determinants of obesity and cardiovascular disease precursors in a sample of children at-risk for the development of obesity. Lambert and colleagues (2012) previously described the study design and sampling protocol in detail. Children were identified as being at-risk for the development of obesity if at least one of their biological parents was obese (BMI $\geq$30 kg/m^2 or waist circumference >102 cm men, >88 cm woman); children were not recruited based on their own weight status. Exclusion criteria included diagnosis of serious psychopathology or medical condition; use of anti-hypertensive medication; or, following a restricted diet (<600 kcal/day). All children were Caucasian of Western European ancestry to reduce genetic admixture. The ethics review board of the CHU Sanite-Justine Hospital approved the QUALITY Cohort study. Informed consent and assent were obtained from parents and children, respectively.

Measures

Cortisol. Saliva samples were collected at awakening and +30 minutes post-awakening, before lunch, before dinner, and before bed on one weekend day. Saliva samples were collected using the Salivette sampling device (Salimetric, Inc.). Participants were instructed not to eat,

drink, or brush their teeth 10min before taking a sample, and to record the date and time of each sample in a daily log. Compliance with this methodology has been previously established (Rotenberg & McGrath, 2014). When saliva collection was complete, all samples were mailed back to the laboratory, where they were packaged in dry ice and couriered to the University of Trier, Germany for cortisol assaying. Cortisol levels are robust to environmental conditions associated with the shipping process (Clements & Parker, 1998). Cortisol levels were determined in duplicate using a competitive solid phase time-resolved fluorescence immunoassay with fluorometric end point detection (DELFIA; Dressendörfer, Kirschbaum, Rohde, Stahl, & Strasburger, 1992). The intra-assay coefficients of variation were less than 11%.

Untransformed cortisol values were used to derive: area under the awakening response relative to ground (AUC_{AG}), dynamic increase of the awakening response (AUC_{I}), and area under the diurnal profile relative to ground (AUC_{TG}; for formulae, see Rotenberg et al., 2012). Maximum sample was determined based on the highest cortisol value for the day. All cortisol measures were normally distributed.

Heart Rate Variability. Continuous electrocardiogram (ECG) recordings were collected with an 8500 Marquette MARS Holter monitor (GE Marquette Medical Systems, Milwaukee, Wisconsin, USA) during a 3hr standardized clinic visit. ECG data were sampled at a rate of 128 Hz, combined with QRS template matching based on 1024 samples/300 sec. The ECG signal was imported into the MARS® Holter Analysis Workstation (GE Marquette Medical Systems, Milwaukee, Wisconsin, USA), where data were formatted for editing, analysis, and interpretation. Input samples were linearly detrended, mean-centered, and tapered using a Hanning window. ECG data were processed according to Task Force Guidelines (1996). Artifact correction was conducted based on a 20% change from the previous signal as a criterion (Kleiger, Miller, Bigger,

& Moss, 1987), as well as visual inspection by a qualified trained technician. A board-certified cardiologist also reviewed all ECG recordings.

Frequency-domain measures of HRV were derived based on power spectral analysis using the Fast Fourier Transformation. ECG data were analyzed in 10 min epochs and averaged across the duration of the recording. Frequency domain measures included: low frequency (LF; 0.04 - 0.15 Hz), high frequency (HF; 0.15 – 0.40 Hz), and the LF/HF ratio. HF is associated with parasympathetic modulation, LF is associated with both sympathetic and parasympathetic modulation, and the LF/HF ratio is associated with the balance between sympathetic and parasympathetic modulation, also referred to as sympathovagal modulation (Cacioppo et al., 1994; Lahiri, Kannankeril, & Goldberger, 2008; Polanczyk et al., 1998; Sztajzel, 2004; Task Force, 1996).

Anthropometrics. Anthropometric measures were derived using standardized protocols by a registered nurse. While the child was dressed in light clothing, a standard measuring tape was used to measure the child's waist circumference midway between the lowest rib and the iliac crest at the end of expiration (World Health Organization, 2008). Without shoes, height was measured using a stadiometer and weight was measured using a calibrated electronic scale. Waist circumference and height were measured twice, to the nearest 0.1 cm. If the difference between the first two measures was greater than 0.5 cm, a third measure was taken. The mean of the two closest measures was used in analyses. BMI Z-score was determined using age- and sex-specific growth charts (Ogdon et al., 2002).

Blood Pressure. After sitting quietly for 5min, resting systolic and diastolic blood pressure was measured on the right arm using an appropriate sized cuff with an oscillometric automated device (Dinamap XL, model CR9340, Critikon Company, FL, USA). While seated,

four blood pressure readings were taken at 1min intervals. The mean of the last three readings was used in analyses.

Metabolic Factors. After fasting overnight, children's blood samples were collected by a registered nurse for measurement of Total cholesterol, LDL, and HDL. Within 30min after collection, blood samples were centrifuged, aliquoted, and placed on dry ice. Lipid concentrations were determined with a Synchron LX20 with Beckman Instruments reagents.

Pubertal Stage. Pubertal stage was scored by a trained nurse based on Tanner's description of pubertal development (Marshall & Tanner, 1969; 1970). Identification of stage was based on pubic hair and breast growth in girls, and pubic hair and genital growth in boys.

Stressful Life Events. Stressful life events were measured using a list of events typically experienced by children (e.g., school problems, parental divorce; Deschesnes, 1998). Children rated the extent to which they were worried or stressed about 14 items over the past 3 months on a 4-point scale (*not at all* to *a whole lot*). The internal consistency of the scale is good (α = .83–.89, Deschesnes, 1998; α = .65, present study). Children were stratified into high vs. low stress exposure groups based on a median split. Children in the high stress group reported feeling significantly more stressed regarding school (t (417) = -12.07, $p < .001$), their weight (t (424) = -6.77, $p < .001$), and their relationships with their friends (t (420) = -9.29, $p < .001$) than children in the low stress group.

Procedure

Children and their parents arrived at the clinic at 7:00 am and completed demographic and health questionnaires. A trained nurse instructed participants on salivary cortisol collection, and provided saliva collection kits for home. The nurse subsequently measured anthropometrics following standardized protocols, collected fasting blood samples, and determined pubertal stage.

Blood pressure was measured after a 5 minute resting period. Children were fitted with a continuous ECG ambulatory monitor between 7:00am and 9:00am, and wore the monitor for approximately 3hrs.

Statistical Analyses

Variables were inspected for normality and outliers to ensure assumptions of the analytic methods were met. Of the initial 536 participants with both HRV and cortisol measures, two participants were excluded as their cortisol data were extreme outliers (>6 SD). Missing cortisol values, due to single saliva samples that were not returned (16%), were imputed using multiple imputation (Enders, 2010). Little's test indicated that missing saliva samples were not missing completely at random (χ^2 [75] = 109.75, $p < .05$), but were likely missing at random. Imputation of missing cortisol values was informed by data from the QUALITY study (e.g., subsequent cortisol samples, age, sex, puberty). Analyses were completed with both the original and imputed datasets. Findings were similar; thus, results based on the imputed dataset are presented for parsimony. Descriptive data (means, standard deviations, minimum, maximum, skewness, kurtosis) were reviewed for all variables. Scatterplots were visually inspected to check distributions of residuals and linearity. Analyses were conducted using IBM SPSS Statistics software (Version 20).

The Hayes PROCESS macro 2.13 (model 3; Hayes, 2013) was used to test the hypothesis that the association between the inter-relation of cardio-autonomic and HPA axis activity with cardiometabolic markers was moderated by exposure to stressful life events. The PROCESS macro implemented sequential regression analyses to test whether the full model, which included the three-way interaction of cardio-autonomic control*HPA axis activity*stressful life events, predicted cardiometabolic markers. The PROCESS macro also conducted simple slopes

analyses to facilitate interpretation of significant interactions. We hypothesized that among children with high exposure to stressful life events, the interaction between cardio-autonomic control and HPA axis activity would be significantly associated with elevated cardiometabolic markers. Measures of cardio-autonomic control were HF, LF, and LF/HF ratio. Measures of HPA axis activity were AUC_{AG}, AUC_{I}, AUC_{TG}, and maximum sample. Analyses were adjusted for developmentally relevant covariates, including age, sex, pubertal stage, household income, parental education, wake time, and BMI Z-score (except for analyses with waist circumference).

Study 3 Results

Participants included 534 children with at least one biological parent who was obese; descriptive statistics are presented in Table 8. Among children, 58.2% were of normal body weight (5-85th BMI percentile), 18.7% were overweight (85-95th BMI Percentile), and 21.3% were obese (>95th BMI Percentile). About half of the children were male (53.9%) and had parents with university education (55.2%); the majority were in the early stages of adrenarche (96.9%). The average household income was $42.8K CAD. Overall, the children's cholesterol (M_{TC} = 3.91 mmol/L, SD = 0.70), lipoprotein levels (M_{LDL} = 2.35 mmol/L, SD = 0.58; M_{HDL} = 1.20 mmol/L, SD = 0.25), blood pressure (M_{SBP} = 93.55 mmHg, SD = 8.02; M_{DBP} = 48.47 mmHg, SD = 5.02), and waist circumference (M_{WC} = 66.94 cm, SD = 11.77) were in the normal range. Children with higher stress were older (t (531) = 2.38, p = .02), had a larger waist circumference (t (531) = 2.08, p = .04), higher diastolic blood pressure (t (531) = 2.92, p = .004), and higher LF/HF ratio (t (530) = 2.70, p = .007), than children with lower stress. Table 9 displays the correlations among cardiometabolic markers and covariates, and the partial correlations with HRV, cortisol, and stressful life events. Correlations between HRV and cortisol measures are presented in Table 10.

Inter-Relation of HRV and Cortisol, Stressful Life Events, and Cardiometabolic Markers

The hypothesis that stressful life events would moderate the association between the inter-relation of HRV and cortisol with cardiometabolic markers was largely supported. Model results of the three-way interactions (HRV*cortisol*stress), are presented in Table 11.

Cholesterol. Three-way interactions between HRV*cortisol*stress were significantly associated with Total and LDL cholesterol for the inter-relations of all HRV and cortisol measures, except LF/HF ratio. HDL cholesterol was significantly associated with the three-way interactions for all HRV measures with AUC_I, and LF with maximum cortisol. Among children exposed to more stressful life events, simple slopes analyses revealed that the inter-relation of lower HF and higher AUC_I was associated with higher Total (β_{simple_slope} = .15, p = .06) and HDL cholesterol (β_{ss} = .17, p = .04). The inter-relation of higher HF with lower AUC_I was not associated with Total or HDL cholesterol (β_{ss} = -.02, p = .74, and β_{ss} = .04, p = .56, respectively). Finally, the HRV*cortisol inter-relation was not associated with LDL cholesterol (Low HF: β_{ss} = .13, p = .11; High HF: β_{ss} = -.03, p = .73). A similar pattern of results emerged when the simple slopes analyses were conducted for the significant interactions between LF and HF HRV and the remaining cortisol measures (AUC_{AG}, AUC_{TG}, maximum sample; results not shown for parsimony). Altogether, among high-stress children, the pattern of lower parasympathetic modulation (LF, HF) with higher cortisol awakening response (AUC_{AG}, AUC_I) or diurnal cortisol (AUC_{TG}, maximum) was associated with higher Total and HDL cholesterol.

Among children exposed to fewer stressful life events, the inter-relation of higher HF and higher AUC_I was associated with higher Total (β_{ss} = .29, p = .004), LDL (β_{ss} = .22, p = .02), and HDL cholesterol (β_{ss} = .17, p = .06). The inter-relation of lower HF with lower AUC_I was not associated with Total, LDL, or HDL cholesterol (β_{ss} = -.13, p = .12; β_{ss} = -.10, p = .21; β_{ss} = -.12,

p = .14; respectively). A similar pattern of results emerged when simple slopes analyses were conducted for the significant interactions between LF, HF, and LF/HF ratio and the remaining cortisol measures (AUC_{AG}, AUC_{TG}, maximum sample; results not shown for parsimony). Among low-stress children, the pattern of higher parasympathetic modulation (LF, HF) or lower sympathovagal modulation (LF/HF ratio) with higher cortisol awakening response (AUC_{AG}, AUC_{I}) or diurnal cortisol (AUC_{TG}, maximum) was associated with higher Total, LDL, and HDL cholesterol.

Blood Pressure. Three-way interactions between HRV*cortisol*stress were not significantly associated with systolic and diastolic blood pressure. Among children exposed to more stressful life events, the inter-relation of HF and AUC_{I} was not associated with systolic (Low HF: β_{ss} = -.06, p = .41; High HF: β_{ss} = -.01, p = .89) or diastolic blood pressure (Low HF: β_{ss} = .03, p = .70; High HF: β_{ss} = .09, p = .22). Among children exposed to fewer stressful life events, the inter-relation of HF and AUC_{I} was not associated with systolic (Low HF: β_{ss} = .00, p = .97; High HF: β_{ss} = -.01, p = .89) or diastolic blood pressure (Low HF: β_{ss} = -.02, p = .80; High HF: β_{ss} = -.02, p = .81). (The pattern of the results with the interactions of LF, HF, and LF/HF ratio and AUC_{AG}, AUC_{TG}, maximum sample was similar; not presented for parsimony.) Post-hoc analyses indicated that lower parasympathetic modulation (LF, HF) and higher sympathovagal modulation (LF/HF ratio) was related to higher systolic and diastolic blood pressure, irrespective of their interactions with cortisol levels or stressful life events (see Table 9).

Central Adiposity. Three-way interactions between HRV*cortisol*stress were significantly associated with waist circumference, but only for LF and HF with AUC_{I}, and LF with AUC_{TG}. Among children exposed to more stressful life events, simple slopes analyses revealed that the inter-relations of lower LF and HF with lower AUC_{I} were associated with larger

waist circumference (β_{ss} = -.19, p = .01; β_{ss} = -.19, p = .01; respectively). The inter-relations of higher LF and HF with higher AUC_I were not associated with waist circumference (β_{ss} = .06, p = .38; β_{ss} = .06, p = .43; respectively). Finally, simple slopes analyses indicated that the inter-relation of lower LF with lower AUC_{TG} (β_{ss} = -.09, p = .18), and the inter-relation of higher LF with lower AUC_{TG} (β_{ss} = -.13, p = .17) were not associated with waist circumference. Among high-stress children, the pattern of lower parasympathetic modulation (LF, HF) with lower cortisol awakening response (AUC_I) was associated with a larger waist circumference.

Among children exposed to fewer stressful life events, the inter-relation of lower LF with lower AUC_{TG} was associated with larger waist circumference (β_{ss} = -.21, p = .03). The inter-relation of higher LF with higher AUC_{TG} was not associated with waist circumference (β_{ss} = .14, p = .15). Finally, simple slopes analyses also indicated that the inter-relations of lower LF and HF with higher AUC_I (β_{ss} = .03, p = .74, β_{ss} = .02, p = .77; respectively), and the inter-relations with higher LF and HF with lower AUC_I (β_{ss} = -.08, p = .41; β_{ss} = -.08, p = .38; respectively) were not associated with waist circumference. Among low-stress children, the pattern of lower parasympathetic modulation (LF) with lower diurnal cortisol (AUC_{TG}) was associated with a larger waist circumference.

Study 3 Discussion

Previous research has implicated the two main branches of the stress response system - the autonomic nervous system and HPA axis - in the etiology of cardiometabolic risk. To date, the majority of this research has exclusively considered *either* autonomic *or* HPA axis activity. This singular strategy overlooks the physiological coordination and interconnectedness of the autonomic nervous system and HPA axis (Reyes et al., 2005; Reyes et al., 2008; Ziegler et al., 1999). Additionally, the moderating influence of stressful life events on the association between

cardiometabolic markers and the stress response system has largely been overlooked. The current study addressed these gaps by examining whether the associations between the inter-relation of autonomic and HPA axis activity with cardiometabolic markers was moderated by stressful life events exposure in a targeted sample of children at-risk for obesity.

The results of the present study largely support the hypothesis that the inter-relation between the autonomic nervous system and HPA axis would be associated with cardiometabolic markers among children with high stress. Consistent with this hypothesis, the inter-relation of parasympathetic modulation with the cortisol awakening response or diurnal cortisol was associated with elevated cholesterol levels (Total, LDL, HDL) among children with high stress. Relatedly, the inter-relation of parasympathetic modulation and the cortisol awakening response was also associated with waist circumference among children with high stress. Contrary to expectations, parasympathetic modulation was singularly associated with systolic and diastolic blood pressure, regardless of cortisol levels or stressful life events. These blood pressure findings were unanticipated given previous reported findings of blood pressure's negative association with HRV (Farah et al., 2014, Zhou et al., 2012; c.f. Guizar et al., 2005), and positive association with cortisol (Guzzetti et al., 2014; Prodam et al., 2013; Soriano-Rodriquez et al., 2010; c.f. DuBose & McKune, 2013; Hill et al., 2010). Our findings may be partly accounted for by salient demographic differences, as the children in the present study were young (M_{Age} = 9.12) and mostly pre-pubescent (78.2%), and may not have accumulated sufficient wear and tear on their vasculature system despite being an at-risk sample.

The pattern of results that emerged from interpretation of the significant three-way interactions was intriguing and supported the notion of the moderating role of stress exposure. Among children with high stress, the pattern of lower parasympathetic modulation with higher

cortisol awakening response or diurnal cortisol was associated with higher cholesterol levels (total, LDL, HDL). This pattern of results was consistent with previous adult and child studies, which found that lower cardio-autonomic control (Christensen et al., 1999; Thayer & Fischer, 2013; c.f., Zhou et al., 2012), and higher morning cortisol (DuBose & McKune, 2013; Guzzetti et al., 2014; Prodam et al., 2013; Weigensberg et al., 2008) were singularly associated with greater Total and LDL cholesterol levels. Notedly, this pattern of results was inconsistent with previous child studies reporting no relation between morning cortisol and HDL levels (Guzzetti et al., 2014; Prodam et al., 2013; Weigensberg et al., 2008). This inconsistency may be accounted for by cortisol measurement differences, as prior studies serum cortisol in the morning hours, irrespective of time of awakening. Despite these inconsistences, an overall pattern of heightened stress response activity was linked to higher cholesterol levels among children exposed to greater stressful life events.

For central adiposity, the observed pattern of significant results was unanticipated. Based on previous child and adult studies, we expected that the inter-relation of lower cardio-autonomic control (Farah et al., 2014; Farah & do Prado, 2013; Zhou et al., 2012) with higher cortisol levels (Barat et al., 2007; Hill et al., 2011; Steptoe et al., 2004) would be associated with larger waist circumference. Instead, among children with high stress, the pattern of lower parasympathetic modulation with *lower* cortisol awakening response was associated with a larger waist circumference. Others have also reported this counterintuitive finding of low cortisol awakening response cross-sectionally and prospectively associated with higher BMI in adolescents (Ruttle et al., 2013). There are at least two possible explanations for this unexpected significant association between low cortisol awakening and larger waist circumference. First, although adipose tissue stimulates the release of cortisol, over time, sustained exposure to elevated cortisol

levels promotes down-regulation of the HPA axis (Ahima & Flier, 2000). Adipose tissue stimulates the release of cortisol by initially secreting signaling molecules (e.g., cytokines) which induce the release of cortisol (Mohamed-Ali, Pinkey, & Coppack, 1998), and then by converting cortisol to inactive metabolites, thereby interfering with the cortisol negative feedback loop (Bjorntorp & Rosmond, 2000). Second, animal and human models of chronic stress indicate that exposure to greater stressful life events results in the down-regulation of the HPA axis and low cortisol (Fries, Hess, Hellhammer, & Hellhammer, 2005). Thus, the low cortisol awakening observed in the present study may be evidence of a blunted awakening response; however, this is speculative given that evidence of an earlier prolonged period of elevated cortisol is required to establish bluntening. Prospective, longitudinal studies beginning in early childhood are needed to consider these possibilities.

To the best of our knowledge, there is only one study that previously considered the inter-relation of the stress systems and its relation with cardiometabolic markers. Our earlier study (Rotenberg et al., 2015) tested the inter-relation and cardiometabolic markers in a population-based sample of healthy children. Consistent with the current findings, the results of our earlier work demonstrated the inter-relation of lower parasympathetic modulation and higher cortisol awakening response was associated with higher Total and LDL cholesterol, among children with high stress. Further, systolic and diastolic blood pressure were associated with lower parasympathetic modulation, irrespective of cortisol levels or stress exposure. In contrast to the current findings, the inter-relation between cardio-autonomic control and the cortisol awakening response was not associated with waist circumference. Rather, lower cortisol awakening response was singularly associated with a larger waist circumference, among children with high stress. It is plausible these differences findings may be accounted for sample demographics, as

the present sample had less advanced pubertal status (78.2% vs. 42% Rotenberg et al., 2015) and larger weight status (21.3% vs. 7.4% obese, Rotenberg et al., 2015). The present findings, in the context of previous findings, together suggest that children exposed to greater stress with a pattern of dysregulated stress response systems have elevated cardiometabolic risk.

Children with lower stress exposure evidenced a different pattern of results. Among children with low stress, higher parasympathetic modulation together with higher cortisol awakening response/ diurnal cortisol was associated with higher cholesterol levels. For waist circumference, the inter-relation of parasympathetic modulation and diurnal cortisol, but not cortisol awakening response, was associated with waist circumference. Specifically, among children with low stress, lower parasympathetic modulation together with lower diurnal cortisol was related to larger waist circumference. This pattern of results suggests that exposure to stressful life events may alter the inter-connection and coordination across the stress systems. For instance, among children with low stress, greater cardiovascular risk was associated with an asymmetric pattern (high cardio-autonomic control and high cortisol), but this pattern differed among those with greater exposure to stress. Among children with high stress, greater cardiovascular risk was associated with a symmetric pattern (low cardio-autonomic control and high cortisol). Longitudinal studies are needed to examine this issue further, and to monitor the developmental trajectories of these stress response patterns.

Overall, the current study highlights the importance of examining the inter-relation and coordination across the stress response system when considering the relation between stress and cardiovascular health. Traditionally, most studies relating stress to cardiometabolic markers include a measure of either cardio-autonomic control *or* HPA axis activity (DuBose & McKune, 2013; Hill et al., 2010; Zhou et al., 2012); however, as evidenced by the current study, this does

not capture the whole picture. In the present study, considering the inter-relation between the stress systems revealed patterns that were related to greater cardiometabolic risk. Accounting for the inter-relation between the stress systems may reconcile inconsistencies within the child literature regarding their association with certain cardiometabolic markers. For instance, waist circumference has been positively (Barat et al., 2007; Hill et al., 2011) negatively (Ruttle et al., 2013), and unassociated (Guzzetti et al., 2014; Soriano-Rodriguez et al., 2010; Weigensberg et al., 2008) with morning cortisol. The present findings suggest the extent of cardio-autonomic modulation may account for these inconsistencies. More precisely, waist circumference was negatively associated with cortisol when cardio-autonomic control was low, but unassociated with cortisol when cardio-autonomic control was high. Future studies examining how stress "gets under the skin" should consider the patterning or inter-relation between stress systems to more comprehensively characterize the association between stress and health.

The present findings provide limited support for existing theories in stress physiology including the Polyvagal Theory (Porges, 1995; 2007a) and Neurovisceral Integration Model (Thayer & Lane, 2000; Thayer & Sternberg, 2006) that emphasize the role of the parasympathetic nervous system in regulating the activity of the HPA axis. Consistent with these models, among children exposed to high stress, inter-relations with low parasympathetic inhibition (i.e., parasympathetic modulation) were associated with deregulated HPA axis activity and elevated cardiometabolic risk. Specifically, among children with high stress, HPA axis activity was related to cholesterol levels (Total, LDL, HDL) and waist circumference in the context of lower parasympathetic modulation, but unrelated to cholesterol levels and waist circumference in the context of higher parasympathetic modulation. The pattern of results among children with low stress was inconsistent with these theoretical models. Specifically, inter-

relations with high parasympathetic inhibition were associated with deregulated HPA axis activity and elevated cardiovascular risk, suggesting that additional pathways are involved in the regulation of the HPA axis.

Four limitations in the current study merit discussion. First, the current study was cross-sectional; thus, we were unable to address causality or consider the developmental trajectories of the patterns between the inter-relations, stress, and cardiometabolic markers found in this study. However, as an early study examining the association of the inter-relation of the stress systems with stress exposure and cardiometabolic markers, the present findings raise questions for future study. Namely, researchers should consider investigating the patterning of the stress response systems and their relation to cardiometabolic markers using prospective, longitudinal designs Second, an interaction term was used to characterize the inter-relation between the stress systems. Other analytical and mathematical approaches may be more suitable to represent their aggregate and interactive effects. Future studies should consider the used of advanced modeling techniques such as multi-level growth curve modeling or cluster analyses in order to identify individual differences in stress response patterns. Andrews, Ali & Pruessner (2013) recently proposed using the ratio of salivary alpha amylase over cortisol to account for the inter-relation between stress systems. Third, stress exposure was based on self-reported stressful life events over the past three months, which may not have been an adequate measure or sufficient period of time to characterize stress. Although this self-report questionnaire has established reliability and validity, the use of other stress measures, such as ecological momentary assessment, perceived stress, and/or laboratory stress reactivity, would also be of interest. Fourth, cortisol measures were based on a single day of weekend saliva collection. Our previous work has established that a single day of saliva collection yields only moderate reliability for certain measures of HPA axis

activity (i.e., maximum sample, AUC_{TG}, AUC_{AG}). At least two days of saliva collection are necessary to derive more reliable measures for most measures of HPA axis activity (AUC_I; Rotenberg, McGrath, Roy-Gagnon, & Tu, 2012). In the present study, findings related to AUC_I paralleled the findings of the remaining cortisol measures, suggesting that the present findings are tenable; however, future studies should collect saliva sample over a longer period of time. Despite these four limitations, the use of a multi-system perspective is a novel and timely approach to examining the relation between stress and health. The autonomic nervous system and HPA axis are structurally and functionally inter-connected; thus, considering the inter-relation between stress systems is more physiologically representative. Future studies should use additional measures of stress, employ prospective designs, and measure autonomic and HPA axis activity concurrently to capture the inter-relation between stress systems, and characterize their link between cardiometabolic markers across the lifespan.

In summary, the present study extends current knowledge regarding the relation between the stress response system and cardiometabolic precursors by supporting existing theories on the functional inter-relation between the stress systems, and emphasizing the importance of context, especially exposure to stressful life events. Aligned with the emerging emphasis on a multi-system perspective, the current findings indicate that considering the inter-relation of autonomic and HPA axis activity, rather than examining the singular associations alone, may provide deeper insight into how stress "gets under the skin". These findings indicate that the patterning of the stress response system is related to the emergence of cardiovascular precursors early in the life course.

Table 8

Descriptive Statistics for Demographics, Risk Factors, and Cortisol and HRV Measures

	Total Sample (n = 534)		High Stress (n = 276)		Low Stress (n = 257)	
	M (n)	SD (%)	M (n)	SD (%)	M (n)	SD (%)
Demographics						
Age*	9.12	.92	9.21	.90	9.02	.92
Female	(247)	(46.1%)	(134)	(48.6%)	(113)	(44.0%)
Adrenarche						
Stage 1	(419)	(78.2%)	(204)	(73.9%)	(213)	(82.9%)
Stage 2	(100)	(18.7%)	(64)	(23.2%)	(35)	(13.6%)
Stage 3	(16)	(3.0%)	(8)	(2.9%)	(8)	(3.1%)
Stage 4	(1)	(0.2%)	-	-	(1)	(0.4%)
BMI *z*-score						
Underweight	(10)	(1.9%)	(3)	(1.1%)	(7)	(2.7%)
Normal weight	(312)	(58.2%)	(162)	(58.7%)	(150)	(58.4%)
Overweight	(100)	(18.7%)	(45)	(16.3%)	(53)	(20.6%)
Obese	(114)	(21.3%)	(66)	(23.9%)	(47)	(18.3%)
Parental Education						
No high school degree	(6)	(1.1%)	(3)	(1.1%)	(3)	(1.2%)
High school degree	(31)	(5.8%)	(19)	(6.9%)	(12)	(4.7%)
Community college degree	(201)	(37.5%)	(105)	(38.3%)	(95)	(37.0%)
University degree	(296)	(55.2%)	(147)	(53.6%)	(147)	(57.2%)
Household Income ($CAD)	42.8K	18.5K	42.0K	18.9K	43.7K	18.1K
Waketime	7:28	1:00	7:29	1:00	7:28	1:00
Stress Level (0-25)	3.87	4.08	6.69	3.87	0.84	0.82
Cardiometabolic Markers						
Total Cholesterol (mmol/L)	3.91	0.70	3.95	0.68	3.87	0.71
Low Density Lipoprotein (mmol/L)	2.35	0.58	2.40	0.57	2.30	0.59
High Density Lipoprotein (mmol/L)	1.20	0.25	1.18	0.24	1.21	0.26
Systolic Blood Pressure (mmHg)	93.55	8.02	94.05	8.25	92.99	7.80
Diastolic Blood Pressure (mmHg)*	48.47	5.02	49.08	4.92	47.82	5.08
Waist Circumference (cm)*	66.94	11.77	67.91	12.36	65.80	11.02
Cortisol Measures						
AUC_{AG} (nmol/L)	5.00	2.52	5.15	2.74	4.85	2.26
AUC_{I} (nmol/L)	0.56	1.47	0.51	1.44	0.61	1.49
AUC_{TG} (nmol/L)	44.16	23.60	45.54	25.82	42.57	21.01
Maximum (nmol/L)	11.87	6.69	11.94	6.55	11.84	6.87
HRV Measures						
LF (ms^2)	1297.31	805.88	1298.86	870.43	1298.42	736.35
HF (ms^2)	915.66	689.31	874.63	677.96	961.75	702.39
LF/HF Ratio*	1.70	0.69	1.77	0.72	1.61	0.64

Note. AUC_{AG}, area under curve relative to ground for awakening response; AUC_{I}, area under curve relative to increase; AUC_{TG}, area under curve relative to ground for the day; LF, low frequency; HF, high frequency.

*Denotes significant difference between high vs. low stress group.

Table 9

Correlations with HRV, Cortisol, Stressful life events, and Covariates

	Total Cholesterol	LDL	HDL	SBP	DBP	Waist Circumference
HRV Measures[a]						
LF	-.04	-.04	.01	**-.08***	**-.18****	-.05
HF	-.05	-.04	-.01	**-.08***	**-.17****	**-.10***
LF/HF	.02	.02	.01	**.09***	.06	**.22****
Cortisol Measures[a]						
AUC_{AG}	**.12***	.05	**.16****	-.01	.00	-.01
AUC_{I}	.05	.04	.06	-.02	.02	-.04
AUC_{TG}	**.10***	.05	.08	-.01	-.02	-.08
Maximum	**.12****	.06	**.15****	-.03	.01	-.06
Stressful Life Events[a]	.05	.08	-.04	.05	**.12****	**.09***
Covariates[b]						
Age	**.09***	.07	-.03	**.26****	**.12****	**.29****
Sex	**.12****	**.14****	**-.12****	**-.09***	.04	.02
Adrenarche	-.02	-.01	**-.14****	**.16****	**.10***	**.28****
BMI *z*-score	**.10***	**.14****	**-.29****	**.31****	**.32****	**.84****
Parent Education	.02	-.08	**.16****	-.04	**-.09***	**-.11****
Household Income	-.04	-.01	**.14****	**-.10***	-.06	**-.10***
Waketime	**.10***	.08	.01	.00	.00	**.14****

Note. AUC_{AG}, area under curve relative to ground for awakening response; AUC_{I}, area under curve relative to increase; AUC_{TG}, area under curve relative to ground for diurnal profile; LF, low frequency; HF, high frequency. Bold values indicate significance: $^{*}p < .05$ $^{**}p < .01$

[a]Partial correlations control for: age, sex, pubertal stage, parental education, and household income; BMI *Z* score was a covariate in all analyses except for waist circumference.

[b]Zero-order correlations

Table 10
Correlations between HRV and Cortisol

	1	2	3	4	5	6	7
HRV Measures							
1. LF	-						
2. HF	**.86****	-					
3. LF/HF Ratio	**-.19****	**-.49****	-				
Cortisol Measures							
4. AUC_{AG}	.03	-.02	**.09***	-			
5. AUC_{I}	.00	-.05	.01	**.11****	-		
6. AUC_{TG}	-.01	.00	-.01	**.67****	-.07	-	
7. Maximum	.03	-.00	.02	**.81****	**.36****	**.63****	**-**

Note. Zero-order correlations are presented.
$^{*}p < .05$, $^{**}p < .01$

Table 11
Three-way Interactions predicting Cardiometabolic Markers

		Total Cholesterol			LDL			HDL			SBP			DBP			Waist Circumference		
HRV	Cortisol	β_{3int}	F	R^2	β_{3int}	F	R^2	β_{3int}	F	R^2	β_{3int}	F	R^2	β_{3int}	F	R^2	β_{3int}	F	R^2
LF	AUC_{AG}	**-.14****	4.62	.11	**-.11***	4.30	.11	-.05	7.47	.17	.01	9.15	.20	.00	7.70	.17	-.04	7.05	.15
	AUC_I	**-.14****	3.55	.10	**-.11****	3.80	.09	**-.09***	6.22	.15	-.01	8.66	.19	-.01	7.65	.17	**.09***	7.26	.16
	AUC_{TG}	**-.14****	3.99	.10	**-.10***	3.96	.10	-.08	6.45	.15	-.02	8.81	.20	.01	7.64	.17	**-.10***	7.68	.16
	Maximum	**-.15****	4.46	.11	**-.12****	4.33	.11	**-.09***	7.05	.16	.00	8.97	.20	.01	7.67	.17	-.02	7.46	.16
HF	AUC_{AG}	**-.12****	4.49	.11	**-.10***	4.29	.11	-.02	7.38	.17	.04	9.29	.20	.03	7.44	.17	-.01	7.06	.15
	AUC_I	**-.14****	3.73	.09	**-.12****	3.90	.10	**-.10***	6.36	.15	.02	8.70	.19	.02	7.23	.17	**.09***	7.45	.16
	AUC_{TG}	**-.11***	3.88	.10	**-.10***	3.96	.10	-.02	6.22	.15	-.00	8.91	.20	.01	7.41	.17	-.06	7.53	.16
	Maximum	**-.14****	4.29	.10	**-.12***	4.28	.10	-.07	6.90	.16	.04	9.11	.20	.05	7.45	.17	.01	7.38	.16
LF/HF	AUC_{AG}	.02	3.62	.09	.04	3.77	.09	-.02	7.16	.16	-.02	9.24	.20	.05	6.68	.15	-.02	8.79	.18
	AUC_I	.06	2.68	.07	.04	3.13	.08	**.11***	6.30	.15	-.04	8.72	.19	.01	6.39	.15	-.08	9.24	.19
	AUC_{TG}	.01	3.29	.08	.01	3.60	.09	-.02	5.97	.14	-.05	8.99	.20	.02	6.51	.15	.01	9.08	.19
	Maximum	.01	3.27	.08	.01	3.60	.09	.03	6.67	.15	-.06	9.15	.20	.02	6.56	.15	-.01	8.86	.18

Note. All analyses controlled for age, sex, adrenarche, parental education, household income, & wake-time. BMI *Z*-score was a covariate in all analyses except for waist circumference. β_{3int} = standardized beta coefficient for three way interaction between cort*hrv*stress. The main effects and two-way interactions between cort*hrv, cort*stress, and hrv*stress were also included in the model (results not shown for parsimony). AUC_{AG} = area under awakening curve relative to ground. AUC_I = area under awakening curve relative to increase. AUC_{TG} = area under diurnal curve relative to ground. LF = low frequency. HF = high frequency. Bold values indicate significance: $^{\pm}p<.07$ $^{*}p<.05$ $^{**}p<.01$

GENERAL DISCUSSION

This research program addressed whether the inter-relation between the stress response systems is related to cardiovascular precursors during childhood and adolescence. Most child research to date relating the stress response system to stress or cardiovascular precursors has measured *either* autonomic or HPA axis activity. This is in part due to the fact that these studies largely come from two separate bodies of stress physiological research. However, there is convincing evidence to suggest that the common practice of considering only a singular stress response system is counterintuitive given that the autonomic nervous system and HPA axis are structurally inter-connected and functionally work together to respond to stress (Andrews et al., 2013; Itoi et al., 2004; Reyes et al., 2005). Based on this evidence, the goal of this research program was to integrate previous stress physiology findings by examining whether considering the inter-relation of stress systems would extend the current understanding of how stress impacts cardiovascular precursors in children and adolescents. In the following sections, the major findings from the three studies are summarized. Next, comparisons between the studies and a synthesis of the findings across studies is presented. Then, the present results are discussed in the context of the existing literature. Finally, strengths and limitations, as well as suggestions for future research, are discussed.

Summary of Results

Study 1. Previous child studies that examined whether the autonomic nervous system and HPA axis were related to perceived stress have led to inconsistent findings, and none considered the inter-relation between these stress systems. Thus, Study 1 was a timely investigation of the inter-relation of the stress systems. Study 1 showed that the inter-relation between cardio-autonomic control and the cortisol awakening response accounted for perceived stress better than

the associations with either system alone. Specifically, the pattern of greater sympathovagal modulation with higher cortisol awakening response was related to higher perceived stress. The inter-relation between stress systems accounted for a small (2-4%), but unique portion of the variance in perceived stress. In fact, when measures of sympathovagal modulation and cortisol awakening response were simultaneously included in the models without their interaction term (i.e., paired), these paired model did not better account for perceived stress than the associations with either system alone. It was the inter-relation between the systems that was important. Thus, Study 1 supported consideration of the inter-relation when examining the association between stress and the stress response system.

Study 1 also highlighted the importance of cardio-autonomic control in regulating the HPA axis. Cardio-autonomic control is an index of inhibitory processes mediated by parasympathetic modulation. Low cardio-autonomic control represents dysregulation of the stress response system (increased sympathetic and HPA axis activity) and poorer adaptation to stress. High cardio-autonomic control represents intact regulation of the stress response system and better adaptation to stress. In Study 1, in the presence of lower cardio-autonomic control (high LF, LF/HF ratio), higher cortisol awakening response was related to higher perceived stress, but in the presence of higher cardio-autonomic control (low LF, LF/HF ratio), there was no association between cortisol awakening response and perceived stress. This finding contributes to the existing literature by suggesting that reported inconsistencies in the relation between stress and the stress response system may be explained by the interaction between the stress systems. It is pertinent to mention that given the importance of parasympathetic modulation, it was expected that inter-relations with HF, the most prominent measure of parasympathetic modulation, would have been related to perceived stress.

The results of Study 1 fostered questions about the inter-relation between the stress systems. Could the results be replicated in another sample? Would the inter-relation also be associated with cardiovascular precursors? Does contextual stress exposure influence the patterning of the stress response system? These questions led to the development of Study 2.

Study 2. Prior research provided evidence of the singular, independent links between cardio-autonomic control and the cortisol awakening response with salient precursors for cardiovascular disease, including high blood pressure, cholesterol levels, and central adiposity. However, extant literature largely ignored the inter-relation between stress response systems. Extending the results of Study 1, Study 2 examined whether the inter-relation between the stress systems was related to cardiovascular precursors and the potential moderating role of stressful life experiences.

Consistent with Study 1 results, there was also evidence for the inter-relation of cardio-autonomic and HPA axis activity in Study 2. The pattern of lower parasympathetic modulation with higher cortisol awakening response was linked to elevated Total and LDL cholesterol. Curiously, this inter-relation was not related to blood pressure or waist circumference. Instead, lower cardio-autonomic control was related to higher blood pressure, while lower cortisol awakening response was related to a larger waist circumference. These unexpected findings were incongruent with previous studies reporting that HRV and cortisol were related to children's blood pressure and waist circumference (DuBose & McKune, 2013; Farah et al., 2014; Soriano-Rodríguez et al., 2010). At first glance, the significant findings of the association of the inter-relation with Total and LDL cholesterol seemed dubious. However, given that the findings were found across two distinct measures of cardio-autonomic control (LF, HF), and the stress response patterns were similar to Study 1, an alternative explanation may account for these unexpected

findings. Specifically, these inconsistencies may be accounted for by differences with respect to the age of the participants (children 6 years of age; Soriano-Rodríguez et al., 2010; adolescents 16 years of age; Farah et al., 2014), sample types (girls only; DuBose & McKune, 2013; boys only; Farah et al., 2014), or methodology (single sample of cortisol; Soriano-Rodríguez et al., 2010).

The pattern of results observed in Study 2 was moderated by stressful life experiences. Among children with more stressful life experiences, the inter-relation of lower parasympathetic modulation with higher cortisol awakening response was associated with higher Total and LDL cholesterol. Relatedly, children with more stressful life experiences and lower cortisol awakening response also had a larger waist circumference. Among children with fewer stressful life experiences, the inter-relation of parasympathetic modulation and cortisol awakening was not associated with Total and LDL cholesterol, and there was no association between the cortisol awakening response and waist circumference among children with fewer stressful life experiences. These findings indicate that it is the interaction between greater stressful life experiences and the inter-relation between stress systems that confers the greatest cardiovascular risk.

Study 2 findings provided further support for the possible patterning of the stress response system. However, their differences from the existing literature raised some important questions that required further investigation. Is the patterning of the stress response system related to some cardiovascular precursors but not others? Would the association between the inter-relation and cardiovascular precursors be more pronounced in a targeted sample at greater risk for cardiovascular pathology that likely has more cumulative physiological wear and tear?

Study 3. Study 3 sought to replicate and extend Study 2 by examining whether the inter-relation between the cardio-autonomic system and the HPA Axis was associated with cardiovascular precursors in a sample of children at-risk for obesity. Children in the QUALITY Cohort were from similar geographical locations (i.e., same province) and were the same age as the children in population-based sample in Study 2. The QUALITY Cohort was originally designed to investigate of determinants of cardiometabolic risk factors in children. Measures of HRV, cortisol, and stressful life experiences were largely similar across Study 2 and 3. This Cohort afforded a unique opportunity to examine the patterning of the stress response system in a targeted sample.

In Study 3, exposure to stressful life experiences moderated the association between the inter-relation and cardiovascular precursors. Among children with more stressful life experiences, the inter-relation of lower parasympathetic modulation with higher cortisol awakening response and higher diurnal cortisol was related to higher cholesterol levels (Total, LDL, HDL). The inter-relation was also related to central adiposity, however, the specific pattern varied such that lower parasympathetic modulation with *lower* cortisol awakening response was related to a larger waist circumference. Among children with fewer stressful life experiences, a different pattern emerged. The inter-relation of higher parasympathetic modulation with higher cortisol awakening response or higher diurnal cortisol was related to higher cholesterol levels in low-stress children. For central adiposity, the inter-relation of lower parasympathetic modulation with *lower* diurnal cortisol was related to larger waist circumference.

Study 3 revealed an interesting pattern of findings that led to new questions about the possible inter-relation of cardio-autonomic and HPA axis activity. The pattern of the inter-relation was distinct in the context of stress exposure. Previous research has demonstrated that

stressful life experiences are biologically embedded early in life. The current findings suggest that exposure to stressful life experiences could potentially alter the coordination across the stress systems based on the different patterns of HRV and cortisol that emerged. It is plausible that early in life, stress system activity is balanced and complementary, with one system actively responding when the other is offline. Then with increased stress exposure, the balance of the inter-connection between the stress systems may be disrupted such that both systems respond in an attempt to maintain homeostasis. Animal models support the hypothesis that the stress system activity is complementary (Munck, Guyre, Holbrook, 1984; Sapolsky et al., 2000); however, experimental studies examining the impact of stress exposure on the inter-connection between the stress systems could not be identified. Longitudinal studies commencing early in the life course are necessary to tease apart possible developmental patterns of the inter-relation between cardio-autonomic and HPA axis activity.

Comparison and Synthesis of Study Findings

This programmatic line of research contributed new knowledge about the inter-relation of the stress systems. Three substantive contributions of this dissertation are discussed: the utility of the inter-relation; the role of cardio-autonomic control; and, the moderating influence of stress exposure.

First, across all three studies, the inter-relation provided additional insight regarding the associations with stress and cardiovascular precursors in children, over and above the singular contributions of either the cardio-autonomic system or the HPA axis alone. The overall pattern of significant inter-relations largely indicated that joint hyper-arousal was related to poorer health outcomes. The hyper-arousal pattern, or the inter-relation between lower cardio-autonomic control and higher cortisol awakening response was associated with higher perceived

stress in Study 1, higher Total and LDL cholesterol in Study 2, and an adverse cholesterol profile (higher Total, LDL, HDL) in Study 3. A different, asymmetric pattern emerged for waist circumference in Study 3, as the inter-relation of lower cardio-autonomic control with *lower* cortisol awakening response was related to a larger waist circumference. The observation that low cortisol awakening response was associated with a larger waist circumference might suggest bluntening of the cortisol awakening response (Fries et al., 2005; Miller, Chen, Zhou, 2007). Following sustained exposure to elevated cortisol, the HPA axis habituates to these high levels and down-regulates its activity (Fries et al., 2005; Miller et al., 2007). In Study 3, two possible pathways may have resulted in elevated cortisol. First, adipose tissues secrete signaling molecules (e.g., cytokines) that stimulate the HPA axis and the release of cortisol (Mohamed-Ali et al., 1998). Adipose tissue also converts cortisol into its inactive metabolite, which would interfere with the cortisol feedback loop (i.e. cortisol down-regulates its own activation; Bjorntorp & Rosmond, 2000). Second, exposure to chronic stress has also been known to down-regulate the HPA axis (Fries et al., 2005). Thus, the pattern of inter-relation results with waist circumference may represent the down-regulation of the HPA axis and consequent bluntening of the cortisol awakening response. This notion of bluntening is only speculative given that prior evidence of elevated cortisol is requisite to establish bluntening exists.

The inter-relation was unrelated to blood pressure in Study 2 and Study 3. For both studies, low cardio-autonomic control was associated with higher systolic and diastolic blood pressure, irrespective of cortisol or stress exposure. The specific regulatory functions of the autonomic nervous system and HPA axis likely explain these findings. Cholesterol and visceral fat accumulation are both regulated by the independent and interactive effects of the autonomic nervous system and HPA axis (Bjorntrop et al., 2000; Flier et al., 2003; Kreier et al., 2002;

Ottosson et al., 1999; Sapolsky et al., 2000). In contrast, blood pressure is predominately regulated by the parasympathetic and sympathetic branches of the cardio-autonomic nervous system (Guyenet, 2006; Julius, 1991; Malpas, 2010), and cortisol helps to augment these autonomic effects (Sapolsky et al., 2000). It is plausible that certain cardiovascular precursors may be better associated with the inter-relation between stress systems, while others are linked more directly to only one system. Alternatively, the inter-relation may vary across the lifespan or in the context of stress exposure.

Second, all three studies converged to indicate cardio-autonomic control played a central role in the patterning of the stress response system. The patterns of the inter-relations with low cardio-autonomic control (low parasympathetic modulation or high sympathovagal modulation) were associated with adverse outcomes. Interestingly, the patterns of the inter-relations with high cardio-autonomic control (high parasympathetic modulation or low sympathovagal modulation) were not associated with adverse outcomes. In Study 1, the inter-relation of higher sympathovagal modulation (LF, LF/HF ratio) with higher cortisol awakening response was associated with higher perceived stress; however, inter-relations with lower sympathovagal modulation were not associated with perceived stress. Similarly, in Study 2 and 3, the inter-relation of lower parasympathetic modulation (LF, HF) with higher cortisol awakening response was associated with elevated cardiovascular precursors; however, inter-relations with higher parasympathetic modulation were not related to cardiovascular precursors. Curiously, the patterns of the inter-relation with LF varied across the studies: LF and LF/HF ratio had a similar pattern in Study 1 suggesting sympathovagal modulation, while LF and HF had a similar pattern in Study 2 and 3 suggesting parasympathetic modulation. The interpretation of LF remains actively debated (de Gues et al., 2014; Heathers, 2014; Montano et al., 2009; Reyes del Paso et

al., 2013), and Friedman (2007) suggested that sympathetic modulation of cardio-autonomic control might vary based contextual factors. In Study 1, HRV was measured over 13 hours while children engaged in their typical routines at home or in school. In Study 2 and 3, HRV was measured over 3 hours during standardized clinic visits. Thus, measurement considerations that increase sympathetic modulation, such as physical activity and meal consumption, were constrained in Study 2 and 3 (Melanson, 2000; Sakaguchi, Arase, Fisler, & Bray, 1988; Schuit et al., 1999).

Third, exposure to stress moderated the association between the inter-relation and cardiovascular precursors. In Study 2 and 3, the inter-relation between the stress systems was associated with cardiovascular precursors among children with greater stressful life experiences. The observation of stress moderation is also congruent with Study 1, as the inter-relation was associated with higher perceived stress. In line with the Diathesis Stress Model, these findings suggest that the inter-relation between stress systems may be more pronounced in the context of greater stress. The present work focused on a limited period of development and grouped children based on their stressful life experiences. Future studies should evaluate the moderating role of stress across different periods of development and consider more precise measures of stress exposure.

Theoretical Contributions of Study Findings

The study findings related to the inter-relation, cardio-autonomic control, and stress exposure moderation contribute to existing theoretical models. Two prominent theoretical contributions include the observations about parasympathetic inhibition and inter-relation models.

Evidence for the distinguished role of parasympathetic inhibition (high parasympathetic modulation or low sympathovagal modulation) was evident across all studies. These findings

support existing theories that posit parasympathetic inhibition is critical for adapting to stress, including Polyvagal Theory (Porges 1995; 2007a) and Neurovisceral Integration Model (Thayer & Lane, 2000; Thayer & Sternberg, 2006). Although the Polyvagal Theory and Neurovisceral Integration Model differ in their primary theoretical framework (evolutionary vs. dynamical systems), both theories contend that parasympathetic inhibition is necessary to regulate physiological processes and promote healthy adaptation. Current findings largely support these theories. The inter-relations with lower cardio-autonomic control (low parasympathetic modulation or high sympathovagal modulation) resulted in hyper-arousal of the stress response system and greater adverse outcomes. However, the observation in Study 3 that inter-relations with higher-cardio-autonomic control and elevated cortisol resulted in higher cholesterol levels is inconsistent with these theories. Together, these findings suggest that parasympathetic inhibition is necessary but not sufficient for buffering the effects of altered HPA axis activity. Theories on the inter-relation between the stress response system may need to expand and consider additional physiological pathways (e.g., hypothalamic- pituitary-gonadal axis; Tsigos & Chrousos, 2002) that influence the stress response system.

Study findings also support theoretical models suggesting an inter-relation between the stress systems. Bauer et al. (2002) recommended that researchers consider the inter-relation between stress systems rather than view each system as independent, based on two proposed competing theoretical models: Additive and Interactive. The Additive Model suggests activity of the autonomic nervous system and HPA axis are similar (i.e., cortisol primarily augments sympathetic effects), and symmetric activity increases risk. Thus, children are at greater risk of the negative effects of stress when the autonomic nervous system and HPA axis respond in a similar manner (jointly activated or jointly deactivated). This would be represented by the pattern

of low cardio-autonomic control with high HPA axis activity, or high cardio-autonomic control with low HPA axis activity. The Interactive Model suggests activity of the autonomic nervous system and HPA axis are complementary, and asymmetric activity increases risk. Thus, children are at greater risk of the negative effects of stress when the autonomic nervous system acts in opposition to the HPA axis. This would be represented by a pattern of low cardio-autonomic control with low HPA axis activity, or high cardio-autonomic control with high HPA axis activity.

Interestingly, if Bauer's Additive/Interactive Models are considered in the context of the Polyvagal Theory and Neurovisceral Integration Model, there is more support for the Additive Model. Results of the current studies largely support this conclusion. Namely, the symmetric pattern of lower cardio-autonomic control with higher HPA axis activity was associated with greater perceived stress in Study 1, and higher Total and LDL cholesterol in Study 2 and 3. However, there was also some support for the Interactive Model given the asymmetric pattern of lower cardio-autonomic control with lower HPA axis activity was associated with a larger waist circumference among the at-risk sample of children in Study 3. Overall, current findings suggest that joint, symmetrical activation of the stress response system is associated with increased risk for negative mental and physical health outcomes. This body of work represents a notable contribution to the field of stress physiology, which has potential to facilitate understanding of the link between stress and cardiovascular disease.

Limitations and Strengths

The current research program is not without limitations. Four limitations are important to highlight. First, the mathematical approach to represent the construct of the inter-relation between stress systems could be debated. Using the interaction term is only one approach to

investigating the inter-connection between systems. Several approaches to representing the inter-relation between the stress systems (e.g., factor analysis of interaction terms, chi-squares analyses) were explored with Study 1. Other mathematical or statistical approaches not considered may better characterize the inter-relation. Other modeling techniques, such as a cluster analyses, may identify different stress profiles/patterns that represent different developmental trajectories. Advances in computing technology may help elucidate the patterning of the stress response system (e.g., topographical 3D modeling). Further, Andrews et al. (2013) proposed using the ratio of salivary alpha amylase/cortisol to capture the inter-relation between the stress systems. Given that the current work is among the first to examine whether the inter-relation was associated with stress and cardiovascular risk factors, interaction models were a fruitful starting point. Future studies should consider alternative inter-relation modeling techniques.

Second, all studies were cross-sectional and conducted in similar aged children. This permitted a narrow focus on this distinct period early during the lifecourse, but limited inferences that could be drawn. For example, in Study 3, the observation that the inter-relation of lower cardio-autonomic control and *lower* cortisol awakening response was related to larger waist circumference suggests that the cortisol awakening response may have been blunted in the at-risk sample. However, in the absence of longitudinal data demonstrating blunted response following chronic overactivation of the cortisol awakening response, it is not possible to draw that conclusion. Given Barker's hypothesis on the fetal origins of cardiovascular risk (1995), prospective, longitudinal studies are required to understand when and how the inter-relation between stress systems emerges early in the life course. Given that dysregulated stress response activity tracks into adulthood and increases risk for negative health outcomes, prospective

studies are also necessary to examine whether distinct stress response patterns predict risk for eventual cardiovascular disease.

Third, stress exposure was conceptualized differently across studies. Study 1 used a measure of perceived stress over the past 30 days; Study 2 and 3 used a measure of stressful life experiences over the past 3 months. While both are broadly stress constructs that have been associated with the stress response system and physical health problems in children and adolescents (Adam, 2006; van Jaarsveld et al., 2009; Slopen et al., 2013), there are important differences between perceived stress and stressful life experiences. Perceived stress is a measure of daily hassles, and reflects the degree to which a situation is appraised as stressful (Cohen et al., 1983). Stressful life experiences are an index of the frequency of events that are objectively stressful (e.g., parental divorce) over a longer time interval. Study 2 and 3 did not consider subjective appraisal of the stressful events. Further, stressful life experiences were dichotomized to create high and low stress exposure groups in both studies. This strategy was used to facilitate interpretation of the three way interactions between HRV, cortisol, and stress, but dichotomization is known to reduce power. (Notedly, a similar pattern of results was observed when stressful life events were retained as continuous.) It is possible that consideration of the subjective stress rating or the cognitive appraisal of the stressful event may have led to more robust results in Study 2 and 3. Future researchers should consider additional stress measures (ecological momentary analyses, early life adversity, laboratory based stress reactivity) at different time periods (prenatal, infancy, toddlerhood, childhood, adolescence) and for varying time durations (lifetime, past year, past month).

Fourth, the measurement of HRV varied across studies. While the ambulatory monitoring equipment and HRV analytical software were largely similar across studies, Study 1

derived HRV from electrocardiogram recordings worn over 13 hours at home and at school, and Study 2 and 3 derived HRV from recordings during three-hour standardized laboratory visits. Further, resting or naturalistic measures of cardio-autonomic control and HPA axis activity were included rather than measures of stress reactivity in response to specific stressors. Replication of the present work with measures of stress reactivity may facilitate understanding of the inter-relation, especially as stress reactivity patterns (i.e., high reactor, blunted reactor, non-reactor) are important indicators of adaptation and functioning (McEwen & Seeman, 1999). However, ambulatory work to capture the dynamic relation of stress in daily life and the inter-relation are important for ecological validity. To dynamically measure stress and the inter-relation, ecological momentary assessment methods synchronized with physiological assessment is essential. Study 1 did not include a concurrent measure of stress when children collected biological markers at home and at school. Measuring stress throughout the day would have been beneficial, as it would allow better temporal resolution and dynamic patterning with the stress response system. Rapidly advancing wearable technology, such as the iWatch, offers great promise for future research. Finally, heart rate variability is one approach to measuring cardio-autonomic activity. Other valid approaches include impedance cardiography, salivary-alpha amylase, and serum norepinephrine. It would be interesting to consider the patterning of the inter-relation with these alternative measures. Importantly, one key strength of the current studies was that electrocardiogram recording and HRV interpretation adhered to existing methodological guidelines (Task Force, 1996).

Future Directions and Conclusion

The present work adopted a novel approach in considering the patterning of the stress response systems, and how their inter-relation is related to children's cardiovascular health. It

also raised important questions for future research. First, what is the predictive utility of the inter-relation between the stress systems? Can the inter-relation between stress systems predict the development of cardiovascular precursors or eventual disease later in adulthood? Second, is the interaction term the best characterization of the inter-relation between stress systems? Could advanced computer modeling techniques more precisely capture the inter-relation between the stress systems? Third, beyond consideration of group-level differences of the inter-relation, are certain patterns of stress response unique to different individuals that relate to cardiovascular health? Is there a "stress response fingerprint" wherein some people are cardio-autonomic responders and others are HPA axis responders? Is the fingerprint or patterning of the inter-relation malleable to chronic stress exposure? Future research in this area might lead to advancements regarding certain patterns of the inter-relation that confer different developmental trajectories. In conclusion, this programmatic line of research on the patterning of the stress response system represents a timely investigation into the inter-relation of the stress response system and its association with cardiovascular precursors during childhood.

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APPENDIX 1

Paired vs. Inter-relation Model

		Paired Model			Interaction Model			
HRV	Cortisol	β_{cort}	β_{hrv}	R^2	β_{cort}	β_{hrv}	β_{int}	$\Delta R^{2,b}$
LF	AUC_{AG}	**.15***	.03	.19	-.15	**-.40***	**.56****	**.04**
	AUC_I	.12	.05	.19	.01	.02	.13	.01
	AUC_{TG}	.10	.04	.18	.03	-.03	.11	.00
	$Slope_{Max}$[a]	.08	.01	.21	.21	**-.48***	-.29	.00
	$Slope_{Awake}$	-.01	.05	.17	.14	-.09	-.23	.01
	Maximum	**.21****	.01	.21	-.13	**-.42***	**.59***	**.03**
	Bedtime	.07	.06	.18	-.04	-.08	.17	.00
HF	AUC_{AG}	**.16***	-.02	.19	.06	-.20	.22	.01
	AUC_I	.12	-.01	.19	.12	-.01	.00	.00
	AUC_{TG}	.10	-.00	.18	.11	.01	-.01	.00
	$Slope_{Max}$[a]	.08	-.03	.21	.13	-.11	-.10	.00
	$Slope_{Awake}$	-.02	.00	.17	-.04	.03	.04	.00
	Maximum	**.22****	-.03	.21	.18	-.11	.10	.00
LF/HF	AUC_{AG}	**.16***	.03	.19	-.15	-.27	**.42**[±]**	**.02**
	AUC_I	.12	.01	.19	**-.39***	-,12	**.58****	**.04**
	AUC_{TG}	.10	.01	.18	-.01	-.09	.15	.00
	$Slope_{Max}$[a]	.08	.03	.21	.21	**-.38***	-.18	.00
	$Slope_{Awake}$	-.02	.01	.17	.15	-.09	-.20	.00
	Maximum	**.22****	.03	.21	-.19	**-.38**[±]**	**.58***	**.02**
	Bedtime	.07	.01	.17	.20	.14	-.19	.00

Note. All analyses controlled for: age, sex, BMI z-score, adrenarche, parental education, household income, sleep duration, & wake-time. β_{cort}, standardized beta coefficient for the cortisol measure, β_{hrv}, standardized beta coefficient for the HRV measure, β_{int}, standardized beta coefficient for the interaction between the cortisol and HRV measure; AUC_{AG}, area under curve relative to ground for awakening response; AUC_I, area under curve relative to increase; AUC_{TG}, area under curve relative to ground for diurnal profile; Slope $_{max}$ = diurnal slope anchored to maximum sample using regression; LF, low frequency; HF, high frequency. Bold values indicate significance.

[a]Analyses also controlled for maximum cortisol sample, anchor point for the calculation of $Slope_{Max}$.

[b]Change in R^2 from paired model.

[±]$p < .07$ *$p < .05$ **$p < .01$