Anxiety Symptoms and Immuno-endocrine Systems from Childhood to Adolescence: Understanding Reciprocal Change over Time

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Abstract

Anxiety Symptoms and Immuno-endocrine Systems from Childhood to Adolescence: Understanding Reciprocal Change over Time

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Anxiety symptoms may strain underlying biological systems to result in negative long-term health consequences. In addition to generating psychological distress, anxiety symptoms may affect the system's ability to respond optimally to stress, and impede endocrine and immune functioning over time. To date, the literature shows inconsistent findings regarding the nature and directionality of the relationship between anxiety symptoms and biomarkers of the immuno-endocrine systems. To explore how different dimensions of anxiety symptoms (physiological versus cognitive-emotional) interact with and affect the underlying immuno-endocrine systems over time from childhood to adolescence, the current studies examined the following questions: (1) How and in what direction are anxiety symptoms associated with diurnal cortisol rhythms concurrently and longitudinally?; (2) Is there an association between anxiety and diurnal salivary immunoglobulin A (sIgA)?; (3) How and in what direction are anxiety symptoms related to overall sIgA levels?; and (4) Are there feedback mechanisms, whereby anxiety symptoms and immuno-endocrine biomarkers create a chain of sequential cause and effect, with each affecting the other in a transactional sequence over several years?

Data were collected from participants in the Concordia Longitudinal Risk Project between the ages of 9 to 18 using a multi-wave design. Repeated measures of self-report symptom questionnaires, salivary samples of endocrine and immune biomarkers, and demographic information were collected at each data wave approximately three years apart. Hierarchical linear modeling and autoregressive cross-lagged panel designs were used to analyze the data. Results showed that higher physiological anxiety symptoms were concurrently associated with elevated diurnal cortisol, whereas longitudinal results over three years showed that chronic worry and social concerns predicted lower diurnal cortisol, illustrating a more blunted diurnal cortisol profile. Diurnal sIgA results revealed a pattern of activation in children with higher anxiety, specifically, worries and social concerns, than those with lower anxiety. Higher levels of total

anxiety, worries and social concerns also led to lower levels of sIgA, which in turn led to increases in anxiety in an incremental "vicious" cycle from age 9 to 18. Taken together, these findings have important implications for understanding the developmental psychobiology of children's anxiety.

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Contribution of Authors

Denise Ma developed the research questions for the dissertation studies, designed and performed all the statistical analyses, interpreted the results, wrote and edited all chapters in the thesis. Dr. Lisa Serbin contributed to the data collection design within the studies. As the supervisor of Denise Ma, she provided guidance, comments, feedback, and suggestions throughout the research process on the design and interpretation of the analyses. She also read revised drafts of the dissertation manuscripts. Dr. Dale Stack contributed to the design of the data collection and recruitment of participants.

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Chapter 1: General Introduction

Anxiety disorders are among the most common psychiatric conditions, affecting between 5.7% and 20.2% of adults and children across different samples and countries (Beidel & Alfano, 2011; Brown, Campbell, Lehman, Grisham, & Mancill, 2001). While generalized anxiety disorder is the most prevalent in adults, separation anxiety, specific phobia and social phobia are the most frequently occurring anxiety disorders in children and adolescents (Beesdo, Knappe, & Pine, 2009; Costello, Egger, Copeland, Erkanli, & Angold, 2011; Kessler et al., 2010). Even without a diagnosis of anxiety disorder, the experience of persistent anxiety symptoms in childhood and adolescence can be extremely distressful and impair functioning across various domains including mental and physical health (Alonso & Lépine, 2007; Kessler et al., 2007; Merikangas & Kalaydjian, 2007).

Although anxiety symptoms in childhood are often transient and may be part of a normative developmental trajectory, a significant proportion of children continue to experience chronic anxiety symptoms and high levels of distress in adolescence and continuing through to adulthood (Orvaschel, Lewinsohn, & Seeley, 1995). Childhood anxiety symptoms that are chronic, unabated and intense pose significant risk for the development of later anxiety disorders, dysthymia and major depression in adulthood (Goodwin & Hamilton, 2002; Vasey & Dadds, 2001). If left untreated, these persistent anxiety symptoms may strain the underlying biological systems resulting in negative physical health outcomes in the long-term (Farrell, Donovan, Turner, & Walker, 2011; Rohrmann, Hennig, & Netter, 2000; Zvolensky & Smits, 2007).

To date, we have a limited understanding of the developmental psychobiology of anxiety symptoms in relation to aspects of the endocrine and immune systems over time (Gerra et al., 2003; Martin, Ressler, Binder, & Nemeroff, 2009). Findings from the developmental psychopathology literature are unclear about the longitudinal sequence of children's anxiety symptoms from childhood continuing through to adolescence and early adulthood. Given the high prevalence rates of anxiety symptoms and their burden on mental and physical health across the lifespan, the goal of the current dissertation studies was to clarify the exact nature, sequence and directionality of the transactional relationship between anxiety symptoms and the functioning of the neuro-endocrine (i.e., diurnal cortisol) and immune (i.e., salivary immunoglobulin A) systems over several years in a risk sample. By examining this question, future prevention programs and interventions targeting anxiety symptoms can better identify specific access points

at the cognitive, biological and behavioural levels to reduce anxiety and improve overall mental and physical health across the course of development.

Defining Anxiety

Anxiety is a universal experience and an important emotional response. It has genetic, biological, and social-environmental underpinnings (Eley, 1999; Hettema, Neale, & Kendler, 2001; Martin et al., 2009), with genetic influences accounting for approximately 30% of the variance across different studies (Bolton et al., 2006; Eley, Rijsdijk, Perrin, O'Connor, & Bolton, 2008; Gordon & Hen, 2004). Anxiety is a multidimensional emotional experience that is characterized by arousal, and associated with subjective experiences, somatic sensations, cognitions, and motivational tendencies (Hofmann & Hayes, 2015). Under normal conditions, anxiety functions as a biological warning system that helps individuals to anticipate and avoid potential harm in an evolutionarily adaptive manner. However, anxiety becomes potentially harmful when it is excessive in severity and duration, occurs in situations known to be harmless, or emerges spontaneously without provocation resulting in long-term functional impairments (Hoehn-Saric & McLeod, 1993).

Originally, Lang's (1968) tripartite model of anxiety divided anxiety into three response domains, including cognitive (self-reported), physiological (somatic bodily responses), and behavioural. Each of these three response domains represents separate and potentially independent underlying mechanisms to the overall construct of anxiety. Later, Rachman (2004) defined anxiety as a "tense, unsettling anticipation of a threatening but vague event" or a feeling of uneasy suspense (Rachman, 2004, pg 3). He further stated that anxiety is related to, but different from the underlying mechanisms of fear, which has a particular focus, and is a direct response to an identifiable fear stimulus. The duration of the fear response tends to be limited in time and focus, in contrast to anxiety, which is often pervasive and persistent, with uncertain beginnings and ends resulting in a sustained state of heightened vigilance and physiological arousal.

Although anxiety often follows fear, chronic anxiety can in turn generate and maintain fears creating a cycle of maladaptive response style and coping to perceived threats (Rachman & Taylor, 1993). The persistence of anxiety has been shown to be detrimental to long-term health through possible mechanisms including, but not limited to chronic activation and strain on underlying physiological arousal mechanisms of the autonomic nervous system (B. E. Cohen,

Edmondson, & Kronish, 2015; Roy-Byrne et al., 2008). However, the longitudinal sequence of the relationship between anxiety and the associated biological systems implicated in physiological arousal, notably the endocrine and immune systems, remain to be further explored.

Anxiety Symptom Dimensions

Many children experience sub-clinical levels of anxiety symptoms while only 10% of those reporting symptoms meet diagnostic criteria for any anxiety disorder (Morris & March, 2004). The experience of anxiety symptoms is distressful even without meeting diagnostic criteria for a disorder. Despite this, the preponderance of research to date has focused on clarifying the psychobiology and etiology of specific anxiety disorders (Copeland, Angold, Shanahan, & Costello, 2014; Fox & Kalin, 2014; Hilbert, Lueken, & Beesdo-Baum, 2014), while very little has examined the biological correlates of anxiety symptoms dimensions (Heller, 1990; Heller, Nitschke, Etienne, & Miller, 1997). A better understanding of anxiety from a symptom dimension approach may help inform early prevention of psychological disorders and reduce susceptibility to later physical illnesses.

Anxiety symptoms can be distinguished as being cognitive-emotional (trait) or physiological/somatic (state) (Spielberger, 1972, 1983). Cognitive-emotional symptoms (e.g., worry) are relatively enduring, and capture individual differences in how one perceives the world and the general tendency for an individual to respond anxiously under duress. In contrast, physiological symptoms of anxiety (e.g., increased heart rate, muscle tension, dizziness, nausea, abdominal pain) are more transitory, occur in direct response to threatening stimuli, and endure for only a short duration following the termination or removal of the threat. However, recurrent and chronic experiences of physiological anxiety symptoms may in turn make the individual more hyper-vigilant to the origins of these physical symptoms, triggering cognitive emotional symptoms (i.e., worries), and resulting in chronic and persistent activation of the autonomic nervous system (Stein & Steckler, 2010).

More recent studies examining anxiety from a transdiagnostic and dimensional approach have moved away from the Spielberger's original classification of state versus trait anxiety dimensions, towards conceptualizing anxiety as being composed of anxious apprehension (cognitive-emotional symptoms) and anxious arousal (physiological symptoms) dimensions (Sharp, Miller, & Heller, 2015). Anxious apprehension is predominantly distinguished by a tendency to engage in negative and repetitive thinking, reflecting cognitive aspects of anxiety. In

contrast, anxious arousal consists of an enduring pattern of hypervigilance, sympathetic nervous system hyperarousal and associated physiological symptoms (Nitschke, Heller, Palmieri, & Miller, 1999).

Previous findings from the literature have shown that cognitive-emotional and physiological anxiety symptom dimensions have distinct neurobiological basis, in addition to differential associations with underlying regional brain activity and neural mechanisms (Baeken, Vanderhasselt, & De Raedt, 2011; Heller, 1990; Heller et al., 1997). Several reviews of twin concordance in anxiety have concluded that variance in both anxious symptoms and disorder is accounted for by a combination of both heritability and unique environmental influences (Eley, 1999; Hettema et al., 2001). Studies have shown that cognitive-emotional anxiety symptoms (i.e., trait anxiety) are moderately heritable with genes accounting for 30% to 45% of the variance, with the rest of the variance accounted for by non-shared environment and measurement error (Bolton et al., 2006). Meanwhile, physiological anxiety symptoms were found to be best explained by shared environment environmental factors (Morris & March, 2004). What remains unknown is how anxiety symptom dimensions differentially relate to the underlying immuno-endocrine biological systems over time in children and adolescents.

Anxiety and the Stress Response System

As a starting point, studies have focused on understanding the link between anxiety and the underlying stress response system. A stressor is defined as any event that can activate a physiological response (Selye, 1975). Stress has been conceptualized as an inferred internal state, based on this physiological stress response. Stress requires a stimulus (often termed stressor), which can be environmental or internal (i.e., psychological states), and is intricately linked to subjective negative emotional responses, such as anxiety (S. Cohen, Janicki-Deverts, & Miller, 2007; Lazarus, 1998). Anxiety as an emotional response is closely related to the appraisals of a situation perceived as being threatening or stressful by the individual (Lazarus, 1998; Lazarus & Lazarus, 1996). More specifically, anxiety emerges as a response to unsuccessful coping when the individual judges the perceived threat or stressor to be insurmountable given their coping capacity (Lazarus & Folkman, 1984). Biondi and Picard (1999) further emphasized that "the subjective perception [cognitive appraisal] of the situation [is] a main determinant of the psychoendocrine response pattern in addition to the "objective" characteristics of a given event [stressor]" (pg. 114). As such, anxiety as an emotional response to perceived or actual threat, and

associated with heightened vigilance and physiological arousal, may be implicated in the overall functioning of the stress response system.

Cognitive affective processes in response to stressful situations and/or threat involve aspects of the underlying central nervous system. The thalamus and prefrontal cortex initially integrate important sensory information from the environment to help determine the meaning of the stimuli. These evaluations or cognitive appraisals could then lead to emotional responses through connections from the prefrontal cortex to the limbic system (i.e., hippocampus, amygdala), which are also primary pathways in the activation of the HPA axis (Lovallo, 2015). The capacity to mount an adrenocortical response to threat is essential to survival (Sapolsky, 1992). Contexts that are perceived as novel, unpredictable, uncontrollable, threatening and potentially involve loss and social threats are more likely to activate the stress response system, and are closely associated with the experience of both fear and anxiety symptoms (i.e., worries; anxious apprehension) (Dickerson & Kemeny, 2004). While a certain level of anxiety in response to short-term stress are both healthy and adaptive, chronic anxiety symptoms in response to chronic stress where individuals may experience uncontrollability and social evaluation/threats could result in potential negative long-term effects including pathophysiological changes in the brain, endocrine and immune systems to result in disease (Danese & McEwen, 2012).

Similar to the effects of chronic stress, it is theorized that the subjective experience of chronic anxiety symptoms (as an internal emotional state), may also be associated with physiological changes in the stress system over time (Charmandari, Tsigos, & Chrousos, 2005; Shekhar, Truitt, Rainnie, & Sajdyk, 2005). Specifically, chronic anxiety symptoms that are disproportional in response to a given threat or stressor is akin to responding with full-blow stress response when only vigilance is warranted. Anxious individuals may perceive the world as full of threats (actual and/or perceived) that demand their endless vigilance and coping on a day to day basis. Over time, this tendency may dysregulate adaptive endocrine functioning by placing excessive and prolonged demand on the stress system, to result in increased illness susceptibility in the future (Charney, Buxbaum, Sklar, & Nestler, 2013; S. Cohen, Miller, & Rabin, 2001). In other words, the experience of chronic anxiety is similar to engaging in "fight or flight" response too often and/or at inappropriate times, which may strain the body's stress system to potentially result in negative physical and emotional health consequences over time (Schulkin, McEwen, & Gold, 1994). In the face of short-term distress, the systems are designed to effectively cope with

the distress in an effective and timely manner. However, in the face of chronic arousal and distress, the body begins to show signs of "wear and tear" in its constant efforts to anticipate and adapt to the level of distress in order to recalibrate towards homeostasis (Dieleman et al., 2015; Juster, McEwen, & Lupien, 2010; McEwen, 1998).

Anxiety, HPA-Axis and Cortisol

Studies to date have focused primarily on the functioning and activation of the hypothalamic-pituitary-adrenal (HPA) axis, which has been shown to be dysregulated in anxiety disorders (Faravelli et al., 2011; Risbrough & Stein, 2006). One important component of the HPA axis is the regulatory role of the glucocorticoids (i.e., cortisol) steroid hormones produced by the adrenal gland under the control of the hypothalamus and pituitary gland, which constitute the HPA axis. Central control of glucocorticoid secretion is mainly regulated by select neurosecretory neurons in the hypothalamic paraventricular nucleus (PVN). When activated by stress, neurons in the hypothalamus release corticotropin releasing hormones (CRH) and arginine vasopressin (AVP), stimulating the pituitary gland to secrete adrenocorticotropin hormone (ACTH) into the bloodstream (Leonard, 2005; Tsigos & Chrousos, 2002). In turn, ACTH travels in the blood to reach the adrenal glands located above the kidneys to stimulate the activation of related downstream pathways and secretion of stress hormones (i.e., HPA-axis cascade).

The two main stress hormones include glucocorticoids (i.e., corticosterone in animals; or cortisol in humans), and catecholamines (epinephrine and norepinephrine). The secretion of cortisol and catecholamines in response to stressors are the main mediators in the chain of hormonal events triggered in response to stress. When these two hormones are secreted in response to stress, they act on the body to trigger the "fight-or-flight" response and associated physiological experiences (i.e., increased heart rate; blood pressure). The magnitude of the HPA stress response is typically regulated by both neuronal and hormonal mechanisms to help maintain cortisol levels within tolerable limits (Keller-Wood & Dallman, 1984).

Cortisol secretion as the hormonal end product of the HPA axis plays a crucial role in the organism's ability to adjust and adapt to challenges. Cortisol binds to glucocorticoid receptors that are present in almost every tissue in the body and consequently mediates many metabolic processes including cerebral perfusion rates and local glucose utilization, cardiovascular output and respiration, blood flow redistribution, energy mobilization, and modulation of immune functioning (McEwen & Seeman, 1999). Cortisol mobilizes energy in the form of glucose

metabolism, and at times, doing so at the expense of other biological systems (e.g., reproduction, immunity, and growth) (Sapolsky, Krey, & McEwen, 1986).

Adaptive versus Maladaptive Stress Response. Animal studies in the literature have shown that an adaptive stress response occurs when the system is activated briefly, and in proportion to the stressor magnitude. At the removal of the stressor, the system should shut off accordingly (McEwen, 1998). Stress responses that are acute, moderate, and short-lived are described as "positive stress" because the corresponding physiological responses are adaptive in increasing vigilance and leading to appropriate energy mobilization to cope with actual and/or perceived threat. These adaptive responses typically include increased heart rate and blood pressure, with mild elevations in cortisol levels in reaction to various challenges (e.g., being in a new environment, meeting new people and/or coping with a degree of unpredictability). In these contexts, elevations in cortisol as a physiological response are adaptive and healthy, provided that the activation of HPA axis is short-term in duration.

In contrast, when there is chronic activation of the stress system, without any buffering to appropriately halt this activation, then cortisol secretion is deemed to be more harmful. Possible factors that contribute to chronic stress and associated dysregulation in adaptive cortisol release include recurrent abuse, neglect, neighbourhood violence, and adverse and/or at-risk environments. These in turn are found to be associated with persistent increases in stress hormones to result in altered levels of brain chemicals. These changes have been associated with disruptions in the internal physiology and normative functioning of the developing brain (Miller, Chen, & Zhou, 2007). Deficits in learning, memory, and emotion regulation may result, possibly culminating in stress-related mental health disorders such as anxiety.

Chronic exposure to adversity including low socioeconomic status, natural disasters, maltreatment, and war have all been studied in relation to their adverse effects on cortisol production (Katz, Pellegrino, Pandya, Ng, & DeLisi, 2002; Lupien, King, Meaney, & McEwen, 2001). Observed in both humans and primates, empirical studies have shown that offspring exposed to deprivation, neglect and abuse reported lower basal levels of cortisol (Gunnar & Donzella, 2002). Possible mechanisms to help explain the lowered basal levels of cortisol involve down-regulation of the HPA axis at the level of the pituitary in response to chronic CRH drive from the hypothalamus (Fries, Hesse, Hellhammer, & Hellhammer, 2005). In addition, a second mechanism has been studied in relation to hypersensitivity to glucocorticoids (Yehuda, 2006).

Real or interpreted threats to homeostasis initiate both the sympathetic-adrenal-medullary (SAM) axis to release catecholamines, and the HPA-axis to release cortisol to mobilize the energy needed for appropriate fight-or-flight responses (Sapolsky, Romero, & Munck, 2000). While adaptive acutely, chronic over-activation of both the SAM and HPA-axis results in chain reactions and subsequent changes in interconnected biological systems. This potentially leaves the individual more susceptible to stress-related diseases over time (Korte, Koolhaas, Wingfield, & McEwen, 2005; Lupien et al., 2006; McEwen, 1998). The allostatic load model has been proposed to assess physiological dysregulation that occur when adaptive homeostatic mechanisms are shifted towards abnormal ranges following prolonged secretion of stress hormones associated with chronic stress. Allostasis defines health as a dynamic state of responsiveness to adapt to and appropriately cope with the changing demands from the environment (Sterling, 2004). In relation to chronic stress, the allostatic load model describes the "wear and tear" experienced in the body when recurrent threats leads to a recalibration of the body in order to respond to them appropriately (McEwen & Stellar, 1993). This in turn may result in "mal-adaptations" on interdependent and related systems (Juster et al., 2010).

Diurnal Cortisol Rhythm. Basal HPA axis activity also follows a distinct diurnal rhythm with the highest cortisol production occurring during the second half of the night with peak cortisol levels occurring in the early morning. In addition, there is a rapid increase of cortisol levels in the morning shortly after awakening (circadian peak) when a surge in cortisol initiates waking activities and prepares the body for the demands of the day (Klimes-Dougan, Hastings, Granger, Usher, & Zahn-Waxler, 2001). This cortisol awakening response (CAR) is a physiological response to awakening, typically occurring 20 to 30 minutes post-awakening, when cortisol release into the bloodstream results in a sharp increase ranging between 38% to 75% of awakening levels (J. Pruessner et al., 1997; Wilhelm, Born, Kudielka, Schlotz, & Wüst, 2007). This increase is inhibited by intake of low-dose dexamethasone (Ebrecht et al., 2000), which is a synthetic glucocorticoid imitating negative feedback signals from circulating cortisol to ACTH-secreting cells of the pituitary, which suggests that the CAR may be driven by hormonal release from the pituitary.

Cortisol gradually declines throughout the day from late afternoon to lower evening levels (circadian trough) when activity is diminished, before showing abrupt elevations after the first few hours of sleep (S. Edwards, Clow, Evans, & Hucklebridge, 2001; Kirschbaum &

Hellhammer, 1994). The diurnal elevation of cortisol is believed to be a "wake-up" signal to increase activity and hunger in nocturnally and diurnally active animals species (McEwen & Stellar, 1993). Levels of morning cortisol and the CAR response are found to be more heritable, stable, and biologically predetermined in a given individual, than afternoon and evening cortisol secretion levels, which are more labile and sensitive to environmental influences (i.e., mood, sleep, food) (Bartels, de Geus, Kirschbaum, Sluyter, & Boomsma, 2003; Shirtcliff et al., 2012; Van Hulle, Shirtcliff, Lemery-Chalfant, & Goldsmith, 2012).

The CAR has been found to be a distinct phenomenon that is superimposed on the diurnal rhythm of cortisol given its significant incremental linear effect in cortisol concentrations in the early morning upon awakening (Clow, Thorn, Evans, & Hucklebridge, 2004; Fries, Dettenborn, & Kirschbaum, 2009). It is also a reliable measure of the reactivity of the HPA axis, being the focus of studies not only with healthy individuals, but also in relation to disorders including cardiovascular, autoimmune, atopic, allergic, and psychiatric diseases (Clow et al., 2004; Wust et al., 2000). The diurnal rhythm of cortisol secretion is important and has been found to have associations with immune functioning (Turner-Cobb, Rixon, & Jessop, 2011; Wolf, Nicholls, & Chen, 2008), sleep (Gribbin, Watamura, Cairns, Harsh, & LeBourgeois, 2012), mental and physical health (Essex et al., 2011; Johnson, Bruce, Tarullo, & Gunnar, 2011; Ruttle et al., 2011), and learning and memory (Quas, Yim, Edelstein, Cahill, & Rush, 2011; Saridjan et al., 2013). Increased CAR was found in subjects experiencing chronic stress and worries (Schlotz, Hellhammer, Schulz, & Stone, 2004), work overload (Schulz, Kirschbaum, Prüßner, & Hellhammer, 1998), and increased stress early in the day (Williams, Magid, & Steptoe, 2005). In addition, alterations in CAR is possibly related to anticipation of upcoming demands of the day (Rohleder, Beulen, Chen, Wolf, & Kirschbaum, 2007).

Furthermore, the physiological mechanisms regulating acute stress-induced cortisol production is distinct from circadian fluctuations in cortisol output. Circadian influences on physiological systems are transmitted by the body's endogenous central pacemaker, the suprachiasmatic nucleus (SCN). Diurnal cortisol and the circadian rhythm of the HPA axis is predominantly controlled by the SCN, which influences adrenocortical activity through input to the paraventricular nuclei of the hypothalamus (Buijs, Van Eden, Goncharuk, & Kalsbeek, 2003; Dickmeis, 2009; Kalsbeek et al., 2006). The diurnal secretion of cortisol are associated with changes in underlying ultradian rhythm, which are pulses of cortisol secretion that occur about

once an hour rather than in response to stressors (Lightman, 2008). Moreover, the HPA axis secretes ACTH and cortisol during non-REM sleep when the hippocampus is inhibited (Wilhelm et al., 2007). However, it is not the only regulatory pathway for cortisol secretion, with pituitary-derived ACTH also being an important mechanism in animal studies (Ulrich-Lai, Arnhold, & Engeland, 2006).

One of the most consistent findings from the literature show that the hippocampus appears to be implicated in the regulation of the CAR (Fries et al., 2009; M. Pruessner, Pruessner, Hellhammer, Pike, & Lupien, 2007). The hippocampus is a region of the brain paradoxically known for its inhibitory effect on HPA axis activity, possibly relating to the regulation of CAR prior to awakening (Herman & Cullinan, 1997; Herman, Ostrander, Mueller, & Figueiredo, 2005). Furthermore, the role of the hippocampus in the regulation of the CAR is also supported by the anatomical and functional pathways linking the hippocampus to the SCN (Krout, Kawano, Mettenleiter, & Loewy, 2002).

The CAR has been examined within the context of other related physiological processes implicated and initiated by the awakening process. Although the CAR is modulated by circadian influences, it also reflects phasic psychophysiological processes specific to sleep-wake transition (Wilhelm et al., 2007). Distinct from stress-induced cortisol production, the CAR may be initiated by the awakening process, and the switching of brain circuitry underlying the transition between sleep and consciousness (Sil'kis, 2009). Furthermore, a role for the SCN in regulating the CAR in human studies is closely influenced by sensitivity to light. Specifically, morning awakening in total darkness has been found to reduce the dynamic of the CAR relative to morning awakenings in light (Scheer & Buijs, 1999). Clow and colleagues (2010) provide a detailed review and summary of the range of physiological regulatory influences implicated in the regulation of both pre-awakening and post-awakening CAR, which is beyond the scope of the current dissertation.

Diurnal Cortisol and Anxiety. Dysregulations and changes in the diurnal cortisol rhythms have been reported in the literature showing mixed and at times, contradictory results with anxiety (Dierckx et al., 2012; Dietrich et al., 2013; Doane et al., 2013; Van den Bergh, Van Calster, Puissant, & Van Huffel, 2008). Confusing and contradictory findings arise in these studies with some researchers finding no HPA axis abnormalities or only weak associations at best in children with anxiety disorders or symptoms (Dietrich et al., 2013; Puig-Antich et al.,

1989). Others have found that children with anxiety disorders exhibited significantly lower night-time cortisol compared to depressed and healthy controls (Feder et al., 2004). On the other hand, some studies have reported the opposite finding in that adolescents with anxiety disorders exhibited elevated cortisol levels (Forbes et al., 2006). Longitudinally, a recent study by Dierckx et al. (2012) found that persistence of an anxiety disorder was associated with higher secretion of daytime cortisol, but also a blunted, decreased morning cortisol rise (slope). Furthermore, children and adolescents with resistant anxiety disorders who did not respond to treatment over a year had shown the most flattening of their morning cortisol rise. This longitudinal study identified aspects of elevations and blunting in terms of diurnal cortisol dysregulation relating to anxiety (Dierckx et al., 2012), further igniting the debate about the nature and sequence of the relation between anxiety and diurnal cortisol secretion.

In children and adolescents, an association between internalizing problems, including anxiety symptoms, and changes in cortisol secretion has been reported in numerous studies showing divergent results (Dietrich et al., 2013; Goodyer, Herbert, Moor, & Altham, 1991; Hastings et al., 2011; Heim, Ehlert, & Hellhammer, 2000; Kallen et al., 2008; Ruttle et al., 2011; Shirtcliff & Essex, 2008; Suzuki, Belden, Spitznagel, Dietrich, & Luby, 2013). While some cross-sectional studies have reported higher levels of internalizing symptoms to be associated with higher cortisol levels (Cicchetti & Rogosch, 2001; Pérez-Edgar, Schmidt, Henderson, Schulkin, & Fox, 2008), other studies have reported lower levels of cortisol (Granger, 1998). Additionally, mid-afternoon cortisol levels showed the most significant day effect and the highest correlation with internalizing disposition (S. Li, 2009). Within the longitudinal literature, elevated basal cortisol tended to precede the development and onset of later internalizing symptoms in children more consistently (Smider et al., 2002). Differences in the reported results could be potentially associated with the nature and duration of the stressors examined (Spies, Margolin, Susman, & Gordis, 2011), the level of symptomology endorsed (i.e., clinical versus non-clinical samples) (Klimes-Dougan et al., 2001), sex differences (Schiefelbein & Susman, 2006) and additional environmental factors including but not limited to differences in socioeconomic status (Lupien et al., 2001). However, the longitudinal sequence of the dysregulation of diurnal cortisol rhythms in relation to specific anxiety symptom dimensions over time in childhood and adolescence remains unclear.

Biological Plausible Models and Mechanisms Linking HPA Axis Dsyregulation and Anxiety

Negative emotions, including but not limited to the experience of anxiety symptoms are associated with the activation of HPA axis in animal studies (Landgraf, Wigger, Holsboer, & Neumann, 1999). HPA axis hyperactivity has been a consistent finding amongst individuals with anxiety disorders including panic (Abelson, Khan, Liberzon, & Young, 2007), social anxiety (Condren, O'Neill, Ryan, Barrett, & Thakore, 2002), and generalized anxiety disorder (Martin et al., 2009). In animal studies, Wistar rats bred for high anxiety-related behaviours also showed signs of stress vulnerability including hyper-reactivity of the HPA axis in response to stressors (Landgraf et al., 1999). Cortisol has lipophilic properties, which make these adrenal hormones able to cross the blood-brain barrier to enter the brain and influence brain function, behaviour, and emotional responses (including anxiety) by way of binding to different receptor types in the brain. A few of the main structures implicated include the hippocampus, amygdala, and frontal lobes given their important roles in learning, memory, and emotion regulation (Lupien et al., 1999).

Cortisol modulates memory of emotionally arousing experiences. Emotional memory is hypothesized to play a crucial role in the pathogenesis and symptomatology of anxiety (Sapolsky, 1992; Sapolsky et al., 2000). Existing theories of the etiology of anxiety argues that increased release of glucocorticoids and neuropeptides, specifically, corticotropin-releasing factor (CRF), during fear increases the excitability of fear circuits to play a role in the organization of pathological anxiety in both humans and animals (Rosen & Schulkin, 1998; Bale et al., 2000). CRF is also known to mediate many of the behavioural effects of stress and is involved in stress-induced anxiety (Adamec & McKay, 1993). HPA axis activation is initiated by CRF from the hypothalamus, contributing to the hypothesis that hypothalamic CRF over-expression may contribute to HPA axis hyperactivity observed in those with depression and anxiety. Furthermore, elevated CRF in cerebrospinal fluid is also observed in individuals with both mood and anxiety disorder, adding to the evidence base of its role in HPA dysregulation (Flandreau et al., 2012). The hyperexcitability of the CRH-HPA system is thought to form an important biological diathesis for the development of anxiety disorders and may sensitize the organisms to later environmental stressors (Adamec & McKay, 1993).

Hyperactive HPA axis from stress in turn has been associated with increased turnover of serotonin in the prefrontal cortex, nucleus accumbens, amygdala and lateral hypothalamus.

Activation of the serotonergic systems may stimuluate anxiogenic and anti-anxiety pathways depending on the type of serotonin receptors stimulated. More specifically, stress and cortisol acts on the expression of 5HT1A and 5HT2A receptors, which are relevant to the psychopathology of anxiety (Leonard, 2005).

Existing literature shows that anxiety disorders are associated with abnormalities in the HPA axis, which are distinct from those consistently found in relation with mood disorders, notably in depression (Mathew, Price, & Charney, 2008; Risbrough & Stein, 2006; Sharp et al., 2015). Anxiety is characterized by hypocortisolemia, supersuppression after dexamethasone, and increased numbers of glucocorticoid receptors. In contrast, depression is characterized by hypercortisolemia, non-suppression after dexamethasone, and decreased numbers of gluco-corticoid receptors. These differences have led to the development of a potential "neuroendocrine continuum" model where a general desensitization of CRF receptors at the pituitary, limbic (amygdala), cortical and hippocampal levels may be linked to repeated stressful events (Boyer, 2010).

Anxiety, Stress and Immunity

The secretion of cortisol and mobilization of the HPA axis has cascading effects downstream in associated biological systems, including the immune system. The endocrine and immune system are interrelated through bidirectional associations. Exposure to stress triggers the release of CRF and vasopressin via activation of the parvicellular neurons from the paraventricular nucleus (PVN) in the hypothalamus. These hormones then stimulate the synthesis of polypeptide precursor pro-opiomelanocortin (POMC) products, which include corticotropin, to interact with the immune system (Jessop, 1998; Lipton & Catania, 1998). Activation of the HPA axis is also seen in responses to stress involving activation of aspects of the immune system. The cytokine interleukin-1 induces the secretion of CRF and corticotropin via the interleukin-1 type I receptors (Sapolsky, Rivier, Yamamoto, Plotsky, & Vale, 1987), stimulating glucocorticoid secretion, and thus interacting with the negative feedback mechanism (Turnbull & Rivier, 1999).

The immune system is a wide-ranging and integrated network of related structures that maintain health. The various immune components work together to defend the body against external pathogens and aid in eliminating internal cells that are causing damage (Bosch, Ring, de Geus, Veerman, & Amerongen, 2002). While short-term stress and anxiety may be adaptive and lead to redistribution of immune cells to areas where they can act quickly and efficiently against

pathogens, the continued secretion of cortisol in response to chronic arousal leads to bone demineralization, shifts in metabolism and compromised immune system functioning over time (Daruna, 2012; Sapolsky, 1992). Studies have shown that in general, acute stress that are limited in duration (i.e., laboratory tasks) generally "up-regulate" or increase innate immunity. At heightened levels, cortisol also plays an anti-inflammatory role for infection or injury to enable lymphocyte circulation in the body, which is essential for "fight or flight" response (Wilson, 2001). In contrast, chronic stress has been associated with decreased overall circulating immune cells and suppressed immunity functioning associated with glucocorticoid resistance (S. Cohen & Herbert, 1996; Segerstrom & Miller, 2004). However, not all components of the immune system functioning are suppressed. Production of proinflammatory cytokines is one aspect enhanced in response to the experience of acute stress (S. Cohen et al., 2012).

Psychoneuroimmunology models linking psychological variables and mental health to immune functioning and illness susceptibility have focused on direct innervation of the central nervous system, hormonal pathways, in addition to behavioural changes (S. Cohen & Herbert, 1996; Kiecolt-Glaser, McGuire, Robles, & Glaser, 2002). Individuals experiencing negative mood states, including anxiety, in response to stressful events often engage in poor health practices (i.e., smoking, poor dietary choices) and poor sleeping habits, which may in turn have immunosuppressive effects on their overall health (Kiecolt-Glaser & Glaser, 1988). A handful of studies investigating the relations between anxiety and immunity have found that anxious mood is associated with decreased NK activity (Locke et al., 1984) and decreased proliferative response to both phytohemagglutinin (PHA) and concanavalin A (Arranz, Guayerbas, & De la Fuente, 2007; Linn, Linn, & Jensen, 1981). Antibody levels were also found to be higher on days when individuals reported positive mood than on days when they reported higher negative mood states (Stone et al., 1994).

Temporary experience of anxiety as a direct emotional response to perceived threats, activate autonomic and endocrine systems to divert resources away from nonessential processes such as immunity and digestion toward systems (e.g., musculature, brain) needed to execute defense against perceived threats in the short term (Charney et al., 2013). Animal studies with mice models have suggested a hyper-reactivity of at least one aspect of immune functioning following short-duration stressors. The augmentation of delayed-type hypersensitivity (DTH) responses in the skin appeared to be mediated through glucocorticoid and epinephrine stress

responses (Dhabhar & McEwen, 1999). A similar finding has also been found in human studies where socially inhibited individuals produced an increased DTH skin response after experiencing high social engagement (S. W. Cole, Kemeny, Weitzman, Schoen, & Anton, 1999). Academic stress in addition to other commonplace and short-term stressors are associated with changes in a variety of immune activities (Marucha, Kiecolt-Glaser, & Favagehi, 1998).

Studies to date have shown that prolonged intrusive ruminations following trauma or natural disasters have associations with maladaptive psychological functioning and may be associated with immune dysregulation (Baum, Cohen, & Hall, 1993). Negative mood, including anxiety, was associated with reduced NK cell lysis in women (Valdimarsdottir & Bovbjerg, 1997). The effects of chronic stress over time on the immune system functioning has not been examined to date in children and adolescents over the course of key developmental transitions. Drawing from the above literature, the current dissertation studies will attempt to highlight the longitudinal associations between anxiety symptoms and one specific aspect of mucosal immunity, that of salivary immunoglobulin A.

Anxiety and Mucosal Immunity: Salivary Immunoglobulin A

One specific aspect of the immune system is mucosal immunity, which include the functioning of salivary immunoglobulin A (sIgA), among other components. SIgA is a non-specific antibody found in high concentrations on mucosal surfaces and barriers of the body (i.e., tears, saliva, gastrointestinal tract, respiratory epithelium and the gut) (Cunningham-Rundles, 2001; Marcotte & Lavoie, 1998; Yel, 2010). As the most abundant class of antibodies found in the intestinal lumen of humans and most mammals, sIgA is a “first line of defense” against invading organisms and play a key role in the prevention of infectious diseases (Cerutti & Rescigno, 2008; Hereman, 2014; Woof & Kerr, 2006). SIgA concentration is typically at 20% of the typical adult value by 1 year of age and peak between age 31 to 40, while showing a slight decrease in healthy subjects between the age of 61 to 70 (Jafarzadeh, Sadeghi, Karam, & Vazirinejad, 2010; Weemaes et al., 2003).

SIgA defends against foreign antigens by preventing attachment to or penetration of body surfaces by invading microorganisms, clearing antigens and pathogenic microorganisms from the intestinal lumen, and modulating the balance between pro- and anti-inflammatory states (Jacob, Pastorino, Fahl, Carneiro-Sampaio, & Monteiro, 2008; Mantis, Rol, & Corthésy, 2011; Woof & Kerr, 2006). Lower levels of sIgA have been shown to be a risk factor for susceptibility to

infections, asthma, allergies, increased upper respiratory infections, periodontal disease and caries (E. Edwards, Razvi, & Cunningham-Rundles, 2004). In contrast, higher levels of sIgA indicate immunocompetence (Deinzer & Schüller, 1998; Lawrence, 2002; Volkmann & Weekes, 2006). A limited number of studies have shown diurnal variations of sIgA in adults with concentration peaking at awakening, then decreasing for the next 4 hours, while remaining relatively constant towards early evening (Hucklebridge, Clow, & Evans, 1998; Miletic, Schiffman, Miletic, & Sattely-Miller, 1996; Zeier, Brauchli, & Joller-Jemelka, 1996).

SIgA levels have been associated with psychological states and negative emotions including anxiety (Bosch et al., 2002; Hucklebridge et al., 1998; Phillips et al., 2006). On the one hand, some studies have shown that short-term anxiety and laboratory stress led to temporary increases in sIgA levels in participants (Dhabhar, 2009; Dhabhar et al., 2010; Hucklebridge et al., 2000). Other studies have reported that negative mood is associated with decreased sIgA concentrations, which in turn predicted higher susceptibility to physical illnesses (Bosch, Ring, & Amerongen, 2004; Deinzer, Kleineidam, Stiller-Winkler, Idel, & Bachg, 2000; F. Li, Han, Ren, & Luo, 2008; Tsuboi et al., 2008).

In comparison, the existing literature on the effects of longer term stress yielded more consistent results. Chronic stress including academic exam stress and anxiety (F. Cohen et al., 1999), burden of care giving (Bauer et al., 2000), and cumulative negative daily hassles have all been found to be immunosuppressive, leading to increased susceptibility to infections and respiratory illnesses (F. Cohen et al., 1999). To date, very little is known about the relationship between children's persistent anxiety symptoms and the effects on their developing mucosal immunity over the span of several years. It is important to understand the longitudinal relationship between anxiety symptoms and immunity especially in children from a longitudinal framework to help prevent illness susceptibility, which may be impacted by both anxiety and underlying immune dysregulation.

Early Environmental Risk Factors for Anxiety

Although the focus of the current dissertation studies is to understand the psychobiology of anxiety from a symptom dimensional approach, the contextual framework around the child's early life and current environment also matter. As with all psychological disorders, a transactional interaction between genetic, biological and environmental factors contributes to the etiology and developmental trajectory of anxiety symptoms and disorders. The biopsychosocial

model conceptualizes clinical disorders, such as anxiety, as a combination of physiology (e.g., biology, genetic inheritance); psychology (e.g., learning, cognitive styles, personality traits); and social/contextual variables (e.g., social support, interpersonal relationships, environmental influences) (Blascovich & Tomaka, 1996). To understand the developmental psychobiology of anxiety in childhood and through to adolescence, environmental and contextual factors must also be taken into account.

Specifically, early life adversity, adverse family environment (e.g., family disruption, parent-child interactions, attachment), low socioeconomic status, family history of mental illness, parental educational attainment, amongst others have consistently been found to be risk factors for higher incidences of anxiety symptoms and emotional disorders later on in a child's life (Andrews, Charney, Sirovatka, & Regier, 2009; Essex, Klein, Slattery, Goldsmith, & Kalin, 2014). The effects of these stressful environmental risk factors on the developing brain in turn interact with and possibly are even mediated by a range of neurochemical and neuroendocrine pathways, including the glucocorticoid and immune systems (Nusslock & Miller, 2015). It is posited that early adverse experiences may be associated with persistent sensitization of stress-responsive neural circuits (Kaufman, Plotsky, Nemeroff, & Charney, 2000). Studies have shown that physical (i.e., food deprivation) and psychological (i.e., restraint, overcrowding, neglect) variables, in addition to unpredictable and random presentation of stressful stimuli in early life induce a state of permanent alertness and preparedness to mount a prompt and appropriate behavioural and physiological response. This in turn may be associated with the persistent activation of the HPA axis and associated systems, including immune functioning, resulting in deleterious effects on well-being over time (Essex et al., 2011, 2014; Stein & Steckler, 2010). As such, early environmental factors interact, shape and may even "program" functioning of the biological systems and the development of anxiety symptoms, serving as an important contextual backdrop to the questions examined in the current studies (Bale et al., 2010; O'Connor, Heron, Golding, & Glover, 2003).

Concordia Longitudinal Risk Project

Given that early environmental and contextual variables are associated with dysregulation in biological systems and are risk factors for the development of later anxiety symptoms and disorders, we might better understand individual differences in the psychobiology of anxiety using a risk sample where normative developmental trajectories are thwarted (Serbin & Karp,

2008). The data collected for the current dissertation was from the Concordia Longitudinal Risk Project (CLRP), which is an intergenerational study that began in 1976, involving children from disadvantaged neighbourhoods in and around Montreal, Quebec to explore questions of intergenerational transfer of risk for psychopathology within families (Ledingham, 1981; Schwartzman, Ledingham, & Serbin, 1985; Serbin et al., 1998). These original participants are now adults and many are parents with children of their own. A subsample of the offspring of the original participants has been followed over time and is the primary focus of the current studies.

These children may be at higher risk for the later development of psychopathology and physical health problems because of the higher than average exposure to cumulative risk factors and early life stress over the course of development. Some of these risk factors are at the level of the family environment (e.g., higher rates of father absence; lower family incomes; higher rates of receiving welfare), while others include neighbourhood characteristics (i.e., higher poverty), educational attainment (e.g., higher school drop-out rates), and elevated parental psychopathology (e.g., higher aggression and withdrawal), and poorer parent-child relationships (Granger, 1998; Pougnet, Serbin, Stack, & Schwartzman, 2011; Serbin & Karp, 2004).

Several empirical studies have also shown allostatic load or signs of "wear and tear" in disadvantaged youths. Specifically, higher allostatic load has been linked to cumulative risk factors including crowding, housing issues, family conflicts, violence, and maternal high school education attainment when children are at age 9 (Evans, 2003). Biological embedding of adversities during sensitive periods in development could in turn affect physiological expressions of the stress response system to stressors being processed differently as either positive, tolerable or toxic stress (Shonkoff, Boyce, & McEwen, 2009). This may in turn relate to experiences of anxiety symptoms and other mental health sequelae.

Interestingly, not all children of the original participants in the CLRP project were affected by the "risk" status and the cumulative early life stressors in the same way, as evidenced by the normative distribution of anxiety symptoms within the current sample (i.e., presence of low, average to high levels of anxiety were reported by the children). As such, the current studies were able to examine and compare differences between and within individuals on both anxiety symptoms and underlying endocrine and immune correlates, while also controlling for the confounding effects of relevant contextual variables such as maternal educational attainment, socioeconomic status, and family income.

Bidirectional Interactions between Mental Health and Biology

The relations between psychological and physical disorders are likely to be bidirectional and involve multiple feedback loops. Affective disturbances can influence underlying biological, behavioural, cognitive and social pathways to result in physical disorders and illness behaviours later on in development (S. Cohen & Rodriguez, 1995). Mental health has been found to shape aspects of individual's biology in ways that may increase risk for the development of both physical health and mental health disorders. Especially relevant to the current intergenerational risk sample, exploring the longitudinal bidirectional relationships between anxiety symptoms (mental health) and underlying biology will help further disentangle what increases risk, and the risk trajectories for later problems in adulthood (Fergus & Zimmerman, 2005).

Early childhood adversity has been found to be associated with changes in cortisol levels, with increased cortisol elevations associated with later development of major depression (Halligan, Herbert, Goodyer, & Murray, 2004). Studies have shown that early life experiences increases basal secretion of cortisol in adult life, in addition to the HPA axis reactivity to stress, which in turn may be a biological risk for the onset of various stress-related psychological disorders. Trait and state changes in negative mood (e.g., worry/anxiety, anger/frustration, depressed mood) were found to be related to increases in cortisol throughout the day suggesting ongoing transactions that occur between adolescents' daily emotional experiences and cortisol levels (Adam, 2006). At the same time, dysregulation and alterations in biological functioning, in this case, diurnal cortisol and/or mucosal immunity, can also have a bidirectional and transactional effect on anxiety symptoms by way of feedback mechanisms (Gunaratne, Lloyd, & Vollmer-Conna, 2013; Nusslock & Miller, 2015; Walker, Dantzer, & Kelley, 2013).What is currently missing in the literature is an extension of these daily sampling studies to an extended time frame, capturing key transitions in child and adolescent development. In understanding how aspects of biology (HPA axis; immunity) interacts with mental health symptoms in a bidirectional and transactional manner over several years, within the context of a risk sample, this would help clarify both risk and protective factors from a developmental perspective.

The Current Dissertation: Research Questions

After an extensive literature review, the current dissertation studies aimed to address several specific questions and important gaps that exist in our understanding of the developmental psychobiology of anxiety. First, *what is the longitudinal association and*

directionality of the relationship between children's anxiety symptoms in childhood and 1) the HPA-axis system, specifically, diurnal cortisol variation; and 2) aspects of the immune system, specifically, sIgA levels, over several years? A limited number of longitudinal studies currently exist in the literature examining mental health symptoms and/or internalizing symptoms generally, which leaves the question of directionality and transactional feedback loops unanswered in relation to the specific anxiety symptom dimensions (Essex et al., 2011; Shirtcliff & Essex, 2008). In clarifying and understanding better the long-term association between anxiety symptoms and components of each of these two systems separately, it will help pave the way for establishing hypotheses in future studies that will integrate multiple related systems in our understanding of the psychobiology of anxiety.

Longitudinal Transactional Feedback Loops.

As mentioned above, psychological symptoms, physical health and the underlying biological systems interact in a bidirectional and transactional manner. Psychological disorders and the experience of negative emotion, such as anxiety may be perceived as being distressful by the individual, which could have associations with underlying biological systems and possibly relate to stress-related immune alterations (Hucklebridge et al., 2000; Kemeny & Laudenslager, 1999; F. Li et al., 2008; Miller & Cohen, 2001; Rabin, 1999; Segerstrom & Miller, 2004)**.** Given this reciprocal relationship and expanding upon the first question of longitudinal sequence and directionality, we asked, *is there evidence of transactional feedback loops whereby anxiety symptoms and biomarkers of the endocrine and immune system are components of a chain of sequential cause and effect, mutually regulating each other over several years?*

Transactional feedback loops are reciprocal processes involving at least two systems, commonly used in studies of the interactions between individuals and their surrounding contexts that may result in an interrelated and mutually regulated sequence of changes observable in both systems (Bronfenbrenner & Morris, 2006; Cicchetti, 1993; Sameroff, 1975). Anxiety symptoms and biomarkers are posited to be components of a chain of sequential cause and effect, with each affecting the other in a circular, feedback loop (e.g., A→ B → A) (Thomas, Thieffry, & Kaufman, 1995). Furthermore, these transactional feedback loops can be described as either "vicious" (i.e., positive or augmenting feedback loops) or "virtuous" (i.e., negative feedback loops). A vicious cycle is characterized by the continued perpetuation of the effects, whereas a virtuous cycle is self-correcting over time. To our knowledge, these feedback mechanisms have

not been tested empirically given the cotemporaneous design of the majority of the studies within the literature to date.

Differential Associations of Anxiety Symptoms Dimensions

Third, *are there differential concurrent and longitudinal associations between specific anxiety symptom dimensions (i.e., physiological versus cognitive-emotional anxiety symptoms) and dysregulation of both cortisol and sIgA functioning?* A few existing studies have noted differential associations between anxiety symptom dimensions and functional brain associations (Heller et al., 1997). It is possible that cognitive emotional anxiety symptoms and physiological anxiety symptoms differentially impact the underlying functioning of the endocrine and mucosal immunity systems. Others have also stressed the importance of examining psychological disorders from a transdiagnostic perspective with a focus on symptoms, rather than focusing exclusively on diagnostic categories of disorders (Sharp et al., 2015). Because the experience of sub-clinical anxiety symptoms is extremely common and distressful in childhood, a closer exploration of the etiology of anxiety by way of symptom dimensions is warranted in relation to the underlying biological correlates.

Overview of Studies

To address each of the overarching research questions above in detail, two studies were included in the dissertation. Study 1 focused on the association between anxiety and diurnal cortisol (endocrine system), while Study 2 examined anxiety and sIgA (mucosal immunity). Specifically, Study 1 explored the concurrent and longitudinal associations of physiological anxiety and cognitive emotional anxiety symptoms with diurnal cortisol over two consecutive waves of data collection between ages 9 to 12, and again from age 12 to 15. Utilizing multi-level modeling to test a traditional cross-lagged panel design equivalent (see Figure 1 for a conceptual model of the design), we explored the sequence and directionality of the relationship between anxiety symptoms and diurnal cortisol over time.

Similarly, Study 2 explored the association between anxiety symptoms dimensions and diurnal sIgA when the children were between ages 9 to 12 (Study 2a), improving upon the design of the first study. Study 2 extended the first study by including a third wave of data collection (ages 15 to 18) to examine the longitudinal associations between anxiety symptoms and aggregated total levels of sIgA (Study 2b). By incorporating this third consecutive wave of data collection, the design of Study 2b allowed for the examination of transactional feedback loops

between anxiety symptoms and sIgA from age 9 to 18, to highlight transactional sequence of the relationship over time. The importance of including a third wave of consecutive data collection allowed for clarifications to the questions of "Which comes first?" and "What happens next?" in understanding the relationship between children's anxiety symptoms and overall sIgA levels.

In summary, the current studies were intended as a first step to disentangling the developmental psychobiology of the anxiety in its association with the endocrine and mucosal immunity systems from a symptom dimensional approach. A better understanding of the differences between anxiety symptoms dimensions in their respective associations to the underlying biological systems may inform future prevention and intervention programs to better ensure optimal psychological and physical health of the individual over the lifespan. In understanding the transactional and longitudinal sequence between anxiety symptoms and diurnal cortisol and sIgA, results from the current studies may contribute to our understanding of the complex interrelations between psychological symptoms and biological correlates over several years during the formative years of childhood through to late adolescence. These studies will be a starting point in helping to inform future research hypotheses regarding more complex associations between anxiety, physical health and illnesses, and multiple related biological systems.

Chapter 2: Study 1

How Children's Anxiety Symptoms Impact the Functioning of the HPA Axis over Time: A Cross-Lagged Panel Approach Using Hierarchical Linear Modeling

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Abstract

Anxiety symptoms in childhood and adolescence can have a long-term negative impact on mental and physical health. Although numerous studies have shown dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis (which controls cortisol secretion) is associated with anxiety disorders, it is unclear how and in what direction children's experiences of anxiety symptoms, which include both physiological and cognitive-emotional dimensions, impact the functioning of the HPA axis over time. We hypothesized that higher physiological anxiety would be cotemporaneously associated with hypercortisolism, while cognitive-emotional symptoms may be more chronic, reflecting trait-like stability, and thus would predict hypocortisolism over time. The sample included 120 children from the Concordia Longitudinal Risk Research Project, who were followed in successive data collection waves approximately 3 years apart from early childhood through mid-adolescence. Between ages 10-12 and 13-15, children completed self-report questionnaires of anxiety symptoms, and provided salivary cortisol samples at 2-hour intervals over two consecutive days. Results from hierarchical linear modeling showed that higher physiological anxiety symptoms were concurrently associated with hypercortisolism, involving cortisol levels that remained elevated over the day. In contrast, longitudinal results over the three years between data collection waves showed that chronic worry and social concerns predicted hypocortisolism, showing a low and blunted diurnal cortisol profile. These results have implications for broadening our understanding of the links between anxiety, the stress response system, and health across the course of development. Implications for developmentally appropriate prevention programs for children and youth are discussed.

Keywords: anxiety, diurnal cortisol, stress, HPA axis, developmental psychobiology

How children's anxiety symptoms impact the functioning of the HPA axis over time: A cross-lagged panel approach using hierarchical linear modeling

Anxiety disorders are highly prevalent and negatively impact both mental and physical health across the developmental lifespan. An epidemiological review reported prevalence rates of anxiety disorders, in which symptoms cause ongoing distress and interfere with normal life activities, to be between 6.1% and 14.8% in children aged 2 through 8 years, and 10.3% to 12.2% in adolescents aged 13 through 18 years across studies (Costello et al., 2011). Anxiety symptoms include both physiological and cognitive-emotional dimensions, which may affect physical and psychological well-being even without meeting diagnostic criteria for a given anxiety disorder. The widespread prevalence of anxiety in childhood and its detrimental long-term effects on psychological and physical health suggest reciprocal relations with underlying physiological mechanisms involving the body's stress-response and arousal systems (Asbrand, Blechert, Nitschke, Tuschen-Caffier, & Schmitz, 2016; Chen, Raine, Soyfer, & Granger, 2015). From a developmental psychobiological perspective, understanding the nature and sequence of the relationship between anxiety symptoms and dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis may disentangle transactional processes in which anxiety symptoms interact with and shape underlying neuroendocrine processes across development.

Dimensions of Anxiety Symptoms

Anxiety is a universal experience and an important emotion that has genetic (Minelli & Maffioletti, 2014), biological (Newman, Llera, Erickson, Przeworski, & Castonguay, 2013) and environmental bases (Rapee, 2012). Typically, it functions as an aspect of the biological warning system that helps individuals to anticipate and avoid potential threat. However, anxiety becomes abnormal when it is excessive in severity and duration, occurs in situations known to be harmless, or emerges spontaneously without apparent provocations, resulting in chronic arousal of the stress response system (Dieleman et al., 2015).

Anxiety consists of physiological or "anxious-arousal" (e.g., stomach-aches, heart palpitations, sweating, shakiness, nausea, and shortness of breath) and cognitive-emotional or "anxious-apprehension" (e.g., fear, worry, concerns, unease, dread) dimensions. These two symptom dimensions are hypothesized to reflect different facets of anxiety and are associated with unique patterns of underlying brain activity (Burdwood et al., 2016). Similarly, these anxiety dimensions can be examined in relation to HPA axis and diurnal cortisol rhythm from a

dimensional approach (Sharp et al., 2015). However, how physiological and cognitive-emotional anxiety symptoms may differentially relate to the HPA-axis functioning and diurnal cortisol rhythm in children and adolescents remains unclear to date.

HPA Axis and Diurnal Cortisol

The hypothalamic-pituitary-adrenal (HPA) axis is the primary mammalian system regulating stress response and arousal processes. Any short- or long-term psychological or physical experiences can threaten the homeostasis (e.g., internal balance) and strain the underlying systems (de Kloet, Joels, & Holsboer, 2005). Chronic strain imposed on the HPA-axis have detrimental effects on both long-term physical and psychological health (Lovallo, 2015). Optimal cortisol secretion to allow for an effective stress response follows a circadian diurnal rhythm with basal levels of cortisol being the highest in the mornings shortly after awakening (Clow et al., 2010). Following this cortisol awakening response (CAR), cortisol levels gradually decline throughout the day to lower evening levels when activity is diminished. Optimal diurnal cortisol rhythm impacts children's immune functioning (Turner-Cobb et al., 2011), sleep (Zeiders, Doane, & Adam, 2011), mental and physical health (Essex et al., 2011), and learning and memory (Keller, El-Sheikh, Vaughn, & Granger, 2010).

Diurnal cortisol can become dysregulated when the HPA axis is over-reactive or hypersensitive to stress (hypercortisolism), and/or showing signs of "wear and tear" and down-regulation in response to chronic strain or cumulative stress loads over time (hypocortisolism) (Fries et al., 2005; Juster et al., 2010). Although acute stressors or threats are often associated with temporary over-secretion, a blunting of the HPA axis has been observed after chronic strain or over-arousal as the system tries to adapt and modulate in response to the strain, and may be indicative of more serious chronic dysregulation in children and adolescents (Dieleman et al., 2015). Early life adversity, chronic risk factors and stress, including lower socioeconomic status, income levels, maternal education, parenting, trauma, and abuse amongst others, have all been found to be associated with the dysregulation of the HPA-axis and diurnal cortisol, although showing inconsistent patterns of results in relation to hyper- or hypocortisolism (C. B. Stroud, Chen, Doane, & Granger, 2016; Vliegenthart et al., 2016).

Additionally, individuals' subjective experiences of cognitive-emotional and physiological anxiety symptoms can reciprocally influence underlying biological processes in a bidirectional manner (Hastings et al., 2011). The experience of chronic psychological symptoms

over time could also result in prolonged distress for the individual, and in turn alter the optimal functioning of the HPA-axis and diurnal cortisol rhythm, and aspects of the fear circuitry (Dieleman et al., 2015; McTeague & Lang, 2012). At present, it is not clear which problem anticipates the other, or whether there is an ongoing transactional relation between specific anxiety symptom dimensions and dysregulation of the underlying HPA axis.

Anxiety and Dysregulation in Diurnal Cortisol Rhythm

More specifically, concurrent and longitudinal associations between children's and adolescents' internalizing problems (which include anxiety symptoms) and the disruption of diurnal cortisol rhythms has been reported in numerous studies (e.g., Dietrich et al., 2013; Ruttle, Armstrong, Klein, & Essex, 2014). Furthermore, depression is strongly associated with disruptions of the HPA-axis functioning (Doane et al., 2013). Given that anxiety is comorbid with and often precedes depression, it is hypothesized that anxiety symptoms may play a role in the sensitization of the underlying HPA-axis to later stress (Ruttle, Armstrong, et al., 2014).

A review of the current literature show inconclusive findings with some researchers reporting no or weak HPA axis abnormalities in children with anxiety symptoms (Dietrich et al., 2013), while others have found that children with anxiety disorders exhibited significantly lower night-time cortisol compared to depressed and healthy controls (Feder et al., 2004). In contrast, other studies have reported that adolescents with anxiety disorders exhibited elevated cortisol levels (Forbes et al., 2006). A limited number of prospective studies to date have explored the developmental relations between psychological symptoms and diurnal cortisol rhythm in early childhood and across the transition into adolescence showing conflicting results of blunting (Shirtcliff & Essex, 2008) and high/sustained cortisol elevations (Laurent, Gilliam, Wright, & Fisher, 2015).

These inconsistencies and contradictory findings in relation to anxiety and diurnal cortisol may be explained in part by differences in physiological versus cognitive-emotional dimensions of anxiety symptoms (McTeague & Lang, 2012). Cognitive-emotional symptoms are relatively enduring, and capture individual differences in how one perceives the world and the general tendency for an individual to respond anxiously under duress to cues in his or her environment. In contrast, physiological symptoms of anxiety (e.g., increased heart rate, muscle tension, dizziness, nausea, abdominal pain) are more transitory, occur in direct response to threatening stimuli, and endure for only a short duration following the termination or removal of the threat

(Hodges, 2015). Previous research has found that physiological anxiety symptoms are more prevalent in younger children than in adolescents, while cognitive-emotional anxiety symptoms are endorsed more by girls than boys (Gullone, King, & Ollendick, 2001), which suggest differential influences and developmental trajectories of these two dimensions. Other studies, albeit from the adult literature, have also explored the relations between physiological versus cognitive-emotional anxiety in relation to both stress response and the activation of the immune system supporting the view that chronic experience of anxiety symptoms may impact the underlying biological and neurological systems (Dietrich et al., 2013). To date, it remains unclear how and in what direction physiological and cognitive-emotional anxiety symptoms differentially relate to dysregulation of diurnal cortisol rhythms.

The Current Study

Current research shows an association between the experience of anxiety symptoms and diurnal cortisol rhythms in children and adolescents. However, the exact nature and sequence of this relation remain poorly understood especially spanning from childhood to adolescence. In addition, studies have shown that cognitive-emotional versus physiological dimensions of anxiety symptoms are distinct and relate differently to various health outcomes. However, this relation has not been examined specifically in relation to diurnal cortisol dysregulation. It is important to disentangle the developmental associations between anxiety symptoms and diurnal cortisol rhythms in children and adolescents given that anxiety is a precursor to many other serious psychological and physical health problems in adulthood (Roy-Byrne et al., 2008).

To address these limitations and clarify the inconsistencies in the literature, the goals of the current study were: 1) To examine how cognitive-emotional versus physiological symptoms of anxiety differentially relate to the dysregulation in diurnal cortisol rhythm concurrently and longitudinally; 2) To understand the nature of the relationship between dimensions of anxiety symptoms and dyregulation in cortisol rhythm as being either hypercortisolism or hypocortisolism; and 3) To examine the sequence of the relationship between anxiety dimensions and patterns of dysregulation in diurnal cortisol over time, clarifying whether anxiety symptoms in childhood predict changes in diurnal cortisol rhythms in adolescence, or, conversely, whether a dysregulated diurnal cortisol rhythm in childhood predicts later anxiety symptoms. Although the focus of the current study is on understanding the developmental psychobiology of anxiety symptom dimensions, given the comorbidity between anxiety and depression reported in the

literature (Schleider, Krause, & Gillham, 2014), children and adolescents' depressive symptoms were also included and controlled for in all analyses.

A multi-level, two-wave cross-panel design with repeated assessments of both anxiety symptoms and diurnal cortisol over three years was used to address these issues. We hypothesized that concurrent anxiety symptoms would be associated with hypercortisolism in childhood and adolescence. Over time, however, the experience of chronic anxiety symptoms would strain the HPA-axis, leading to down-regulation. We therefore expected that chronic and persistent anxiety symptoms would be associated with hypocortisolism (blunting) of the diurnal cortisol rhythm three years later in adolescence.

Because physiological anxiety symptoms have been found to be more transient and indicative of immediate and short-term autonomic arousal processes closely tied to the fear/threat response circuitry, we further hypothesized those physiological anxiety symptoms, specifically, would predict concurrent over-reactivity of cortisol at each wave of data collection. In contrast, cognitive-emotional dimensions of anxiety would predict hypocortisolism three years later because of their stability over time as a "trait-like" construct characterizing the individual's propensity to respond anxiously to real or perceived threats. As such, cognitive-emotional anxiety symptoms may be more enduring over time, reflecting maladaptive cognitive appraisals of situations as being stressful even in the absence of threats, which in turn lead to chronic strain on the stress response system to result in hypocortisolism.

Method

Description of Sample: Concordia Longitudinal Risk Project

Participants in the current study were participants in the Concordia Longitudinal Risk Project (CLRP), a multigenerational longitudinal study of families from disadvantaged backgrounds beginning in 1976. The project began with the screening of a large community-based sample and the selection of over 1700 French-speaking families and their children attending grades 1 (age 6-7), 4 (age 9-10), or 7 (age 12-13) from 22 public schools serving economically disadvantaged neighbourhoods in Montreal, Quebec, Canada. Ledingham (1981) and Schwartzman et al. (Schwartzman et al., 1985) provided detailed description of the original sample population and procedures. Subsets of these participants have been followed up and screened approximately every three years on various observational, interview-based, health, education and social functioning measures (see Serbin et al., 1998).

Study Participants

Participants in the current study were the offspring of original participants in the CLRP. The data analyzed was collected as part of a larger on-going study with approval from the Institutional Review Board of Concordia University. Participating families were French speaking from primarily French-Canadian backgrounds, with fewer than 5% from Latin American, Haitian, or other ethnic backgrounds. Demographic variables including age, children's sex, income and socioeconomic status, and maternal education levels were controlled in all the analyses and found to be statistically non-significant in terms of both main effects and interactions with the predictors in preliminary analyses.

Although 120 participants consented to the saliva sampling procedure, only those who were able to provide sufficient saliva to assay for cortisol (minimum 4 samples per day across 2 days) were included in the present analyses (n = 77 at Wave 1; n = 56 at Wave 2). Out of the 77 participants at Wave 1, 31 were male and 46 were female (M_{age} = 10.79, SD_{age} = 0.88), and at Wave 2, 26 were male and 30 were female (M_{age} = 13.79, SD_{age} = 1.22). The participants who completed the salivary samples did not differ from those who did not complete the procedures in terms of family income, maternal education, neighborhood disadvantage, or welfare enrollment (analyses of representativeness within the sample; all p values >.10).

In the current sample, the average income of the participating families was $56,218.95 at Wave 1, which is below the reported Canadian national ($66,550) and Quebec ($61,780) median income levels for that period (Statistics Canada, 2013). Although the original CLRP sample is a biased or risk sample in terms of the original participant selection process, many of the indicators of risk (e.g., income, socioeconomic status, maternal education attainment) were controlled for and found to be non-significant predictors in the current analyses. Table 1 shows means, standard deviations, and Pearson correlations of all demographic (control variables) and symptom measures (predictors).

Procedure

Data were collected in two waves spaced approximately 3 years apart for each child. The first data collections took place between 2002 and 2005 (Wave 1) and the second collection three years later, between 2005 and 2008 (Wave 2). Informed written consents were obtained from participants and their parents or legal guardians prior to their participation in the study. Participants received a small honorarium for their time and involvement.

Salivary cortisol sampling. Diurnal cortisol was assessed non-invasively using saliva samples, which reflect the plasma concentration of the non-protein bound active portion of cortisol (Kirschbaum & Hellhammer, 1994). At Waves 1 and 2, participants were instructed to provide saliva samples on two consecutive days using salivettes at specified target times throughout each day (i.e., upon awakening, 30 minutes post-awakening, followed by every 2 hours until bedtime). They were asked to refrain from eating within the 30 minutes prior to each sampling. Participants were instructed to remove the cotton swab from a plastic vial, chew on it for 30-45 seconds until it was saturated with saliva before placing it back in the vial, touching it as little as possible. Samples were kept frozen until they were assayed for total amount of cortisol at the Douglas Hospital Research Laboratories in Montreal, Quebec, Canada. Cortisol was assessed in duplicate with a salivary enzyme immunoassay kit (Salimetrics, State College, PA). The detection limit of the assay (ED_{80}) was .01ug/dl and the mean intra-assay variability coefficients were 16.3% at Wave 1 and 10.4% at Wave 2. The inter-assay variability coefficients were acceptable at less than 15% at both time points (Schultheiss & Stanton, 2009). In order to normalize the distributions, raw cortisol values were log-transformed and the remaining outlying scores were winsorized to within three standard deviations of the mean. Participants also completed daily diaries on each of the two days of sampling recording actual times of saliva sampling, mood, stress, health, food consumption including time of eating, exercise, morning awakening, and bedtime. Medication intake was also recorded with 17.2% of the participants at Wave 1, and 11.2% at Wave 2 report taking medications.

Symptom Measures

At Waves 1 and 2, children completed self-report measures of anxiety symptoms. Because of the comorbidity and associations reported in the literature between anxiety and depression, the current study also included a measure of depressive symptoms to control for its possible confounding effects along with all other relevant control variables (see Table 1 for the Pearson correlations between anxiety and depressive symptoms).

All measures in the current study were administered in French. Translated versions of English measures were created through a back-translation process when published French-language versions were not available. Back-translated measures used in the current study included the Revised Children's Manifest Anxiety Scale (RCMAS) (Reynolds & Richmond, 1985a; Turgeon & Chartrand, 2003) and Children's Depression Inventory (CDI) (Kovacs, 1985;

Mack & Moor, 1982).

Anxiety symptoms. Participants completed the self-reported 37-item Revised Children's Manifest Anxiety Scale (RCMAS) (Reynolds & Richmond, 1985a) at Wave 1 and 2, which assessed the level and nature of their anxiety symptoms. The RCMAS yields a total anxiety summary score, in addition to three subscale scores in specific domains of worry/oversensitivity (e.g., *"I am afraid of a lot of things")*, social concerns/concentration (e.g., "*Other people are happier than I")*, physiological/somatic anxiety (e.g., *"Often I feel sick in the stomach.")*. Overall raw scores of 19 and above (*T* scores $\geq$ 60) are indicative of children who are experiencing clinically significant levels of anxiety (Stallard, Velleman, Langsford, & Baldwin, 2001). In the current sample, only 2.6% of the children at Wave 1 and 2.9% at Wave 2 had scores of above 19 (see Table 1 for means and standard deviations). Relative to the normative sample according to sex and age of the children, the anxiety scores of the current sample of participants reflected non-clinical levels of anxiety symptoms generalizable to beyond only clinical or borderline/sub-clinical samples (Gerard & Reynolds, 2004).

Recent normative studies examining the psychometric properties of the RCMAS in various samples of children and adolescents reported high internal consistency for its total score in addition to each of its subscales in diverse samples (Turgeon & Chartrand, 2003; Varela & Biggs, 2006). In the current study, the Cronbach alphas for total anxiety ranged from 0.84 at Wave 1 to 0.83 at Wave 2, indicating strong internal consistency. The alpha reliabilities for specific sub-scales of worry/oversensitivity (Wave 1 $\alpha = 0.78$; Wave 2 $\alpha = 0.82$), physiological/somatic anxiety (Wave 1 $\alpha = 0.61$; Wave 2 $\alpha = 0.60$), and social concerns/concentration (Wave 1 $\alpha = 0.61$; Wave 2 $\alpha = 0.68$) also indicate overall satisfactory internal consistency across administrations.

Depressive symptoms. The Children's Depressive Inventory (CDI) (Kovacs, 1985) is a 27-item self-report scale suitable for assessing depressive symptoms in children aged 7 to 17. Depressive symptoms are controlled for in the current analyses as a possible confounding variable given its comorbidity with anxiety symptoms. Each item consists of three self-report statements graded in severity from 0 (least severe) to 2 (most severe). Children are asked to indicate which statement best matched how they have been feeling in the past two weeks (e.g., *I am sad once in a while (0); I am sad many times (1); I am sad all the time (2).* Total scores of 19 and above on the CDI (*T* scores $\geq$ 65) indicate clinically significant levels of depression (Masip,

Amador-Campos, Gómez-Benito, & del Barrio Gándara, 2010). In the current study, 7.9% of the children at Wave 1 and 6.0% at Wave 2 scored within the clinical range in the current sample (see Table 1 for means and standard deviations). Depressive symptoms scores, in terms of means and standard deviations within the current sample are comparable to those reported in community samples (Masip et al., 2010). Recent studies have reported the CDI as a reliable and valid measure across different cultural samples as well validating its use for screening purposes and in comparison to structured interviews. (D. A. Cole & Martin, 2005; Matthey & Petrovski, 2002; Timbremont, Braet, & Dreessen, 2004) In the current study, the Cronbach alphas of total CDI scores ranged from 0.81 at Wave 1 to 0.83 at Wave 2 across the two administrations, indicating strong internal consistency.

Data Analytic Plan

Data in the current study were analyzed using Hierarchical Linear Modelling (HLM), Version 7.01 (Bryk & Raudenbush, 1992; Raudenbush, Bryk, & Congdon, 2004). Longitudinal multilevel models captured not only how variables change over time, but also how those changes are associated with trait-level (i.e., between-persons) and state-level (i.e., within-person) factors. They are ideal when the assumption of independence may be violated as is in the case of repeated measures from the same individual over time. HLM models change over time in outcome variable by estimating a curve for each individual. Each curve conveys information about an individual's baseline (i.e., the intercept), and his or her change across time (i.e., the slope) while taking into consideration other between-person contextual factors that may serve as additional explanatory variables and/or confounds (Shirtcliff & Essex, 2008). HLM is also well suited to capture the strong diurnal slope of repeated measures of cortisol even within smaller sample sizes, since it is capable of estimating a curve based on the available data values present while extrapolating other missing data points based on this curve to maximize degrees of freedom (Hruschka, Kohrt, & Worthman, 2005).

Three-level hierarchical models were constructed to examine how dimensions of anxiety symptoms predicted changes in both the intercept and diurnal cortisol slopes, concurrently and longitudinally across three years. The statistical design conceptually resembled that of a traditional cross-panel balanced design best suited to examine bidirectional relations between variables to infer the sequence and directionality of the effects. Given the complexity of the data involving multiple repeated measurements of diurnal cortisol within the same individual across

two waves, in addition to the nonlinear diurnal rhythms, a cross-panel equivalent was tested using HLM (see Figure 1).

Model specification. Three-level hierarchical linear models partitioning within-the-day (i.e., diurnal), day-to-day (i.e., across two days), and between individual (i.e., individual differences in anxiety symptoms) sources of diurnal cortisol variability were used to test our hypotheses. Four random intercept and slopes models were specified in the current study to examine the concurrent associations between dimensions of anxiety symptoms and diurnal cortisol at Wave 1 and Wave 2, and their longitudinal sequence and associations over time. A series of steps were followed for model specifications in the current study (Bryk & Raudenbush, 1992). First, an unconditional random intercept model was constructed with only the outcome variable (log-transformed diurnal cortisol) entered to examine whether there is significant variability in cortisol to be explained at each of the levels. Results showed significant within ($p <$ .01) and between-person effects ($p < .01$) on cortisol variability that remain to be further explored. We then proceeded with the addition of theoretically relevant predictor variables at each of the three levels to further explain diurnal cortisol variation.

Level 1 specification included “time since waking" (TSW) as a within-the-day predictor capturing the diurnal rhythm of cortisol variation. The intercept reflects the overall baseline level of cortisol upon awakening for each person. The models also included both quadratic (TSW^2) and cubic (TSW^3) functions of TSW to allow for the examination of curvature in individual slopes across the day. An additional variable representing the "cortisol awakening response" (CAR) was also included as a within-person predictor of cortisol variability. Samples provided approximately 30 minutes post-awakening were identified as CAR using a dummy code (1 = CAR, 0 = not CAR). Additional predictors included daily subjective ratings of mood, stress, health, food intake, and exercise, which were all statistically non-significant predictors of diurnal cortisol intercept or slopes ($p > .10$), and were removed from the models to preserve parsimony.

Level 2 captured day-to-day variability in cortisol intercept and the diurnal slopes across the two consecutive days of cortisol sampling, with individual’s report of medication usage on each day of cortisol sampling (1 = Medication; 0 = No Medication) entered as a predictor. No statistically significant associations between medication use and cortisol variability were found ($p > .10$).

Level 3 specification captured between-persons variations in diurnal cortisol intercept and

slopes. Control variables of age, sex (0 = Male, 1 = Female), maternal education, and income were first entered into all models. No significant main effects were found in relation to these variables ($p > .10$). Next, depressive symptoms were added as a control variable. Including depression in the models did not change any of the existing statistical significant associations between anxiety symptom dimensions and diurnal cortisol. In the current analyses, children's depressive symptoms were not significantly associated with any concurrent or longitudinal changes in diurnal cortisol intercepts or slopes once children's anxiety symptoms were entered into the model as a between-persons predictor.

Results

Concurrent Associations: Physiological Anxiety Symptoms Predicted Hypercortisolism

At Wave 1, a three-level hierarchical linear model separated within-the-day (n = 1259 samples; total df = 1259), day-to-day (n =150 days; total df = 150), and between individual (N = 77; total df = 77) sources of diurnal cortisol variability. Time since waking in terms of its linear slope ($p < .000$), quadratic function ($p < 0.05$), and CAR ($p < .000$) were significant predictors of diurnal cortisol variability. At Level 2, there was substantial variability in cortisol intercept levels across the two days, χ^2 (72) = 336.55, $p< .001$. At Level 3, cortisol intercept levels varied between individuals, χ^2 (74) = 169.01, $p< .001$ and the slopes varied linearly (TSW), χ^2 (74) = 126.59, $p <.001$; quadratically (TSW^2), χ^2 = 123.86, $p < .001$; and cubically (TSW^3), χ^2 (74) = 122.01, $p < .001$, suggesting that each individual had their own diurnal slope and the cortisol variability was significant different from one person to the next. At Wave 1, 64.2% of the cortisol variability was explained by individual's diurnal rhythm (i.e. within the day fluctuations), while day-to-day fluctuations accounted for 9.4% of the total variance in cortisol. Furthermore, 26.4% of the cortisol variability was still left to be accounted for by between individual fluctuations in dimensions of anxiety symptoms, after controlling for sex, age, income, medications, and depressive symptoms.

Concurrently at Wave 1, children who reported higher total anxiety symptoms had *lower* morning cortisol intercept (β = -0.0109, t = -2.36, $p <.05$) but hypercortisolism over the day, with larger increases in CAR, followed by a moderate decline in cortisol levels during the day (linear: β = 0.0098, t = 2.90, $p <.01$; quadratic curvature: β = -0.0012, t = -2.13, $p <.05$). Compared to children with low to average overall anxiety symptoms, those with higher anxiety showed a disrupted diurnal cortisol rhythm that remained elevated and blunted a few hours after

awakening. More specifically, higher physiological anxiety symptoms were associated with dysregulated cortisol that remained high and elevated throughout the day (linear trend: β = -0.0236, t = 2.77, p <.01; quadratic trend: β = -0.0032, t = -2.25, p <.05) (see Figure 2, left panel).

At Wave 2, a similar model separated within-the-day (n = 652 samples; total df = 652), day-to-day (n = 112 days; total df =112), and between individual (N = 56; total df = 56) sources of cortisol variability. All time-related variables were found to be significant predictors of variability in cortisol (ps < .001). At Level 2, the model at Wave 2 did not show substantial variability in cortisol intercept levels between the two days. At Level 3, cortisol intercept levels varied across individuals, χ^2 (52) = 112.58, p< .001 and the slopes varied linearly, χ^2 (52) = 95.57, p <.001; quadratically, χ^2 (52) = 93.18, p = .001; and cubically, χ^2 (52) = 89.08, p = .001. Within the day fluctuations accounted for 81.7% of the total variance, and between individual fluctuations accounted for 18.3% of the total variance in cortisol levels.

Concurrently at Wave 2, higher physiological anxiety symptoms were also concurrently associated with *lower* morning cortisol intercept (β = -0.0224, t = -3.31, p <.05), again showing hypercortisolism (linear slope: β = 0.0126, t = 2.90, p <.01) that remained elevated throughout the day compared to those who reported low to average levels of physiological anxiety (see Figure 2, right panel). These results further showed that physiological anxiety symptoms were concurrently associated with hypercortisolism both in childhood and in adolescence. In contrast, cognitive-emotional symptoms of anxiety (worry and social concerns dimensions) were not found to be statistically significant concurrent predictors of diurnal cortisol at Wave 1 or 2.

Longitudinal Associations: Wave 1 Cognitive-Emotional Anxiety Symptoms Predict Wave 2 Hypocortisolism

Additional models examined the longitudinal predictions and directionality of the relation between diurnal cortisol and children's dimensions of anxiety symptoms. First, we examined how Wave 1 anxiety dimensions predicted Wave 2 diurnal cortisol intercept and slope by specifying Wave 2 cortisol as the outcome variable while entering Wave 1 anxiety symptoms as between-persons predictors. All time-related variables were found to be significant predictors of variability in cortisol (ps < .05). At Level 3, cortisol intercept varied across individuals, χ^2 (36) = 86.52, p< .001, and the slopes varied linearly, χ^2 (36) = 65.46, p <.01; quadratically, χ^2 (36) = 62.68, p < .01; and cubically, χ^2 (36) = 59.18, p < .01. Day-to-day fluctuations accounted for 12.5% of the total variance in level of cortisol, within the day fluctuations accounted for 45.7% of the total

variance, and between individual fluctuations accounted for 41.8% of the total variance in cortisol levels. Over time, between individual differences may be better able to capture the more stable aspects of the diurnal rhythm in cortisol above and beyond cotemporaneous within-the-day variations, which are less stable as longitudinal predictors of cortisol variability over time.

Longitudinal results showed that youths who had more cognitive-emotional anxiety symptoms, specifically, higher worries/oversensitivity at Wave 1 had *lower* morning cortisol intercept three years later (β = -1.0023, t = -19.85, p <.001). They also had more dysregulated diurnal slope across the day, showing larger increases in the cortisol awakening response, followed by a steep and rapid decline in cortisol than compared to children with low to average levels of worry/oversensitivity (see Figure 3, left panel).

Furthermore, significant effects of linear (SD = 0.0952; χ^2 (34) = 63.88, p <.01), quadratic (SD = 0.0132; χ^2 (34) = 61.75, p <.01), and cubic slopes (SD = 0.0005; χ^2 (34) = 59.34, p <.01) were found showing that cognitive-emotional anxiety symptoms of worry/oversensitivity at Wave 1 predicted random variations in within-person diurnal slopes three years later at Wave 2. In contrast, physiological anxiety symptoms at Wave 1 did not predict significant effects on diurnal cortisol slopes at Wave 2.

Similarly, youths with higher social concerns or worries, which capture another facet of the cognitive-emotional symptom dimension of anxiety at Wave 1 also had *lower* morning cortisol and overall blunting of the diurnal cortisol rhythm three years later (β = -1.0081, t = -20.31, p <.001). They showed hypocortisolism compared to those with low to average social concerns/worries. Individuals with higher social concerns had levels of cortisol that remained elevated during the day following CAR, showing a more blunted slope (linear trend: β = -0.0218, t = -1.82, p <.08). Individuals varied in terms of the linear (SD = 0.0893; χ^2 (34) = 60.63, p <.01, quadratic (SD = 0.0125; χ^2 (34) = 58.96, p <.01) and cubic (SD = 0.0005; χ^2 (34) = 55.95, p = .10) bends of their cortisol slopes in a random intercept and slope model (see Figure 3, right panel).

Longitudinal Associations: Wave 1 Diurnal Cortisol Associated with Wave 2 Physiological Anxiety Symptoms

Last, we examined whether Wave 1 diurnal cortisol would in turn predict Wave 2 anxiety dimensions (following the procedure in Shirtcliffe (2008) study). All time-related variables were significant predictors of cortisol variability (ps < .05). Cortisol intercept varied across

individuals, χ^2 (52) = 122.21, p< .001. Day-to-day fluctuations accounted for 8.5% of the total variance, within the day fluctuations accounted for 50.4% of the total variance, and between individual fluctuations accounted for 41.1% of the total variance in cortisol levels.

Longitudinally, children with lower morning cortisol intercept at Wave 1 showed the highest levels of physiological anxiety symptoms at Wave 2 (β = -0.6703, t = -18.21, p <.01) after controlling for depressive symptoms and physiological anxiety symptoms at Wave 1 (i.e., baseline levels). Children with a less reactive and steep cortisol awakening response at Wave 1 had higher physiological anxiety symptoms three years later (see Figure 4).

Significant random effects of linear (SD = 0.0681; χ^2 (52) = 70.98, p <.05) and quadratic (SD = 0.0103; χ^2 (52) = 69.66, p <.05) bends were found indicating individual variations in diurnal slopes across time (individual differences) in relation to their own levels of physiological anxiety symptoms.

Summary of Results

The current study examined the nature and sequence of the associations between dimensions of anxiety symptoms and diurnal cortisol dysregulation concurrently and over three years from childhood to adolescence using HLM to model a multi-level cross-lagged design equivalent. Results supported our hypotheses by showing that physiological anxiety symptoms were associated with hypercortisolism that remained elevated throughout the day concurrently in childhood (Wave 1) and adolescence (Wave 2). Regarding the sequence of the relation between anxiety symptom dimensions and diurnal cortisol over time, longitudinal results supported the hypothesis that cognitive-emotional symptoms of anxiety, specifically, worry-oversensitivity and social concerns symptoms at Wave 1 predicted hypocortisolism and lower overall morning cortisol intercept three years later at Wave 2. Finally, dysregulation in diurnal cortisol rhythm in childhood (i.e., lower overall morning cortisol and less reactive CAR) at Wave 1 was associated with higher reported physiological anxiety symptoms three years later at Wave 2.

Discussion

The current study examined the nature and directionality of the relationship between physiological and cognitive-emotional anxiety dimensions and the underlying HPA-axis, specifically, diurnal cortisol rhythms from childhood through mid-adolescence. Using a longitudinal cross-lagged design incorporating multi-level modeling, the present study examined the following questions: 1) How do physiological and cognitive-emotional symptoms of anxiety

differentially relate to diurnal cortisol rhythms concurrently and longitudinally? 2) What is the exact nature of the relationship between children's anxiety symptom dimensions and diurnal cortisol rhythms concurrently and over several years? and 3) What is the sequence of the relationship between anxiety symptom dimensions and possible diurnal cortisol dysregulation over three years?

Regarding the first question, a unique contribution of the current study is the finding that physiological anxiety symptoms had stronger concurrent associations with diurnal cortisol and predicted hypercortisolism than cognitive-emotional dimensions of anxiety. However, physiological symptoms of anxiety in childhood did not predict long-term effects on diurnal cortisol rhythms three years later in adolescence. In contrast, cognitive-emotional dimensions of anxiety, specifically, worries and social concerns were predictive of long-term associations with diurnal cortisol three years later and reflected hypocortisolism. These results suggest that there may be differential associations between anxiety symptoms dimensions and the underlying HPA functioning, complementing the existing research showing differences in regional brain activity and neural mechanisms in relation to these two anxiety symptom dimensions (Sharp et al., 2015). To our knowledge, the current results are the first to explicitly examine how dimensions of anxiety symptoms relate concurrently and longitudinally to diurnal cortisol rhythms in children and adolescents.

One way to understand this difference is that physiological symptoms of anxiety tend to be situation- or stressor-dependent, directly related to the physiological arousal mechanisms and processes, and typically do not persist after the removal of the threat or perceived threat (Forgays, Sosnowski, & Wrześniewski, 1992). These physiological symptoms of anxiety are more closely associated with fight or flight response, which are typically short-term activation of the autonomics system and fear circuitry. In contrast, cognitive-emotional anxiety symptoms of worry or social concerns are more pervasive and consists of cognitive appraisals that are related to the perceived coping capacities of the individual in response to objective and perceived stress or threats (Lazarus, 1993). These cognitive emotional symptoms of anxiety may be more enduring, and lead individuals to experience chronic hypervigilance and apprehension even in the absence of threat or stressor (Adam, 2006; Eysenck, 1997). They also tend to be more stable and long-term, shaping how the individual perceives and responds to stress in their environment. Over time, cognitive-emotional symptoms of anxiety are often described as being uncontrollable

and inescapable, similar to how individuals often perceive and define chronic stress that strain the HPA axis resulting in long-term dysregulation and down-regulation (Aguilera, 2015).

Regarding the question of the exact nature of the relationship between anxiety symptoms and diurnal cortisol rhythm, results from the current study showed evidence of both hypercortisolism and hypocortisolism in relation to anxiety symptoms dimensions. Specifically, concurrent results from hierarchical linear modeling showed that higher overall levels of anxiety, in particular, physiological anxiety symptoms, were associated with lower overall morning levels of cortisol, a steeper cortisol awakening rise and elevated diurnal cortisol levels (hypercortisolism) throughout the day in comparison to individuals with lower levels of physiological anxiety in childhood and in adolescence. However, longitudinally, higher self-reported cognitive-emotional symptoms of worry and social concerns dimensions of anxiety in childhood predicted a more blunted diurnal cortisol profile (hypocortisolism) three years later in adolescence. Children who reported the most worries or social concerns had lower overall cortisol levels in the morning and over the course of the day three years later, with less curvature in their diurnal slopes than compared to those with lower levels of cognitive-emotional symptoms of anxiety.

Theories of HPA-axis and stress reactivity emphasized the detrimental effects of over-activation (hypercortisolism) of the stress-response system (Selye, 2013). However, more recent research has increasingly called attention to the presence of low cortisol or hypocortisolism especially in childhood and adolescence as an important biomarker of dysregulation of the HPA-axis functioning, challenging and refining previous assumptions of how the HPA axis functions (Gunnar & Vazquez, 2001). In the current study, evidence of both hypercortisolism and hypocortisolism was found further illustrating the complex and reciprocal interactions between the biological and psychological aspects of anxiety and the stress-response system.

Furthermore, the evidence of hypo- and hypercortisolism in relation to self-reported levels of anxiety symptoms may be situated within the allostatic theory proposed by Miller and colleagues (Miller et al., 2007). Exposure to stress or threats (actual or perceived), may initially activate the autonomic nervous system and the HPA axis to release cortisol. However, after prolonged exposure to chronic strain (resulting in persistently elevated cortisol levels), the HPA axis shows signs of "wear-and-tear" and down-regulates, resulting in hypocortisolism or blunting of the diurnal cortisol rhythm over time. Initially, physiological anxiety symptoms may act as a

direct and immediate response to threats and activate the stress response system. Because these physiological anxiety symptoms typically abate with the end of the stressor or threat, they may not show persistent associations with cortisol activity in the long-term. However, for some individuals, what remain are the cognitive-emotional anxiety symptoms closely linked to cognitive appraisals that may be chronically activated in the evaluation of daily hassles and situations as being stressful (Lazarus, 1998). When these cognitive-emotional anxiety symptoms become chronic, unabated, and disproportional to the threat or stress encountered as they tend to be for individuals with anxiety disorders, they may in turn strain the underlying HPA-axis and stress response system, leading to a dysregulated diurnal cortisol in the manner of hypocortisolism over time (Sharp et al., 2015).

Regarding the question of the sequence and directionality of the relationship between anxiety symptoms and diurnal cortisol rhythm, the current study found support for a bidirectional and transactional associations between the two. We cannot conclude from the current findings whether anxiety symptoms *cause* dysregulation in diurnal cortisol rhythm, or if pre-existing dysregulation in the HPA axis makes individuals more vulnerable to experiencing anxiety symptoms later on. However, the current results showed a transactional relation in that higher anxiety symptoms predicted overall lower and blunted cortisol rhythm over time, but a dysregulated diurnal cortisol in childhood was also associated with higher physiological anxiety symptoms later in adolescence. It is possible that dysregulation in underlying HPA-axis functioning could be making the children physically ill, with many of the symptoms being mistaken for or confused with physiological anxiety symptoms. It is unclear if potential disruptions in the HPA-axis in childhood is actually making children sick and therefore leading to some of the reported physiological symptoms (e.g., nausea, dizziness, and stomachaches). Future longitudinal examinations of the linkage between the HPA-axis, physiological anxiety symptoms and physical health may be warranted in future studies.

Lastly, the focus of the current study was to examine the developmental psychobiology of anxiety from a symptom-dimension approach, specifically in relation to the HPA-axis and diurnal cortisol rhythms. Controlled in all our analyses are individuals' depressive symptoms. In the current set of analyses, children's depressive symptoms did not significantly predict cortisol variations once anxiety symptoms are accounted for. The significant statistical associations between anxiety symptom dimensions and diurnal cortisol rhythm remained even after including

depressive symptoms in the models as a control variable. There have been extensive studies showing the linkage between stress, elevated cortisol levels and major depression. Given the associations between depressive and anxiety symptoms, their comorbidity as well as the theory that anxiety symptoms tend to precede later onset of depression by possibly sensitising the HPA-axis, future studies may best to examine both facets in more detail in a larger sample of children and adolescents.

Using a multi-level hierarchical modeling design, while incorporating a two waves cross-lagged design incorporating repeated measures of both anxiety symptoms and cortisol over several years, the current study attempted to answer the question of under "what circumstances, for whom, and when under versus overactivation" of the HPA axis is most likely in children and adolescents (Badanes, Watamura, & Hankin, 2011). While the current study has strengths in its methodological design and a unique contribution to the understanding of the relation between dimensions of anxiety and diurnal cortisol rhythm, it is not without limitations. First, additional waves of assessments would have been ideal to address the question of sequence and directionality of the relationship between anxiety symptoms and cortisol over the unfolding of development. Second, although having two time points of repeated assessment combined with multi-level modeling of the data enhanced the power and interpretation of our results, our current sample size was small, limiting the extent of the interpretation and generalizability of the findings. Given the limited sample size, it was not feasible to analyze specific gender effects by separating the sample by sex. However, in all the models, children's sex was controlled for and interactions with predictors did not show significant gender effects in the present study.

Third, the current study solely focused on the HPA axis and cortisol in relation to anxiety symptom dimensions. However, the endocrine system has a multitude of interconnections with many other hormones and biological systems such as the immune system, which is also implicated in stress-response (Marceau, Ruttle, et al., 2013). It is very likely that there are other correlated biological markers that work in tandem with the HPA-axis and cortisol, which were not included in the current study. To gain a comprehensive understanding of the psychobiology of anxiety, it would be ideal for future studies to integrate multiple biomarkers that are associated with anxiety and stress.

Lastly, given that the original CLRP participants constituted a risk sample with exposure to more environmental stress (in terms of lower income, SES and maternal educational levels),

the current results may be limited somewhat in terms of its generalizability. However, these risk factors have been controlled for in the current analyses and found to have non-significant main effects, as well as interaction effects with our predictors of interest. Furthermore, when examining the anxiety and depression symptom scores of the current sample, the children's scores are comparable to the normative data reported in the literature for large-scale community samples. Although the sample may be a biased sample in terms of original sample recruitment, the results from the current analyses may be generalizable to beyond solely a borderline-clinical or clinical sample of participants. Nevertheless, future studies may best replicate the current findings using a larger community sample.

In conclusion, the current study clarified the nature of the relationship between anxiety symptom dimensions and diurnal cortisol patterns over time from childhood through adolescence. The hierarchical multi-leveling approach and the cross-lagged design allowed for a closer examination of the between-persons and within-persons associations of anxiety symptoms and diurnal cortisol variation over several years. Clinical implications of the results highlight the importance of distinguishing cognitive-emotional and physiological anxiety dimensions in the prevention and treatment of anxiety in young children given their differential associations with the underlying stress response system. Proper assessment of anxiety symptoms dimensions is warranted especially in the context of primary health care interventions to ensure proper diagnosis and treatment of the underlying issues. Appropriate early assessments, preventions and interventions of anxiety in children and adolescents will help prevent more serious psychological and physical health issues later in adulthood.

Table 1

Descriptive Statistics and Pearson Correlations for All Predictor Variables at Wave 1 and 2

Variable	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. Child Sex	--															
2. W1 Age	-.13	--														
3. W2 Age	-.16	.76***	--													
4. W1 Fam. Inc.	-.20*	-.02	-.01	--												
5. W2 Fam. Inc.	-.18	-.12	-.18	.63***	--											
6. Mat. Educ.	.06	-.12	-.19*	.38***	.25**	--										
7. W1 Tot. Anx.	.31**	-.04	-.18	-.02	-.11	.05	--									
8. W1 Phys. Anx.	.26**	-.11	-.17	-.03	-.12	-.01	.80***	--								
9. W1 Worries	.30**	-.05	-.17	-.02	-.08	.00	.92***	.59***	--							
10. W1 Soc. Conc.	.17	-.01	-.11	.02	-.12	.09	.76***	.41***	.64***	--						
11. W2 Tot. Anx.	.08	.03	.04	-.06	-.02	-.09	.30**	.21	.35**	.15	--					
12. W2 Phys. Anx.	.04	.01	.10	-.19	-.12	-.11	.19	.17	.21	.00	.73***	--				
13. W2 Worries	.19	.04	-.01	-.01	.01	-.01	.26*	.19	.30**	.11	.89***	.45***	--			
14. W2 Soc. Conc.	-.08	-.00	-.00	.03	.11	-.11	.28*	.14	.34**	.31**	.73***	.31**	.54***	--		
15. W1 Dep. Sxs.	.11	-.10	-.15	-.08	-.15	-.18	.47***	.41***	.38***	.51***	-.05	-.10	-.08	.13	--	
16. W2 Dep. Sxs.	-.06	.01	.01	-.11	-.11	-.24*	.29**	.22*	.25*	.27*	.45***	.27**	.32**	.51***	.30**	--
Means	.55	10.89	13.74	56218.95	54588.07	11.93	9.27	3.41	3.88	1.81	8.43	3.04	3.89	1.54	10.35	8.43
St. Dev.	.50	.94	1.18	31547.31	33326.84	2.42	5.67	2.15	2.89	1.65	5.38	2.09	2.98	1.66	6.58	6.08

Notes. W1, W2 = Wave 1, Wave 2; For child's sex, 0 = male, 1 = female. Fam. Inc. = Family Income in Canadian dollars; Tot. Anx. = Total Anxiety. Phys. Anx. = Physiological Anxiety; Soc. Conc. = Social Concerns; Dep. Sxs. = Depressive Symptoms. St. Dev. = Standard Deviation.

$^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$.

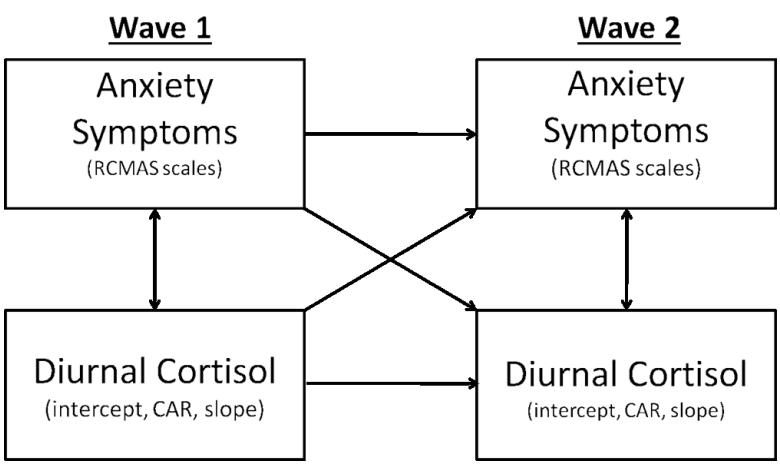

Figure 1. Cross-panel design exploring the sequence and directionality of the relationship between anxiety symptoms and diurnal cortisol rhythms over three years.

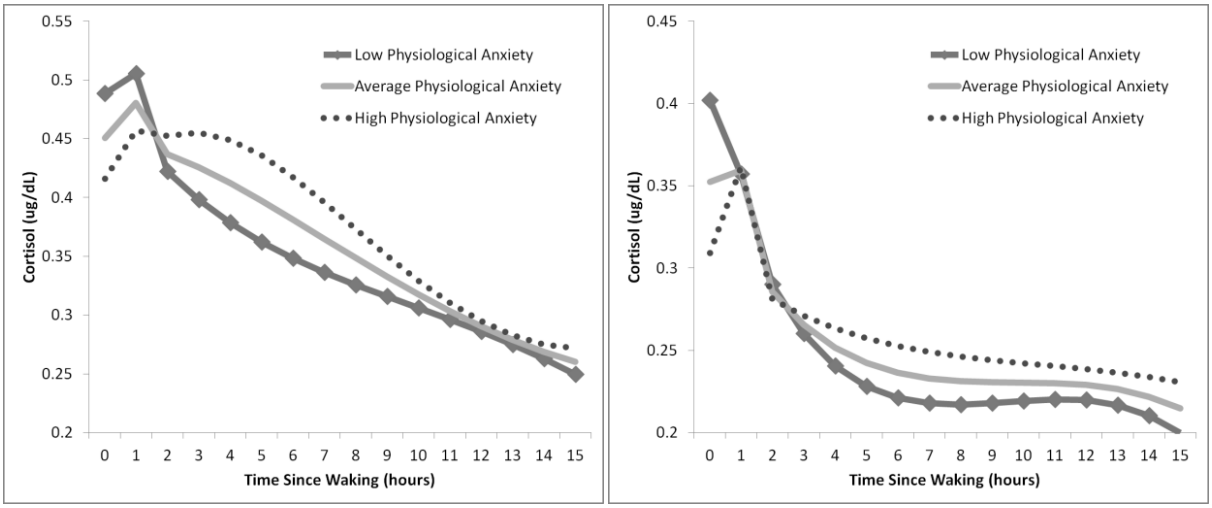

Figure 2. Concurrent associations showing Wave 1 (left panel) and Wave 2 (right panel) physiological anxiety symptoms and diurnal cortisol rhythms.

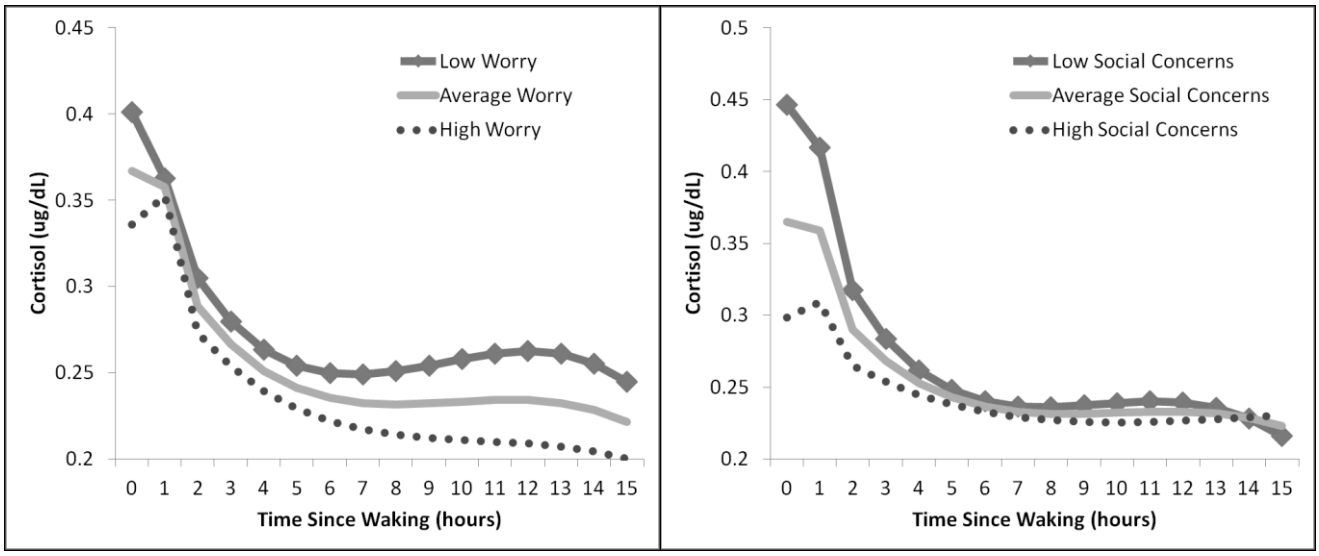

Figure 3. Longitudinal associations between Wave 1 cognitive-emotional anxiety dimensions (Left panel: worries and oversensitivity; Right panel: social concerns) and Wave 2 dysregulated diurnal cortisol three years later.

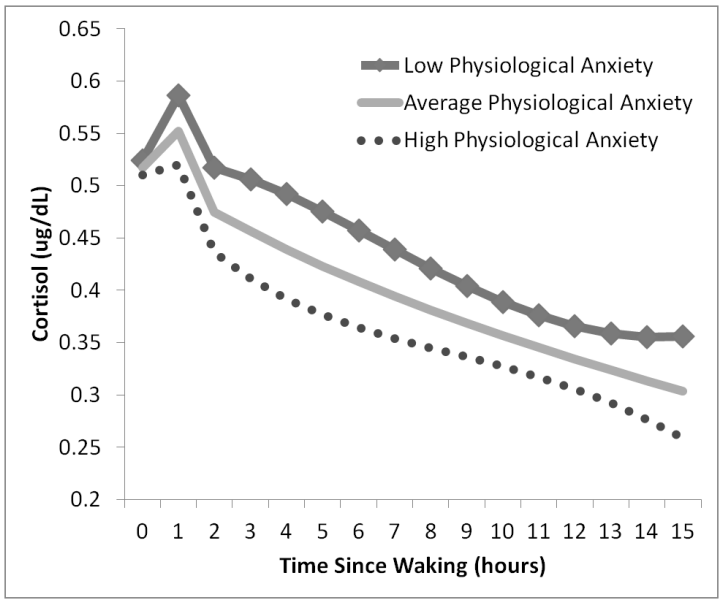

Figure 4. Longitudinal associations between wave 1 diurnal cortisol and wave 2 physiological anxiety symptoms.

Chapter 3: Extending Study 1 and Rationale for Study 2 – The Relationship between Anxiety Symptoms and Salivary Immunoglobulin A (sIgA)

Study 1 focused on understanding the association between dimensions of anxiety symptoms and diurnal cortisol rhythms in children and adolescents over time. Whereas Study 1 highlighted the concurrent and longitudinal associations between anxiety symptom dimensions and diurnal cortisol over two consecutive data waves, the exact sequence of this relation remain unclear extending beyond these two waves. Specifically, we cannot conclude the existence of any transactional feedback loops over time that may exist between anxiety and diurnal cortisol rhythms without an additional third wave of data collection. Although diurnal cortisol samples were not sufficiently available at a third wave to examine this question of mutual regulation in relation to diurnal cortisol rhythms, the two wave design was extended to include three successive waves in Study 2 to examine the relationship between anxiety symptoms and a different yet related biological system, that of mucosal immunity. The inclusion of a third consecutive wave of data collection is an important addition to the study design because it will clarify questions of "which comes first?" and "what next?" in our understanding of the transactional sequence between anxiety symptoms and sIgA.

The immune system is an important complementary system to that of the endocrine system. The secretion of cortisol and mobilization of the HPA axis has cascading effects downstream in various aspects of the immune system. While short-term anxiety may be adaptive and lead to redistribution of immune cells to areas where they can act quickly and efficiently against pathogens, the continued secretion of cortisol in response to chronic arousal leads to compromised immune system functioning over time (Daruna, 2012; Sapolsky, 1992). Studies have shown that temporary experience of anxiety as a direct emotional response to perceived threats, activate autonomic and endocrine systems to divert resources away from immunity toward systems (e.g., musculature, brain) needed to execute effective "fight or flight" defenses in the short term (Charney et al., 2013). However, the toll of experiencing chronic anxiety symptoms over time on the mucosal immune functioning, specifically in relation to salivary immunglobulin A (sIgA) levels, has not been examined to date especially in children and adolescents. A better understanding of chronic strain resulting from the experience of psychological symptoms and its long-term effect on aspects of the immune system may elucidate pathways and mechanisms to the development and maintenance of physical illnesses over time.

Clarifying the relation between anxiety symptoms and sIgA over time will allow us to formulate hypotheses and design studies that will incorporate multiple related biological systems (i.e., aspect of both the endocrine and immune systems) to understand the psychobiology of anxiety in children and adolescents. However, before attempting to integrate multiple theoretically related biological systems, it is important to clarify and examine just how dimensions of anxiety symptoms are associated with each system as a first step to solving the puzzle.

Given the above, the goals of Study 2 were two-fold with results presented in two separate, but related studies (Study 2a and 2b). The first goal of the second study was to examine how anxiety symptom dimensions impact the diurnal rhythm of sIgA in a subsample of children between the ages of 9 to 12 who provided adequate salivary samples of sIgA for the estimation of a diurnal rhythm (i.e., minimum 4 samples per day across 2 days). The second goal of Study 2 was to extend the design and expand the research questions in Study 1 to examine mutual regulation between anxiety symptoms and overall levels of sIgA over time. An additional wave of data collection was included when the children were between the ages of 15 to 18 years to allow for a double cross-lagged panel statistical design. Because overall aggregated daily average of sIgA was used, which is typically how it has been measured in the existing literature, a minimum of three valid saliva samples across two days at each successive wave of data collection were deemed sufficient for the double-cross lagged panel analysis. This sample of 103 participants was therefore larger than that of Study 1 because we were able to include additional participants with fewer salivary samples per day to examine the question of mutual regulation of children's anxiety symptoms and overall sIgA levels over several years.

Taken together, Study 1 and 2 move us closer to a better understanding of the developmental psychobiology of anxiety symptom dimensions. Each study helps clarify the specific relationship between anxiety symptoms dimensions and aspects of the endocrine (i.e., diurnal cortisol) and immune system (i.e., sIgA), respectively. Further, the design of Study 2 also extends Study 1 to examine mutual regulation of anxiety symptoms dimensions and sIgA over a key developmental period spanning the ages of 9 to 18 years. Clarifying and understanding these respective associations independently is an important first step towards future integrative studies that will incorporate anxiety and multiple related systems for a more comprehensive understanding of the biological underpinnings of anxiety in children and adolescents over time. By examining questions of mutual regulation and transactional feedback loops over time, the

sequence of the mutual shaping that may occur between chronic mental health symptoms and aspects of the underlying biological systems will be better elucidated. Understanding the sequence of "which comes first" will in turn help disentangle when and where to intervene to mitigate illness susceptibility over the course of development through to adulthood.

Chapter 4: Study 2

Children's Anxiety Symptoms and Salivary Immunoglobulin A: A Mutual Regulatory System?

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Abstract

Anxiety can impact the immune system resulting in negative health outcomes, especially in children and adolescents. Salivary immunoglobulin A (sIgA) is a first line of defense against foreign antigens, with lowered levels of sIgA indicative of weakened mucosal immunity. Little is known about how anxiety symptoms affect the diurnal rhythm of sIgA secretion, or the longitudinal transactional sequence between anxiety symptoms and sIgA levels in children and adolescents. The goals of the current study were to: 1) explore the contemporaneous associations between children's anxiety symptoms and diurnal rhythm of sIgA; and 2) examine transactional relations between children's anxiety and sIgA levels across successive longitudinal periods from middle childhood (Wave 1; ages 9-12) to early adolescence (Wave 2; ages 12-15), and from early to mid- adolescence (Wave 3; ages 15-18). Hierarchical linear modeling results showed a pattern of over-activation of diurnal sIgA slopes in children with higher anxiety than lower anxiety. Additionally, higher levels of total anxiety, and specifically, worries at Wave 1 significantly predicted lower levels of sIgA three years later at Wave 2. Lowered sIgA levels at Wave 2 in turn predicted higher anxiety at Wave 3, illustrating a "vicious cycle" feedback loop. That is, higher levels of anxiety symptoms were followed by lower levels of sIgA at a subsequent time point, which in turn predicted increases in anxiety in an incremental cycle over time. These findings broaden our understanding of the developmental links between anxiety symptoms, the immune system, and health. Implications for developmentally appropriate prevention programs for children and youth are discussed.

Keywords: anxiety symptoms, immunity, salivary immunoglobulin A (sIgA), psychoneuroimmunology, stress.

Children's Anxiety Symptoms and Salivary Immunoglobulin A: A Mutual Regulatory System?

Studies have shown complex associations between psychological symptoms and the immune system in children and adults (Carlsson, Frostell, Ludvigsson, & Faresjö, 2014). Psychosocial factors, including but not limited to stress, emotions, and social support are linked to the susceptibility and exacerbation of physical illnesses through interactions with the immune system (Kiecolt-Glaser et al., 2002). Developmentally, experiences during childhood have long-lasting effects on the immune system's evolving response capacity and susceptibility to disease. However, a gap exists in our current understanding of how psychological symptoms impact children's immune functioning over the course of development (O'Connor, Moynihan, & Caserta, 2014). The current study examined the association between children's anxiety symptoms and immune functioning, measured by variations in salivary immunoglobulin A (sIgA) levels, both concurrently and longitudinally to better understand the interplay between psychological and physical health.

Children's Anxiety Symptoms and Health

Anxiety disorders are one of the most prevalent psychiatric diagnoses in children and adolescents. A recent epidemiological review reported prevalence rates of anxiety disorders, in which symptoms cause ongoing distress and interfere with normal functioning, to be between 6.1% and 14.8% in young children aged 2 through 8 years, and 10.3% to 12.2% in adolescents aged 13 through 18 years across studies (Costello et al., 2011). Although anxiety is a universal experience and an important emotional response with genetic, biological and psychosocial underpinnings, chronic anxiety symptoms often lead to poorer perceived health, physical illnesses (i.e., infections), and poorer quality of life (Mendlowicz & Stein, 2014).

Anxiety comprises of both cognitive-emotional (e.g., worried, fearful, nervous) and physiological symptom dimensions (e.g., muscle tension, headaches, stomach aches, nausea). Younger children experience and report higher physiological anxiety symptoms, which result in significant distress, social impairments and increased physical health problems (Campo, 2012). These two symptom dimensions are hypothesized to reflect different facets of anxiety and are associated with unique patterns of underlying brain activity (Burdwood et al., 2016). However, the question of how physiological and cognitive-emotional anxiety symptoms may differentially impact immune functioning in children and adolescents remain unclear. Even without meeting

diagnostic criteria for a given anxiety disorder, chronic anxiety symptoms contribute to on-going strain and chronic autonomic arousal to result in negative impacts on long-term health (Pinquart & Shen, 2011). Given the above, it is important to examine children's anxiety symptoms from a dimensional approach, where both cognitive-emotional and physiological symptoms dimensions are explored in relation to immune system functioning (Sharp et al., 2015).

The Immune System: Salivary Immunoglobulin A

Anxiety symptoms influence physical health through interactions with aspects of the immune system implicated in stress response. The immune system is a complex and interactive network involving the brain, neurotransmitters, neuropeptides, secretory glands, and a multitude of specific immune cells. Although no single marker of "immune functioning" can fully reflect immune competence, sIgA is often selected as an indicator of the functional status of the local mucosal immune system due to its non-invasiveness, convenience and widespread use in psychoneuroimmunology research (Obayashi, 2013).

SIgA is a non-specific antibody found in high concentrations on mucosal surfaces and barriers of the body (i.e., tears, saliva, gastrointestinal tract, respiratory epithelium and the gut). As the most abundant class of antibodies found in the intestinal lumen of humans and most mammals, sIgA is a "first line of defense" against invading organisms and play a key role in the prevention of infectious diseases (Hereman, 2014). SIgA defends against foreign antigens by preventing attachment to or penetration of body surfaces by invading microorganisms, clearing antigens and pathogenic microorganisms from the intestinal lumen, and modulating the balance between pro- and anti-inflammatory states (Woof & Kerr, 2006). Lower overall levels of sIgA have been shown to be a risk factor for susceptibility to infections, increased upper respiratory infections, periodontal disease and caries, while higher levels of sIgA indicate immuno-competence (Corthésy, 2013). SIgA secretion in adults and children also follows a diurnal rhythm peaking at awakening, then decreasing for the next four hours, while remaining relatively constant towards early evening (Hucklebridge et al., 1998; Watamura, Coe, Laudenslager, & Robertson, 2010).

Anxiety Symptoms and sIgA: A Mutual Regulation Process

Psychological disorders and the experience of emotional symptoms such as anxiety strain the underlying biological stress systems to trigger stress-related immune alterations (see Segerstrom & Miller, 2004 for a review)**.** At the same time, alterations in the immune system can

also have a bidirectional and transactional effect on anxiety symptoms by way of feedback mechanisms (Nusslock & Miller, 2015).

A number of studies have identified specific stressors or challenges that are associated with changes in diurnal sIgA rhythm in adults and children (Tsujita & Morimoto, 1999; Watamura et al., 2010). However, it is unclear how and in which direction psychological strain and distress associated with the experience of anxiety symptoms may alter aspects of the underlying diurnal rhythm of sIgA in children. This is an important question to examine further given the high prevalence rates of anxiety symptoms in childhood, and the range of negative behavioural and health consequences that are associated with dysregulated circadian rhythms (Barnard & Nolan, 2008).

Previous findings are inconclusive regarding the exact nature and directionality of the relationship between anxiety symptoms and aggregated overall levels of sIgA. On the one hand, some studies have shown that anxiety and laboratory stress led to temporary increases in total sIgA levels (Dhabhar, 2009; Hucklebridge et al., 2000), while others have reported that negative mood is associated with decreased sIgA concentrations, which in turn predicted higher susceptibility to physical illnesses (F. Li et al., 2008; Tsuboi et al., 2008). Most of these studies have only examined the unidirectional association between anxiety symptoms and sIgA levels at a common point in time, leaving questions regarding the longitudinal sequence and possible mutual regulation of anxiety and sIgA over time unanswered.

It is possible that anxiety symptoms and changes in sIgA levels may be components of a chain of sequential cause and effect, with each affecting the other in a circular, feedback loop over time (e.g., Anxiety → Immune Function → Anxiety) (Thomas et al., 1995). Transactional feedback loops are reciprocal processes involving at least two systems, commonly used in studies of the interactions between individuals and their surrounding contexts that may result in an interrelated and mutually regulated sequence of changes observable in both systems (Bronfenbrenner & Morris, 2006; Cicchetti, 1996). These feedback loops can be described as either "vicious" (i.e., positive or augmenting feedback loops) or "virtuous" (i.e., negative feedback loops). A vicious cycle is characterized by the continuation or amplification of deleterious effects, whereas a virtuous cycle is self-correcting over time. Examining possible mutual regulatory feedback mechanisms in relation to anxiety symptoms and sIgA over time may

help disentangle the contradictory findings within the current literature and elucidate longitudinal transactional processes between psychological symptoms and immunity.

In addition, chronic experience of psychological symptoms such as anxiety may strain underlying immuno-endocrine systems. Consistent with the allostatic load theory, coping with chronic strain requires an adaptation of neuroendocrine, immunological and autonomic systems resulting in potential cumulative wear and tear on these systems and down-regulation over time (Juster et al., 2010). This may in turn result in long-term negative health consequences including poor subjective health and higher susceptibility to infections (Ruttle, Serbin, Martin-Storey, Stack, & Schwartzman, 2014). The extent to which children and adolescents' immune system may be affected by the prolonged experience of anxiety symptoms has not been examined to date.

The Present Studies

The objectives of the present paper were addressed in two complementary studies examining differing facets of the relationship between anxiety symptom dimensions and sIgA in children and adolescents. Study 1 explored the contemporaneous associations between anxiety symptom dimensions and diurnal sIgA rhythm in middle-childhood (age 9 to 12). Study 2 examined evidence of feedback loops between anxiety symptoms and aggregated total sIgA levels over three consecutive waves of data collection spanning nine years (age 9 to 18) that would suggest mutual regulation between the two systems.

In Study 1, we hypothesized that children with higher levels of anxiety symptoms (both cognitive-emotional and physiological) would show higher elevations in their diurnal sIgA during the day compared to those with lower anxiety symptoms. Given the associations between anxiety and the autonomic nervous system, we would similarly expect a temporary mobilization of all related systems implicated in mounting a stress response, which includes aspects of the immune system (Moons & Shields, 2015; Sominsky et al., 2013).

In Study 2, we hypothesized a "vicious" cycle or positive feedback loop whereby higher total anxiety symptoms, specifically worries and social concerns, at Wave 1 (ages 9 to 12) would lead to lower sIgA levels at Wave 2 (ages 12 to 15). Lowered levels of sIgA at Wave 2 would in turn predict higher levels of anxiety symptoms at Wave 3 (ages 15 to 18). It was also anticipated that lower sIgA levels at Wave 1 might lead to increases in anxiety symptoms at Wave 2, due to increased likelihood that these children might experience more physical illnesses, which may in

turn increase their anxiety symptoms, and subsequently lead to decreases in sIgA at Wave 3.

Methods for Study 1 and Study 2

The Concordia Longitudinal Risk Project: Original Sample

Participants were part of the Concordia Longitudinal Risk Project, a multi-generational study of families initiated in 1976 (Schwartzman et al., 1985). Representative subsets of the participants and their families have been followed since that time on various observational, interview-based, health, education, social functioning, and emotional symptoms measures. For a more detailed description of the project's original sample and methodology, please refer to Serbin et al. (1998).

Participating families (N = 115) were French speaking from primarily Quebecois (French-Canadian) backgrounds, with fewer than 5% from Latin American, Haitian, or other ethnic backgrounds. At Wave 1 (2002-2005), children were between 9 to 12 years old (M_{age} = 10.79, SD_{age} = 0.92). At Wave 2 (2005-2008), children were in early adolescent years (ages 12 to 15; M_{age} = 13.62, SD_{age} = 1.11), and in the final wave of data collection at Wave 3 (2008-2012), children were in mid-adolescence (ages 15 to 18; M_{age} = 16.95, SD_{age} = 1.27). In the current sample, the average income of the participating families was $59,018 at Wave 1, which is below the reported Canadian national ($66,550) and Quebec ($61,780) median income levels for that period (Statistics Canada, 2013). For sample sizes and characteristics of the specific sub-samples, please see below under Study 1 and Study 2 Participants.

Procedure

The data analyzed in the current study were collected with approval from the Institutional Review Board of Concordia University. All procedures were carried out with the adequate understanding and written informed consent of the children's parents and caregivers prior to their participation.

SIgA sampling procedure and measurement. Participants were asked to collect salivary samples using salivettes on two consecutive school days (8 samples each day) at specified target times throughout each day (i.e., upon awakening, 30 minutes post-awakening, followed by every 2 hours until bedtime). Participants were instructed to remove the cotton swab from a plastic vial, place it in their mouth for 30 to 45 second until it was saturated with saliva, before placing it back in the vial, handling it as little as possible. The salivettes were subsequently assayed for sIgA concentrations at the Douglas Hospital Research Laboratories in Montreal, Quebec, Canada.

Participants were asked to record the exact times that the samples were taken in addition to any illnesses, medications taken, mood, diet, exercise, and naps at each of the salivary sampling times. Unstimulated saliva was used and sIgA concentrations were calculated using a commercially available salivary enzyme immunoassay kit without modification to the manufacturer's recommended protocol (Salimetrics, State College, PA, USA). The assay used 25ul of saliva per determination, has a lower limit of sensitivity of 2.5ug/mL, which is the minimal concentration of sIgA that can be distinguished from 0, and a range of standard curve from 2.5 to 600ug/mL. On average, inter and intra-assay coefficients of variation were less than 15% and 10%, respectively, at each of the three waves of data collection (Schultheiss & Stanton, 2009). Samples were run in duplicate and mean concentration values were calculated for each sample. In order to normalize the distributions, a natural log transformation was applied to raw sIgA concentration values to correct for positive skew in the distribution and the remaining outlying scores were winsorized to within three standard deviations of the mean, consistent with transformation procedures reported in previous studies (Phillips et al., 2006).

Measures

All measures in the current study were administered in French. Translated versions of English measures were created through a back-translation process when published French-language versions were not available. Back-translated measures used in the current study included the Revised Children's Manifest Anxiety Scale (RCMAS) (Reynolds & Richmond, 1985b; Turgeon & Chartrand, 2003) and Children's Depression Inventory (CDI) (Kovacs, 1985; Mack & Moor, 1982).

Control variables. Children's sex (coded as 0 = male; 1 = female), age (as a time-varying covariate), maternal educational attainment (years of education), income, and family socioeconomic status (occupational prestige scores) were included in all analyses as control variables (Fowler, Henry, & Marcal, 2015; McMunn, Kelly, Cable, & Bartley, 2012). Additionally, medications (coded as 0 = no; 1 = yes), and the frequency and type of physical illnesses (e.g., allergies, bronchitis, asthma, colds) were also controlled given their associations with both anxiety symptoms and sIgA (Ruttle, Serbin, et al., 2014).

Although the focus of the current studies is on understanding the relationship between children's anxiety symptoms and sIgA, we also included a measure of children's depressive symptoms to control for its effects given the comorbidity between anxiety and depression, and

the research showing anxiety as a precursor to the development of depression (Cummings, Caporino, & Kendall, 2014). Participants completed the same self-report measures of anxiety and depressive symptom measures at all three waves of data collection. In the current studies, overall anxiety and depressive symptoms were moderately correlated across the three waves (*rs* = 0.30 at Wave 1; 0.43 at Wave 2; and 0.65 at Wave 3; *ps* < .01).

Anxiety symptoms. Participants completed the self-reported 37-item Revised Children's Manifest Anxiety Scale (RCMAS) (Reynolds & Richmond, 1985b), which assessed the level and nature of their anxiety symptoms. The RCMAS yields a total anxiety summary score based on 28 items, which are divided into three anxiety subscale scores in specific domains of worry/oversensitivity (11 items; e.g., *"I am afraid of a lot of things")*, social concerns/concentration (7 items; e.g., "*Other people are happier than I")*, and physiological/somatic anxiety (10 items; e.g., *"Often I feel sick in the stomach.")*. The remaining nine items on the RCMAS constitute the Lie subscale as a validity check on the responses. Items on each subscale were constructed so that they do not overlap with each other in the calculation of subscale scores. Overall raw scores of 19 and above (*T* scores $\geq$ 60) are indicative of participants who are experiencing clinically significant levels of anxiety. The percentage of participants who scored within the clinically significant range of anxiety was low (2.4% at Wave 1; 1.1% at Wave 2; 1.5% at Wave 3). Relative to the normative sample according to sex and age of the participants, the distribution of anxiety scores of the current sample of participants reflected non-clinical levels of anxiety symptoms generalizable to beyond only clinical or borderline/sub-clinical samples (Gerard & Reynolds, 2004).

Recent normative studies examining the psychometric properties of the RCMAS in various samples of children and adolescents reported high internal consistency for its total score in addition to each of its subscales in diverse samples (Turgeon & Chartrand, 2003; Varela & Biggs, 2006). In the current studies, the Cronbach alphas for total anxiety (α = 0.74 – 0.80), worry/oversensitivity (α = 0.78 – 0.82), physiological/somatic anxiety (α = 0.59 – 0.61), and social concerns/concentration (α = 0.61 – 0.70) indicated acceptable internal consistency across the three waves of data collection.

Depressive symptoms. The Children's Depression Inventory (CDI) (Kovacs, 1985) is a 27-item self-report scale suitable for assessing depressive symptoms in children between the ages of 7 to 17. Depressive symptoms are controlled for in the current analyses as a possible

confounding variable given its comorbidity with anxiety symptoms. Each item consists of three self-report statements graded in severity from 0 (least severe) to 2 (most severe). Children are asked to indicate which statement best matched how they have been feeling in the past two weeks (e.g., *I am sad once in a while (0); I am sad many times (1); I am sad all the time (2)).* Total scores of 19 and above on the CDI (*T* scores $\geq$ 65) indicate clinically significant levels of depression (Masip et al., 2010). In the current study, 7.2% of the children at Wave 1, 6.7% at Wave 2 and 3.0% at Wave 3 scored within the clinical range. Depressive symptoms scores, in terms of means and standard deviations within the current sample are comparable to those reported in community samples (Masip et al., 2010).

Recent studies have reported the CDI as a reliable and valid measure across different cultural samples as well validating its use for screening purposes and in comparison to structured interviews (Ivarsson, Svalander, & Litlere, 2006; Steele et al., 2006). In the current studies, the Cronbach alphas for total CDI indicated high internal consistency across all three waves of data collection ($\alpha = 0.81 – 0.83$).

Study 1: Anxiety Symptoms and Diurnal sIgA at Wave 1

Study 1 Subsample

A total of 77 children consented to complete the relevant questionnaires and salivary sampling. Out of these, 51 children (male = 24; female = 27) ranging in age from 9 to 12 years (M = 10.95 years; SD = 0.90) provided sufficient and adequate sIgA saliva samples (i.e., minimum 4 samples per day across 2 days). The saliva samples from the remaining 26 participants were not available because participants did not successfully complete the salivary sampling procedure, contamination of samples during storage, or insufficient amount of saliva available for immunoassays.

Data Analytic Plan

Hierarchical Linear Modelling (HLM), Version 7.01 (Bryk & Raudenbush, 1992; Raudenbush et al., 2004) was used to analyze the data. HLM models change over time in the outcome variable by estimating a curve for each individual based on available values, while extrapolating missing data points from this curve (Hruschka et al., 2005). HLM models convey information about an individual's baseline (i.e., the intercept), and his or her change across time (i.e., the slope) while taking into consideration other between-person contextual factors that may serve as additional explanatory variables and/or confounds (Shirtcliff & Essex, 2008). As such, it

is well suited to capture diurnal slope variations in sIgA.

A three-level HLM model was constructed partitioning within the day (n = 433 samples; total df = 433), day-to-day (n = 94 days; total df = 94), and between individual (i.e., individual differences in anxiety symptoms) (n = 51; total df = 51) sources of variability in diurnal sIgA. A series of steps were followed for model specifications (Bryk & Raudenbush, 1992). First, an unconditional random intercept model was constructed with only the outcome variable (i.e., log-transformed diurnal sIgA) entered to examine whether there is significant variability in sIgA to be explained at each of the levels. Results showed significant within and between-person effects ($p < .001$) on sIgA variability that remain to be further explored. We then specified theoretically relevant predictor variables at each of the three levels to explain diurnal sIgA variation.

Level 1 specification included “time since waking" (TSW) as a within-the-day predictor capturing the diurnal rhythm of sIgA variation throughout the day. Children in the sample showed significant differences in their intercept (β = 1.321; t (50) = 25.87; SE = 0.05; $p < .000$), which reflects baseline levels of sIgA upon awakening for each individual. The models also included both quadratic (TSW^2) and cubic (TSW^3) functions of the linear TSW slope to allow for the examination of non-linear curvature in individual slopes across the day. Time since waking in terms of its linear slope (TSW: β = 0.131; t (50) = 3.11; SE = 0.04; $p < .000$), quadratic (TSW^2: β = -0.025; t (50) = -3.34; SE = 0.008; $p < .01$), and cubic slopes (TSW^3: β = 0.001; t (50) = 3.41; SE = 0.000; $p < .000$) were all significant and accounted for 70.04% of the total variation in sIgA across the day. In other words, a significant proportion of variation in sIgA across the day is associated with its diurnal rhythm. Additional within-the-day predictors of mood, stress, health, food intake and exercise were found to be non-significant predictors of intercept and slopes, and were removed from the models to preserve parsimony (Anderson, 2012).

Level 2 model specification captured day-to-day variability in sIgA intercept and the diurnal slopes across the two days of salivary sampling, with individual’s report of medication usage on each day of sIgA sampling included as a predictor.

Finally, Level 3 specification captured between-persons variations in diurnal sIgA intercept and slopes. Control variables of age, sex, maternal education, socioeconomic status, and health variables (i.e., number of infections; subjective health) were first entered into all models. Next, depressive symptoms were also added as a control variable. Last, predictors of interest, including total anxiety scores, physiological and cognitive-emotional anxiety symptom scores

(i.e., worries and social concerns) were entered into the model.

Study 1 Results

Descriptive statistics. Table 1 summarizes the descriptive statistics including the means, standard deviations, and intercorrelations for all variables at Wave 1.

Figure 1 shows a descriptive linear scatter plot of each child's observed diurnal variation in sIgA over the day in the current sample. Similar to previous findings, the observed diurnal patterns of sIgA showed an increase within the first 4 hours post-awakening, followed by a gradual decrease towards the afternoon, with most children also exhibiting a gradual increase towards the evening.

Control variables. No statistically significant main effects or interactions with predictors of interest were found in relation to age, sex, maternal education, socioeconomic status, and health variables (i.e., infections; subjective health). These variables were also not statistically significant in their associations with diurnal sIgA intercepts or slopes in the current study.

Higher anxiety symptoms predicted over-activation of diurnal sIgA. Between-persons differences in levels of anxiety symptoms had significant effects on the diurnal slopes (linear: β = 0.015; t (49) = 2.53; SE = 0.006; $p < .05$; quadratic: (β = -.001; t (50) = -3.07; SE = 0.000; $p <$ .01) and accounted for 27.08% of the total variance in sIgA levels. Specifically, children with higher total anxiety showed steeper increases in levels of sIgA from awakening until five hours after awakening in comparison to those with lower total anxiety symptoms (Figure 2; upper left panel). Their sIgA levels throughout the day also remained elevated, before decreasing to a level below those with lower anxiety symptoms in the evenings.

In examining the specific dimensions of anxiety symptoms, children's physiological anxiety symptoms did not have statistical significant associations in relation to the overall intercept or slopes of sIgA (Figure 2; top right panel). In contrast, children with higher worries had more elevated sIgA and steeper increases from awakening to five hours afterwards (linear slope: β = 0.022; t (49) = 2.26; SE = 0.009; $p < .05$; quadratic slope: β = -0.002; t (49) = -2.26; SE = 0.001; $p < .05$) than compared to those with fewer worries (Figure 2; bottom left panel). Similarly, children with higher social concerns also showed a similar pattern in terms of steeper increases in sIgA levels over the course of the morning, before showing decreases into the afternoon with evening levels lower than those with average to low levels of social concerns (quadratic slope: β = -0.004; t (49) = -2.56; SE = 0.002; $p < .05$) (Figure 2; bottom right panel).

The statistical significance of the effects of anxiety symptoms dimension on the intercept and slope of diurnal sIgA remained after the inclusion of all control variables described above.

Discussion of Study 1 Results

The goal of Study 1 was to examine how children's experiences of anxiety symptoms, both physiological and cognitive-emotional dimensions, impact the diurnal rhythm of sIgA. Children's diurnal sIgA rhythm followed a similar pattern to that reported in the literature, showing a gradual increase within the first 4 hours post-awakening, followed by a gradual decrease towards the afternoon, with most children also exhibiting a gradual increase towards the evening (Hucklebridge et al., 1998; Watamura et al., 2010). In relation to the main hypothesis of the study, results showed a pattern of activation (as shown by steeper increases in sIgA following awakening to 5 hours afterwards) in diurnal sIgA in children with higher anxiety, specifically, worries and social concerns, than those with lower anxiety. These results are consistent with findings in the literature showing temporary activation of the immune system and increases in sIgA in direct response to immediate or concurrent experiences of distress associated with negative mood and emotional states (Deinzer et al., 2000). In contrast, physiological anxiety symptoms (e.g., muscle tension, headaches, stomach aches, nausea) did not significantly predict diurnal slopes of sIgA in the current sample. Since anxiety symptoms were measured in the same time period as sIgA samples, we do not know from these results whether transactional feedback loops existed between sIgA and anxiety. Study 2 addressed this question by examining the long term sequence and directionality of the relationship between anxiety symptoms and sIgA levels over nine years using a double cross-lagged panel design. Study 2 included three consecutive waves of data collection, obtained at approximately 3 year intervals when the children were between ages 9 and 18 years.

Study 2: Longitudinal Sequence and Transactional Feedback Loops between Anxiety Symptoms and sIgA

Study 2 Participants

A sample of 103 children (48 boys; 24 overlapping with Study 1) participated in study 2. Because Study 2 examined overall aggregated daily averages of sIgA across the two days, over three successive longitudinal time periods, a minimum of 3 valid saliva samples at each wave were sufficient to estimate overall aggregated averages of total sIgA levels. This sample was

therefore larger than that of Study 1 because we were able to include those participants with fewer samples per day.

Missing Data

Data were missing at random at all three waves and resulted from attrition, non-successful completion of questionnaires and/or salivary samples, and insufficient amount of sIgA samples. Full Information Maximum Likelihood (FIML) method was used to estimate missing data in both the predictors and outcomes across all three waves of data collection (Muthén & Muthén, 1998). FIML estimation was found to be superior to list-wise deletion, pairwise deletion and mean imputation methods resulting in less bias and sampling variability (Enders & Bandalos, 2001). Even with large amounts of missing data present for dependent variables (i.e., above 50%), studies have shown FIML to be a good estimation method in providing unbiased parameter estimates (Graham, 2009). The amount of missing data across the sample for all variables of interest ranged between 13% to 35% across the three waves.

Procedure

SIgA samples, symptom measures, and demographic data were collected in three consecutive waves between 2002 and 2012 (ages 9 to 18). At Wave 1 (2002 – 2005), children were between 9 to 12 years (M_{age} = 10.79, SD_{age} = 0.92). At Wave 2 (2005 – 2008), children were in their early adolescent years (ages 12 – 15; M_{age} = 13.62, SD_{age} = 1.11), and at Wave 3 (2008 – 2012), children were in late adolescence (ages 15 – 18; M_{age} = 16.95, SD_{age} = 1.27). Consecutive waves of repeated data collection provided an ideal design in the examination of longitudinal transactional and reciprocal feedback loops between anxiety symptoms and sIgA over several years to test our hypotheses of mutual regulation.

Aggregated total sIgA. Whereas Study 1 explored the diurnal rhythm of sIgA using repeated daily measures of sIgA, Study 2 aimed to replicate and extend what is already known in the literature using overall aggregated levels of sIgA by computing an average daily total from the available samplings of sIgA for each participant in our sample at all three successive waves of data collection. All other control variables remained the same as those described previously in the Methods for Study 1 and Study 2 section.

Data Analytic Plan

Autoregressive double cross-lagged panel modeling (ARCL) (Kenny, 2005) was used to examine the longitudinal transactional sequence and feedback loops between sIgA and anxiety symptoms across the three waves using Mplus Version 7 (Muthén & Muthén, 1998).
The cross-lagged panel design is ideal for examining the longitudinal and reciprocal sequence of relations between two constructs, while also being able to control for contemporaneous associations between the variables, and the stability of the variables over time. Double cross-lagged designs are ideal for clarifying questions of directionality over time and for exploring feedback loops especially when there are more than two waves or time points of repeated measures assessments (Kenny, 2005; Kenny & Campbell, 2014).

The statistical fit of the cross-lagged models were assessed using a combination of model fit indices commonly reported in the literature (Markus, 2012). The chi-square test statistic indicates whether there is a significant difference between the sample means and variance-covariance structure and those from the hypothesized a priori model. Non-significant chi-square fit statistics indicate good fitting models. In addition to the chi-square test statistic, which is sensitive to sample size, several other fit indices were also used. These included the comparative fit index (CFI; Bentler, 1990), the root mean square error of approximation (RMSEA; Kenny, Kaniskan, & McCoach, 2014) and the standardized root mean square residual (SRMR; Bentler, 2007). Generally, CFI values over .90 (Hoyle & Panter, 1995) suggest acceptable fit. RMSEA and SRMR values of .08 or less also indicate adequate fit (Hu & Bentler, 1999).

Study 2 Results

Descriptive statistics. Table 2 provides descriptive statistics on variables of interest at all three waves.

Augmenting feedback loop between total anxiety symptoms and sIgA. Results from the autoregressive double cross-lagged models showed an augmenting feedback loop between total anxiety and sIgA levels over time. This model including total anxiety and sIgA indicated good model fit: $\chi 2$ (5) = 4.19, p = .52, CFI = 1.000, RMSEA = .000 CI_{95}(.000, .125), SRMR=.054. Figure 3 shows the final standardized results. Consistent with our hypothesis, higher total anxiety at Wave 1 predicted lower overall sIgA levels at Wave 2. Diminished sIgA levels at Wave 2 in turn predicted higher overall total anxiety symptoms at Wave 3, while controlling for all contemporaneous associations between the two variables at each wave.

Feedback loops between anxiety symptom dimensions and sIgA. Double cross-lagged models were also utilized to examine whether reciprocal feedback loops were present between specific anxiety symptom dimensions (i.e., cognitive-emotional versus physiological symptoms) and sIgA over several year. Similar to the previous model, an augmenting or "vicious" cycle was found in the transactional relationship between worries and sIgA over time (Fit Indices: $\chi 2$ (5) = 9.68, p = .09, CFI = 0.800, RMSEA = .09, CI_{95}(.000, .185), SRMR=.08). Figure 4 (top panel) shows the final standardized coefficients. Specifically, higher worries at Wave 1 predicted lower sIgA levels at Wave 2. Lower levels of sIgA at Wave 2 in turn led to higher worries at Wave 3 showing an augmenting looping effect between the specific anxiety dimension of worries and sIgA over time. This is in contrast to the social concerns dimension of anxiety, which showed stability over time, but did not have statistically significant transactional feedback loops with sIgA (Figure 4, bottom panel).

The last cross-lagged model examined the association between physiological anxiety symptoms and sIgA, which had excellent fit indices: $\chi 2$ (5) = 1.26, p = .94, CFI = 1.000, RMSEA = .00, CI_{95}(.000, .030), SRMR=.04. Similar to the previous models, results showed higher physiological anxiety symptoms at Wave 1 predicting lower levels of sIgA three years later at Wave 2, indicating compromised immune functioning after controlling for the contemporaneous associations. However, no feedback loop was found extending from Wave 2 to Wave 3 (trend: p = .09). All of these results remained significant after the inclusion of all time variant (i.e., age, infection, subjective health, depressive symptoms), and time-invariant (sex, SES, mother's education) control variables.

Discussion of Study 2 Results

The goal of Study 2 was to examine the longitudinal transactional relationship between anxiety symptoms and sIgA over three consecutive waves from age 9 to 18 and explore any feedback loops that may exist across that period between anxiety and sIgA. Extending Study 1 with the inclusion of three time points of repeated anxiety symptoms and sIgA measures, results from the autoregressive double cross-lagged panel analyses showed an augmenting feedback loop (i.e., "vicious cycle") between total anxiety, specifically, the worries symptom dimension, and sIgA over time. Higher total anxiety and worries in children between ages 9 to 12 predicted lowered immunity by way of diminished sIgA levels when they were 12 to 15 years of age. Lowered sIgA levels in turn predicted higher total anxiety symptoms and worries between ages

15 to 18. These results are the first to show an augmenting feedback loop that may be indicative of mutual shaping and maintenance of both anxiety symptoms and immune functioning over the course of development. In contrast, the social concerns dimension of anxiety did not show any reciprocal feedback loop relationship with sIgA over time although results indicated strong stability of social concerns anxiety symptoms that persist over time.

In contrast to Study 1 results, where physiological anxiety symptoms were unrelated to diurnal slopes or intercepts of sIgA measured at the same wave of data collection, higher physiological anxiety symptoms predicted lower levels of sIgA three years later at Wave 2 in the longitudinal cross-lagged panel design, indicating compromised immunity after controlling for the contemporaneous associations. However, no feedback loop was found extending from Wave 2 to Wave 3 in relation to physiological anxiety, although results come close to statistical significance. These results are among the first to highlight the longitudinal reciprocal relations between anxiety and immune functioning from childhood to late adolescence.

General Discussion

Anxiety symptoms are prevalent in childhood and adolescence, and are precursors to the later development of adult psychopathology and physical health problems (Farrell et al., 2011). A gap exists in our current understanding of how strain and distress from the experience of anxiety symptoms in childhood and adolescence may shape aspects of immune functioning over time (O'Connor et al., 2014). Contemporaneous association between anxiety and lowered levels of sIgA has been reported, with no studies to our knowledge examining the long-term transactional sequence between the two over several years (Vermeer, van IJzendoorn, Groeneveld, & Granger, 2012). This paper aimed to address the above limitations, examining how children's anxiety symptoms shape both the diurnal rhythm of sIgA between ages 9 to 12 (Study 1), and the mutual regulation between anxiety symptoms and total levels of sIgA over three consecutive data waves spanning ages 9 to 18 (Study 2). By including both designs, underlying associations between anxiety symptoms and sIgA levels both in the short- and long-term will be elucidated. Differences in both circadian rhythms and longer-term developmental effects will also help clarify mutual regulatory components between anxiety symptoms and immunity to better inform treatment and prevention.

First, concurrent results showed a pattern of activation where children with higher anxiety, specifically, worries and social concerns showed steeper increases in sIgA over the

morning (i.e., from awakening to five hours afterwards) than compared to those with fewer anxiety symptoms (Hucklebridge et al., 2000). In contrast, physiological anxiety symptoms did not have significant associations with the diurnal slopes or intercept of sIgA in the current sample when measured at the same time point. These results suggest cognitive emotional anxiety symptoms, similar to other contextual stressors, modulated the diurnal rhythm of sIgA secretion.

A novel contribution of the current results is in showing the presence of a "vicious" cycle or augmenting feedback loop between anxiety symptoms, specifically, worries, and total levels of sIgA over three consecutive waves from ages 9 to 18. Consistent with our hypothesis, higher total anxiety, specifically, worries at Wave 1 predicted lower overall sIgA concentration levels at Wave 2. In turn, diminished mucosal immunity at Wave 2 predicted higher total anxiety and worries at Wave 3. In contrast, although children's social concerns dimension of anxiety showed strong stability and persistence over time, there was no evidence of a feedback loop. These results indicate that children with persistent worries over time may be at risk for compromised mucosal immunity, reflected by lowered overall levels of sIgA. The findings further illustrate the mutual regulation between chronic anxiety symptoms and immunity over several years.

In addition, results also showed that higher physiological anxiety symptoms at Wave 1 predicted lower overall sIgA concentration levels at Wave 2. It is possible that physiological anxiety symptoms (i.e., stomach aches, nausea, sweating) reflect the experience of physical pain and illnesses (Campo, 2012). As such, it may be that physiological anxiety symptoms may be confounded with symptoms of physical illnesses. At the same time, these physiological anxiety symptoms are also closely associated with cognitive-emotional anxiety symptoms. It may be that worries as negative cognitive appraisal may make the experience of physiological symptoms worse, which may increase perceived distress and in turn strain the underlying immune systems even more (Lazarus, 1998; Lazarus & Folkman, 1984).

The complementary nature of the studies showed that anxiety symptoms in childhood measured at the same time as sIgA tend to mobilize the immune system in preparation for mounting a general stress response (Bosch et al., 2002). However, chronic and persistent anxiety symptoms that are unabated even in the decline and absence of threats or stressors are no longer adaptive, but strain the immune system over several years (Vermeer et al., 2012). This in turn leads to an adaptation of neuroendocrine, immunological and autonomic systems, similar to the experience of chronic stress, resulting in a down-regulation and suppression of sIgA (Johnston-

Brooks, Lewis, Evans, & Whalen, 1998; Juster et al., 2010). Given these results, the duration of the experience of emotional distress, such as anxiety symptoms, may have differential effects on underlying immune systems.

Strengths and Limitations. The strengths of the current studies include having three consecutive time points of data collection, which spanned a nine year period, capturing a major developmental transition from childhood to late adolescence. Second, the longitudinal design of the study allows for the observation of feedback processes and transactional interactions between anxiety symptoms and sIgA over the course of development. However, several limitations should be taken into consideration in interpreting the results, which may suggest extensions of the current design for future studies.

Although repeated assessments of both anxiety symptoms and sIgA at each wave of data collection enhanced the power of the analyses, the sample size was relatively small, limiting the statistical ability to detect interaction effects. Given that the original Concordia Study participants constituted a risk sample with exposure to higher than average environmental stress (in terms of lower income, SES and maternal educational levels), the current results may be limited in terms of its generalizability. However, these risk factors have been controlled for in the current analyses and found to have non-statistically significant main effects, or interactions with our predictors of interest. Further, the anxiety and depression symptom scores of the current sample are comparable to the normative data reported in the literature for large-scale community samples. Nevertheless, future studies should replicate the current findings using larger samples comprised of ethnically and culturally diverse participants.

Second, the current study only used sIgA as one measure of immunity. A more exhaustive measurement of immune responses is needed in future studies. Multiple cytokines, including interleukin (IL)-4, transforming growth factor-Beta, Il-5, Il-6, and Il-10 are instrumental in stimulating sIgA production. The lack of data for pro-inflammatory cytokines limits the interpretation of the interaction in the larger immune-endocrine network. Future studies will benefit from examining these related immune indicators to elucidate the mechanisms of their interactions.

Third, children's self-reported depressive symptoms were included as a control variable in all the analyses in the current study. Although depression has been shown to be related to inflammatory illnesses (Blume, Douglas, & Evans, 2011), depressive symptoms were not a

significant predictor of diurnal sIgA and did not have any longitudinal feedback associations with overall levels of sIgA over time. In all the models with anxiety symptoms as the predictor, adding depressive symptoms as a control variable also did not change the results in relation to anxiety and sIgA. This suggests that anxiety symptoms in children and adolescents seemed to have a stronger association with sIgA than depressive symptoms in the current sample. Future studies may warrant examining the association between anxiety, depressive symptoms and different aspects of the immune systems in more detail to clarify the underlying mechanisms.

Future directions. The current studies examined the construct of anxiety from a transdiagnostic dimensional approach, focusing on both the physiological and cognitive-emotional dimensions of anxiety in relation to sIgA. What remains to be explored in more detail is the meaning, sequence and directionality of the relationship between these anxiety dimensions over time. Although studies have highlighted the differences between the physiological and cognitive-emotional dimensions, what has not been explored in detail is the transactional sequence between them longitudinally.

Second, anxiety and psychological distress can influence immunological functions through various neuroendocrine mechanisms, which were not examined in the current study. In response to chronic stress, activation of the sympathetic-adrenomedullary system and the hypothalamic-pituitary adrenocortical system can lead to immunosuppression, with one outcome being sIgA deficiency. The relation between anxiety and sIgA found in the current studies may be in part explained by persistent hypersecretion of cortisol, which can lead to a diminished capacity to effectively suppress immune control pathways (Miller, Cohen, & Ritchey, 2002). With a larger sample, the relation between anxiety, neuroendocrine and immune function remains a question to be explored in future studies.

Lastly, given the association reported in the literature between anxiety and poorer health outcomes over time, the possible mediating role of sIgA warrants a closer examination in future studies. It is possible that lowered sIgA levels might explain the association between anxiety symptoms and the onset and frequency of physical illnesses.

Conclusions and Implications for Policy and Prevention

In conclusion, given that anxiety symptoms are especially prevalent in young children and are precursors to negative psychological and physical health outcomes in adolescence and adulthood, preventive intervention should be aimed at decreasing these symptoms early, before

the cumulative and reciprocal effects of feedback loops on neuroendocrine functioning, immune competency, and health can occur. Interventions such as optimizing social support, relaxation training, and mindfulness, which all have been shown to have positive effects in reducing anxiety symptoms and elevate immune functioning could be incorporated into prevention and intervention programs to help children and adolescents cope (Pawlow & Jones, 2005; Uchino, 2006).

In addition, children's anxiety symptoms are often associated with medically unexplained physical symptoms, or functional somatic symptoms (FSS) diagnosed by primary care physicians and pediatricians (Campo, 2012). Therefore, an accurate understanding of anxiety symptoms, both physiological and cognitive-emotional dimensions, and their respective associations with illnesses and health will allow for appropriate delivery of medical interventions in primary care settings. Physician training and additional resources focused on assessing and treating children's chronic anxiety symptoms could be an important preventive avenue for health care policy. Taken together, the current results provide an integrative understanding of how anxiety symptoms and immune functioning mutually regulate each other from childhood through adolescence. These processes may provide an important element in understanding the interrelations between psychological and physical health.

Table 1

Study 1: Means, Standard Deviations, and Pearson Correlations for All Variables at Wave 1

Variables	1	2	3	4	5	6	7	8	9	10	11	12	13
1. Child Sex	--												
2. Child Age	-.21	--											
3. Mat. Educ.	.67	-.13	--										
4. SES	.01	-.06	.34*	--									
5. Medication	.04	.04	.02	-.06	--								
6. Infections	-.18	.22	-.26	-.09	.28*	--							
7. Subj. Health	.13	-.20	.03	.20	-.10	-.10	--						
8. Total Anx.	.13	-.10	.09	.14	.09	-.02	-.04	--					
9. Physio. Anx.	.20	-.17	-.01	.09	.01	-.01	.06	.78***	--				
10. Worries	.10	-.05	.11	.07	.06	.01	-.12	.92***	.53***	--			
11. Soc. Conc.	.00	-.03	.15	.17	.22	-.07	-.03	.77***	.35*	.69***	--		
12. Dep. Sxs.	-.07	.05	-.41**	-.16	.15	.11	-.19	.35*	.23	.35*	.27	--	
13. Mean log sIgA	-.09	-.14	.12	-.05	.01	-.03	-.05	.13	.17	.10	.02	.17	--
Means	.53	10.95	12.41	44.53	.53	1.29	8.92	8.76	3.41	3.65	1.71	10.55	1.42
SD	.50	.90	2.46	11.64	.50	.99	1.16	5.87	2.37	2.96	1.63	6.59	.50

Notes. For child's sex, 0 = male, 1 = female; Mat. Educ. = Maternal Education; SES = Socioeconomic status; Meds = Medication; Subj = Subjective; Anx. = Anxiety; Physio = Physiological; Soc. Conc. = Social Concerns; Dep. Sxs. = Depressive Symptoms; *SD* = Standard Deviations. *$p < .05$; ** $p < .01$; ***$p < .001$. $n = 51$.

Table 2

Study 2: Descriptive Statistics for All Variables from Waves 1 to 3.

	Wave 1		***Wave 2***		***Wave3***	
	M	*SD*	*M*	*SD*	*M*	*SD*
*Sex (male:female)	48:55	-	-	-	-	-
*Yearly family income ($CAD)	59,018	33,324	-	-	-	-
*Socioeconomic Status (SIOPS)	44.00	11.77	-	-	-	-
*Maternal Education (Years)	12.17	2.47	-	-	-	-
Age	10.79	.92	13.62	1.11	16.95	1.27
Log SigA	1.55	.33	1.49	.52	1.76	.28
Total Anxiety Scores (RCMAS)	8.54	5.51	8.16	4.95	9.17	5.73
Physiological Anxiety Subscale	3.26	2.22	2.88	1.94	3.02	2.07
Worries Subscale	3.67	2.87	3.80	2.83	4.41	3.02
Social Concerns Subscale	1.60	1.54	1.49	1.60	1.74	1.74
Total Depressive Sxs (CDI)	9.90	6.48	8.57	6.24	9.16	5.63

Notes. *Time-Invariant Control Variables. *M* = Means; *SD* = Standard Deviation; sIgA = salivary immunoglobulin A; Sxs = symptoms; RCMAS = Revised Children's Manifest Anxiety Scale; CDI = Children's Depression Inventory; *n* = 103.

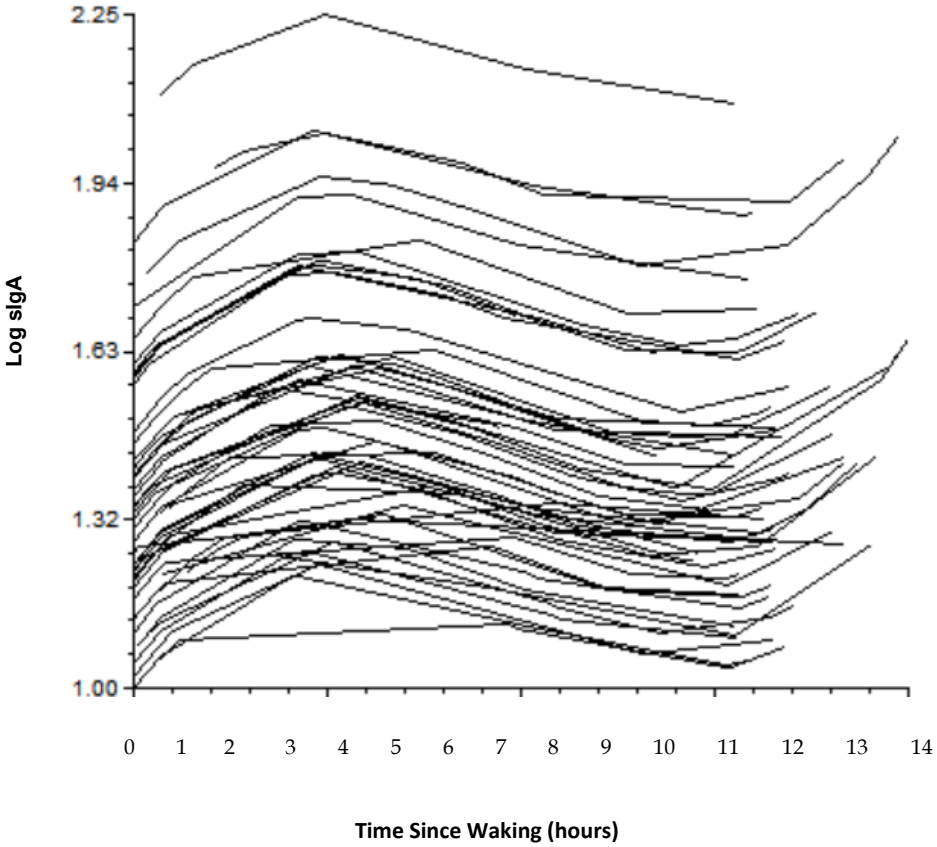


Figure 1. Descriptive linear scatterplot showing observed diurnal variations in children's sIgA levels across the day at Wave 1. $n = 51$.

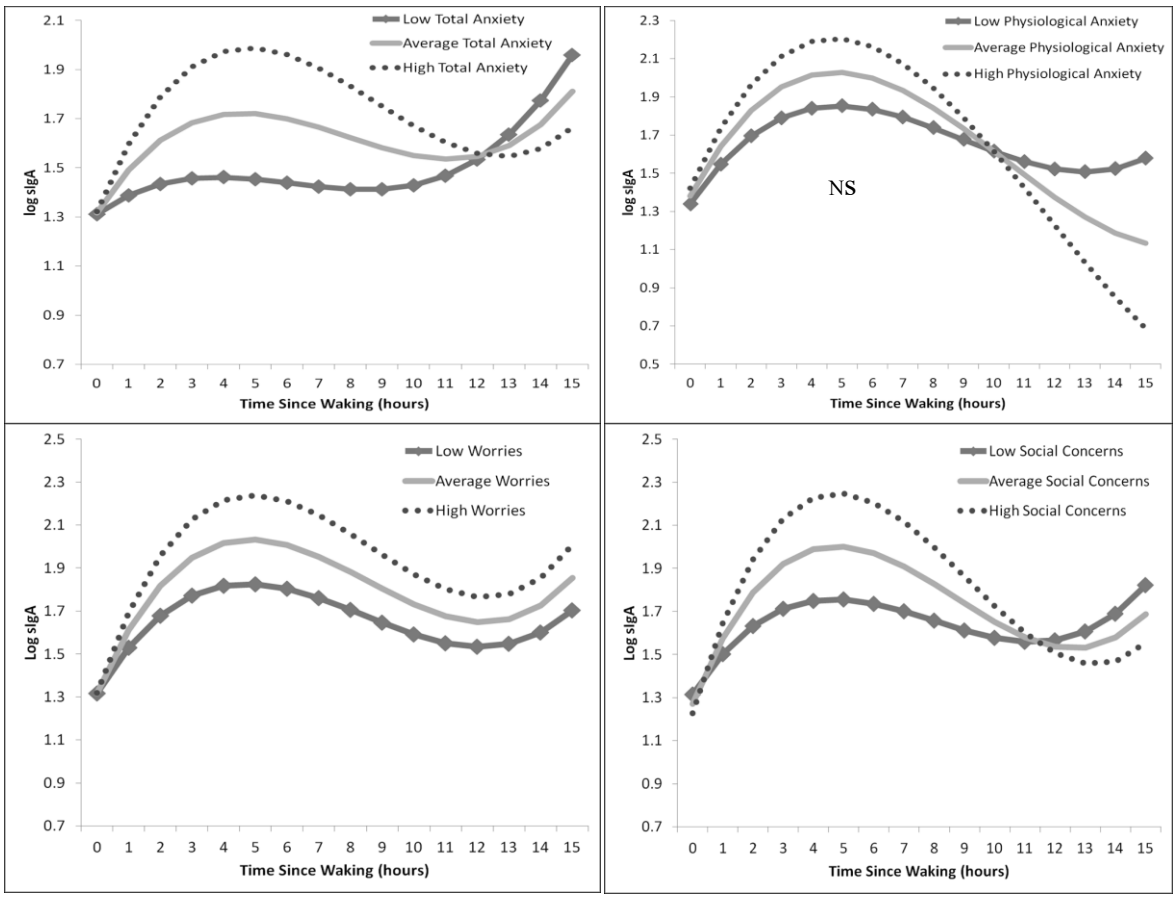


Figure 2. Levels of anxiety symptoms and associated effects on diurnal sIgA variations at Wave 1. Children with higher total anxiety, specifically, worries and social concerns dimensions showed steeper increases over the morning in sIgA in contrast to those with average to low levels of anxiety symptoms across the day. In contrast, physiological anxiety symptoms results did not show significant slopes or intercept effects in diurnal rhythm.

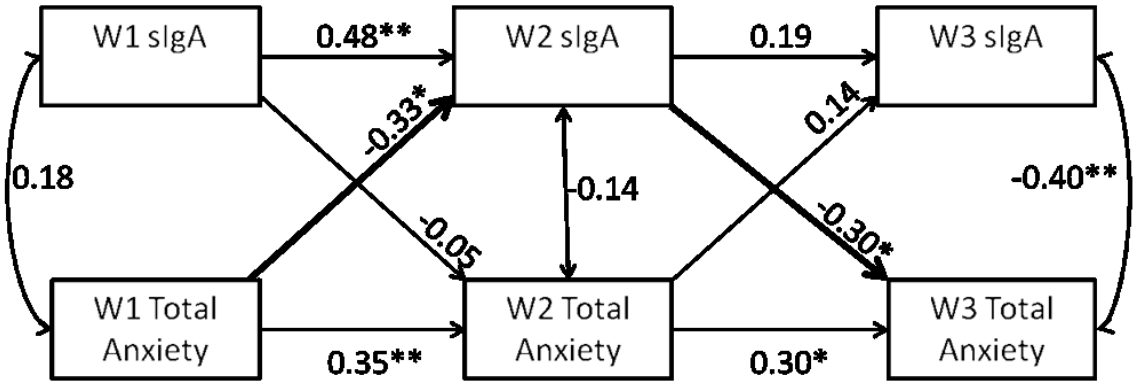


Figure 3. Standardized path coefficients showing a "vicious cycle" feedback loop between sIgA and total anxiety symptoms over three consecutive waves. The augmenting feedback loop is shown in red. Model fit indices: $\chi 2$ (5) = 4.19, p = .52, CFI = 1.000, RMSEA = .000 CI_{95}(.000, .125), SRMR=.054. n = 103.

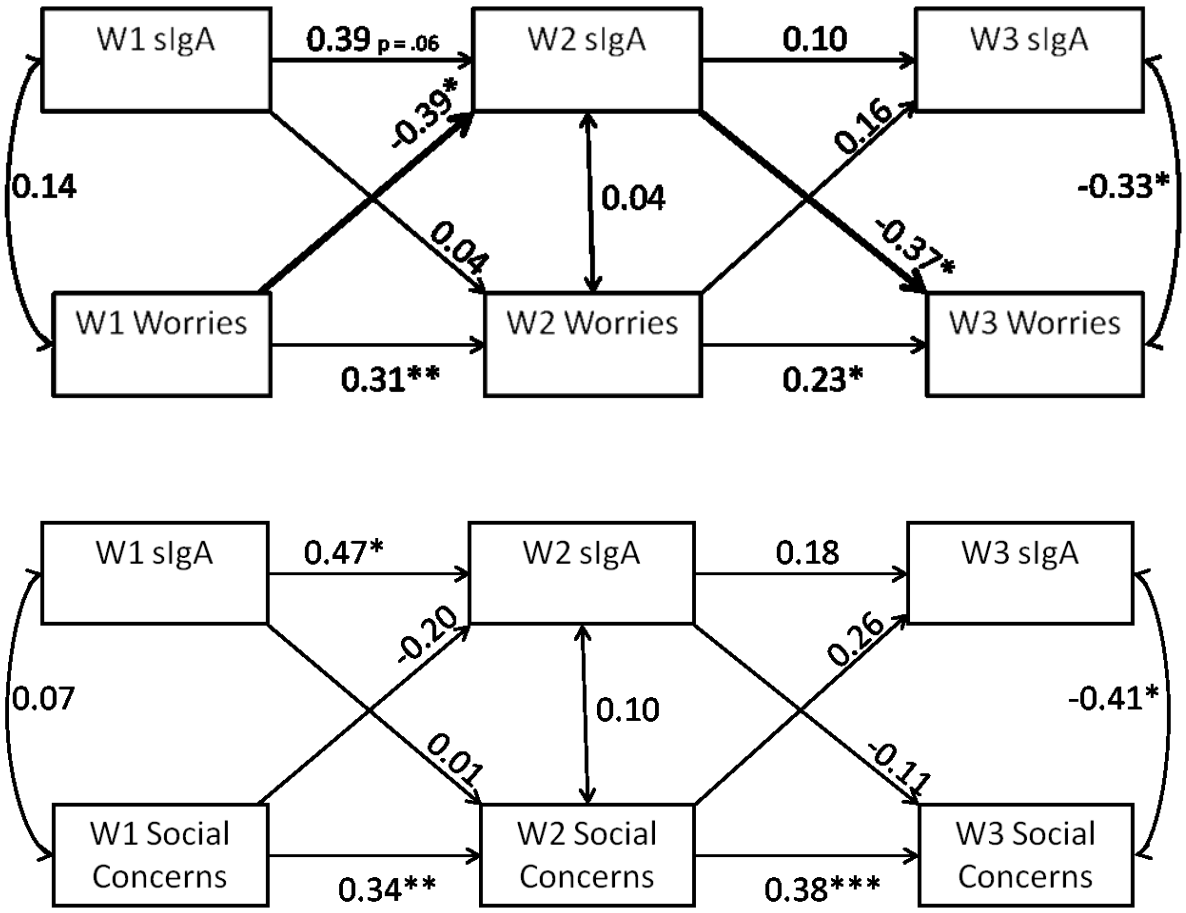


Figure 4. Top panel shows the standardized path coefficients showing a vicious cycle feedback loop between sIgA and worries over three consecutive waves. The augmenting feedback loop is shown in red. Model fit indices: $\chi 2$ (5) = 9.68, p = .09, CFI = 0.800, RMSEA = .09, CI_{95}(.000, .185), SRMR=.08.

The bottom panel displays the standardized path coefficients for the reciprocal relations between sigA and social concerns dimensions of anxiety over time. Model fit indices: $\chi 2$ (5) = 3.30, p = .65, CFI = 1.000, RMSEA = .00, CI_{95}(.000, .110), SRMR=.05. n = 103.

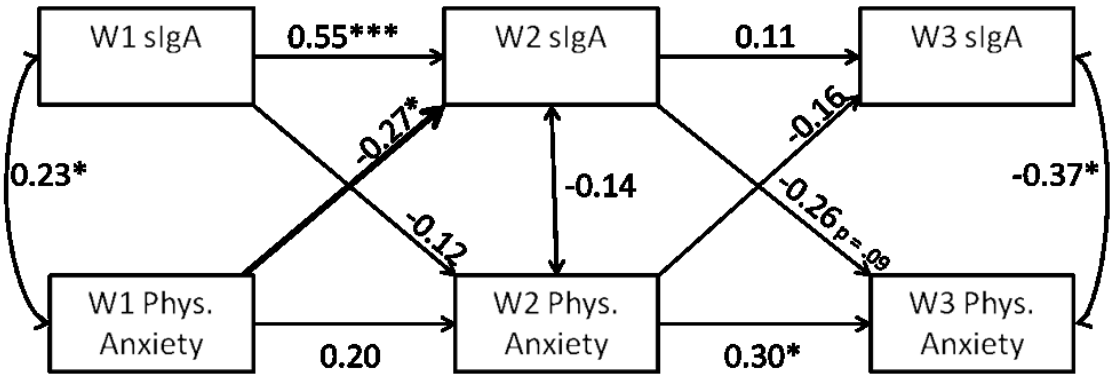


Figure 5. Standardized path coefficients showing the relationship between sIgA and physiological anxiety symptoms over three consecutive waves. Although Wave 1 physiological anxiety symptoms significant predicted lower sIgA levels three years later at W2, no feedback loop was found extending from W2 to W3 (trend; p = .09). Model fit indices: $\chi 2$ (5) = 1.26, p = .94, CFI = 1.000, RMSEA = .00, CI_{95}(.000, .030), SRMR=.04. n = 103.

Chapter 5: General Discussion

The goal of the current dissertation studies was to understand the developmental psychobiology of anxiety symptoms in children and adolescents. The two studies examined the longitudinal sequence of the association between anxiety symptoms and the HPA-axis (i.e., diurnal cortisol) and the related mucosal immunity system (i.e., salivary immunoglobulin A) separately in a sample of children and adolescents from the Concordia Longitudinal Risk Project. Results from these two studies are the first steps in disentangling the longitudinal psychobiological bases of anxiety symptoms. In clarifying the relationship between these systems and anxiety separately, these findings will set the stage for future studies examining the complex interrelations between anxiety symptoms and both the neuroendocrine and immune system over time.

The aims of the current studies were to address three research questions that remain unanswered within the current literature: 1) What is the longitudinal association and sequence of the relationship between children's anxiety symptoms and (1) diurnal cortisol and (2) salivary immunoglobulin A (sIgA), over several years?; 2) Is there evidence of transactional feedback loops whereby anxiety symptoms and biomarkers of the endocrine and/or immune system are components of a sequential chain of cause and effect, showing mutual regulation over several years?; and 3) Are there differential concurrent and longitudinal associations between specific anxiety symptom dimensions (i.e., physiological versus cognitive-emotional anxiety symptoms) and possible dysregulation of diurnal cortisol and sIgA functioning? Findings in relation to these questions would contribute to our current understanding of the transactional relationship between psychological symptoms and biological correlates over the course of development.

Regarding the longitudinal associations between anxiety symptoms and diurnal cortisol, results showed that cognitive-emotional dimensions of anxiety, specifically, chronically elevated worries and social concerns reported by the child, were predictive of blunting or flattening of the diurnal cortisol slopes three years later. In contrast, physiological symptoms of anxiety in childhood did not predict long-term effects on diurnal cortisol rhythms three years later in adolescence. Higher physiological anxiety symptoms did show stronger contemporaneous associations with diurnal cortisol, and predicted elevated diurnal cortisol throughout the day.

These results suggest that cognitive-emotional anxiety symptoms appeared to have long-term effects on the underlying stress system, specifically diurnal cortisol rhythms, similar to the

changes observed in response to chronic stress (Dieleman et al., 2015). Similar to the experience of chronic stress, unabated cognitive-emotional symptoms of anxiety may result in possible chronic arousal of the adrenal system and autonomic system. Cognitive emotional symptoms may lead to hypervigilance on the part of the individual over time, who may be engaged in frequent scanning of their environment for any perceived danger or threat. Over time, this tendency likely will result in over-activation of the autonomic and stress response systems, potentially leading to adrenal fatigue and a "wear and tear" effect similar to that reported in the chronic stress literature (Danese & McEwen, 2012; McEwen & Wingfield, 2010). Although these cognitive-emotional symptoms of anxiety may be initially triggered by perceived and/or actual threats, the "anxious apprehension" that remains afterwards may be incorporated into the cognitive schema of the individual in their appraisal and interpretation of future threats (Burdwood et al., 2016).

Whereas cognitive-emotional anxiety symptoms were associated with blunting of the cortisol rhythm three years later, physiological anxiety symptoms showed stronger concurrent associations with diurnal cortisol. One way to understand this difference is that physiological symptoms of anxiety tend to be situation- or stressor-dependent, more directly reflecting the "fight or flight" fear response related to the short-term activation of the autonomic system and fear circuitry. Although these physiological symptoms typically are short-term in duration, individual differences in cognitive appraisals may shape how individuals interpret and appraise these physical symptoms. Maladaptive or catastrophic cognitive appraisals could in turn exacerbate the experience of the physiological anxiety symptoms and lead to the experience of increased worries and concerns (cognitive-emotional anxiety symptoms) over time.

The endocrine system and aspects of the immune system are involved in signalling, communicating and mutual regulation in order to help maintain homeostasis for the whole system (Sephton & Spiegel, 2003). In order to examine the joint contribution of both systems in the development and maintenance of anxiety in future studies, we need to first clarify just how anxiety symptoms are related to immunity over time. Extending the discussion to the mucosal immune system, the current studies also examined how anxiety symptoms dimensions relate to diurnal sIgA rhythms in children and adolescents. Results showed a pattern of activation in diurnal sIgA in children endorsing higher cognitive-emotional symptoms of anxiety. These results are consistent with findings in the literature showing temporary activation of the immune system and increases in sIgA in direct response to immediate or concurrent experiences of

distress, negative mood and emotional states (Deinzer et al., 2000; Dhabhar et al., 2010). Again, differences emerged in relation to diurnal sIgA along the lines of the two anxiety symptom dimensions. In contrast to cognitive-emotional symptoms of anxiety, physiological anxiety (e.g., muscle tension, headaches, stomach aches, nausea) did not have any associations with diurnal slopes or intercepts of sIgA in the current study.

This may be explained in part by the timing and sequence of the cascading effect downstream that is associated with mounting a stress response. It is likely that physiological anxiety symptoms are more closely related to the initial fight or flight fear response, which is primarily associated with the autonomic changes that are an integral part of the stress response as an immediate physiological reaction to perceived or actual threat. However, the lingering and oftentimes persistent cognitive emotional symptoms of anxiety, which are closely related to the appraisal of the situation (Lazarus, 1998) may persist once the initial fight-or-flight response and associated symptoms have dissipated to exert effects downstream in mucosal immunity. It is possible that physiological anxiety symptoms as a direct response to threats and stress, both trigger and are maintained by cognitive emotional symptoms of anxiety. The exact sequence and transactional relation between the two dimensions of anxiety over time remain to be further explored in future studies.

An important and novel contribution of the current dissertation is in the findings showing an augmenting transactional feedback loop (i.e., "vicious" cycle) between cognitive emotional symptoms of anxiety and overall levels of sIgA over the span of nine years capturing key transitions in development. Consistent with our hypotheses, results from a double autoregressive cross-lagged analyses revealed that higher total anxiety and worries at Wave 1 (age 9-12) predicted lower overall sIgA levels at Wave 2 (age 12-15). In turn, diminished sIgA levels at Wave 2 predicted higher total anxiety and worries symptoms at Wave 3 (age 15-18). These results suggested that elevated and persistent anxiety symptoms, specifically, worries in childhood may have long-term mutual regulatory effects in relation to overall sIgA levels.

Double-cross lagged panel results also showed that higher physiological anxiety symptoms at Wave 1 predicted lower overall sIgA concentration levels at Wave 2. However, diminished sIgA levels at Wave 2 did not further predict higher physiological anxiety symptoms at Wave 3. Given that chronic anxiety symptoms predict poorer long-term health (Farrell et al., 2011), it is possible that these children who are experiencing physiological anxiety symptoms are

also more susceptible to illnesses in the future because of compromised mucosal immunity. Coping with physical illnesses may in turn make these children more anxious and more likely to worry about their physical symptoms, which could exacerbate and maintain the transactional feedback loop over time between anxiety and sIgA. It may be that worries as a negative cognitive appraisal may exacerbate physiological symptoms, resulting in distress and added strain to the underlying systems over time (Lazarus, 1998; Lazarus & Folkman, 1984).

This is in concordance with the perseverative cognition hypothesis (Brosschot, Gerin, & Thayer, 2006; Brosschot, Verkuil, & Thayer, 2010), which examines the moderating associations between worry and ruminations, and health consequences in response to the experience of stressors. In addition to stressful events experienced within our daily lives, sustained cognitive representations or appraisals about these stressful events may relate to prolonged physiological activity. This could in turn increase susceptibility to somatic diseases and exerts a toll on overall health. Worries, rumination and anticipatory stress are found to be associated with cardiovascular, endocrinological, immunological and neurovisceral systems to possibly play a role in terms of risk for future diseases (Verkuil, Brosschot, Gebhardt, & Thayer, 2010).

Taken together, the findings from the dissertation studies highlighted differences between dimensions of anxiety symptoms and their associations with specific aspects of the underlying HPA-axis and mucosal immunity systems. These results are consistent with emerging research showing that physiological and cognitive-emotional anxiety symptoms may have different underlying functional and biological correlates (Burdwood et al., 2016; Sharp et al., 2015). In both studies, cognitive-emotional dimensions of anxiety, in particular worries seemed to have longer-term effects on changes in diurnal cortisol and sIgA levels over time. Children's worries predicted a significant blunting or "flattening" of the diurnal rhythm three years later, in addition to compromised mucosal immunity indicated by lowered sIgA levels over time. These results seem to suggest longer-term negative effects of anxious appraisals (i.e., worries; ruminations) that remain unabated over time, possibly capturing anticipation or a readiness for possible future perceived threats. This may in turn exacerbate the demand placed on both the endocrine and immune systems involved in our stress responses.

Different and more inconsistent across the two studies is the role of physiological anxiety symptoms in relation to changes in underlying diurnal cortisol and sIgA levels, both concurrently and over several years. Concurrently, higher physiological anxiety symptoms significantly

predicted diurnal cortisol that remained elevated throughout the day. Although we observed a similar pattern in diurnal sIgA over the day for those reporting higher physiological anxiety symptoms, this finding was statistically non-significant, limiting the extent of the interpretations at the current time. Longitudinally, physiological anxiety symptoms seemed to be predictive of lowered overall sIgA levels, but were not predictive of diurnal cortisol changes three years later. It is possible that there exists other factors not fully accounted for in the current studies (i.e., physical illnesses and symptoms; environmental factors) that could be better accounting for both the physiological anxiety symptoms and underlying changes in diurnal cortisol and sIgA levels. In summary, the current dissertation studies may be an important starting point in disentangling the psychobiology of anxiety symptoms from a dimensional approach to better understand the etiology of anxiety disorders and the impact on children and adolescents' health over time.

Strengths and Limitations

The current dissertation studies have several methodological strengths, which included the use of repeated measures design across several years to capture longitudinal sequence and time-sensitive associations between anxiety symptoms and underlying biological correlates. By having repeated measures of both anxiety symptom measures and salivary samples of cortisol and sIgA, both contemporaneous and longitudinal transactional associations were modeled using HLM and MPlus. These sophisticated statistical analyses were best suited to capture both between-person and within-person variations and changes that may have otherwise gone unnoticed with traditional cross-sectional designs and analyses.

An additional strength of the studies is the use of different measures of both diurnal cortisol and sIgA for a comprehensive understanding of their respective relationship with anxiety symptoms. Given that sampling of any biomarkers typically is costly and time-consuming, many studies in the literature utilize limited numbers of saliva samples to minimize attrition. In the current studies, measures of intercepts and slopes of both diurnal cortisol and sIgA, cortisol awakening response, in addition to overall aggregated sIgA across several years were available from our participants. With these different aspects of the biomarkers measured and included in the analyses, we were able to examine transactional relations between each of these components and anxiety symptoms over time.

Despite these strengths, several limitations should be taken into consideration for future studies. The current studies examined two specific biomarkers within the endocrine and mucosal

immune systems: diurnal salivary cortisol and sIgA. The interrelations and mechanisms between the endocrine and immune systems are vast and complex, with many different neurotransmitters, biological markers and hormones working together with far-reaching effects on central nervous and peripheral nervous systems (Glaser & Kiecolt-Glaser, 2014). The relation and associations between anxiety symptoms and these other components within both endocrine and immune systems remain to be further explored. Other biological and genetic underpinnings of anxiety such as temperamental factors and the specific role of behavioural inhibition, both of which have been found to be strongly predictive of later anxiety, should also be incorporated in future studies.

Additionally, the functioning of the endocrine system and HPA axis is quite nuanced. For example, DeKloet (1991) and colleagues discussed that optimal cortisol functioning and stress response depends on a balance between mineralcorticoid (MR) and glucocorticoid (GR) receptors. Compared to GR receptors, MR receptors have higher affinity for cortisol/corticosterone and are therefore highly occupied even under basal (i.e., stress-free) conditions. GR receptors become increasingly occupied as circulating corticosteroid levels rise (i.e., during stress responses). MR have been implicated in the appraisal process and onset of the stress response, while GR's predominant role is in the mobilization of energy substrates and most stress-induced changes in behaviour, including anxious-like behaviour to facilitate learning and memory. In addition, differences between circadian versus stress-related regulation of cortisol secretion were also not explored in detail in the current studies. These subtle differences and complexities within the endocrine system in relation to these receptors were not the focal points of the current studies. However, future studies may be best to examine this component to fine-tune the current set of preliminary findings in relation to diurnal cortisol and anxiety symptoms.

The first study of the dissertation included a measurement of the cortisol awakening response (CAR) in our investigation of the relationship between anxiety symptoms and diurnal cortisol secretion over the day. Accurate measurement of the CAR include precise collection of the first waking sample, which is an important indicator of pre-awakening cortisol secretion, in addition to subsequent data points capturing the dynamic linear increase. With the accurate collection of these data points, a composite Area Under the Curve (AUC) capturing the post-awakening period could better elucidate the CAR increase (Clow et al., 2010; Wust et al., 2000). Although participants recorded the time of day associated with each of their saliva sampling, and

this was modelled in all the analyses, we did not have an objective measure of the sampling times. Therefore, we cannot confirm whether the first sample taken is immediately upon awakening. Post-hoc analyses revealed that 70% of the participants did comply with our instructions and took their second saliva sample 20 to 30 minutes post awakening. The possible participant non-compliance with sampling times specified would explain the lack of the CAR dynamic rise observed in some of our results, and consequently affect the data quality in relation to CAR.

Furthermore, studies have shown that prior-day events, in addition to pre-awakening variables (i.e., sleep duration and quality; light exposure during the awakening process) might also influence the CAR rise, which were not fully accounted for in the current study. While physical illnesses, subjective health reports and medication usage were controlled in all the analyses, these measures were self-reported and retrospective, therefore lacking the validity of medically diagnosed illnesses. The current studies also did not examine physical illnesses as an outcome of interactions or mediation between anxiety symptoms and the underlying endocrine and immune systems. These limitations are important to address in future studies as physical illnesses could be an important confound affecting both anxiety, and underlying endocrine and immunological functioning (Roy-Byrne et al., 2008).

Additional confounding variables not controlled for in the current studies included sleep (duration, quantity, and quality), daily hassles, lifetime measures of stress, and season of the year, which all have been found to be related to cortisol and immunity (Kennedy, 2016; Pierre, Schlesinger, & Androulakis, 2016). Furthermore, saliva volume and secretory flow rate were not controlled for in the current studies, which may have affected measurement of salivary cortisol and sIgA (Miletic et al., 1996). Additional genetic (e.g., family history of mental health; children's temperament) and environmental risk factors (e.g., parenting styles) warrant further examination in future studies given their respective association to both anxiety symptoms and aspects of stress response system (Marceau, Ram, et al., 2013).

Although age of the participants was controlled, and was also examined for possible moderating effects in all models and analyses, the current study did not include a measure of pubertal status. It is possible that the same-aged participants were in different stages of pubertal development. Although some researchers have noted that age may be a suitable proxy for puberty status (L. R. Stroud et al., 2009), others have shown the importance of valid measurement of

pubertal status and pubertal timing (i.e., Tanner Stages) in the study of the developmental trajectory of the onset of internalizing disorders, notably depression in adolescents (Angold, Costello, & Worthman, 1998). Pubertal stage and related hormonal measures could be included in future follow-up studies to examine more specifically the role of puberty, including possible interactions between cortisol and sex hormones (Netherton, Goodyer, Tamplin, & Herbert, 2004).

Finally, causal conclusions cannot be drawn from the current results despite the longitudinal nature of the associations. Given the intricate complexity of the interrelationship between the body and the brain, there could be third variables, not included in the current analyses as predictors or control variables, contributing to and explaining the concurrent and longitudinal findings between anxiety symptoms dimensions, and diurnal cortisol and sIgA. Early life developmental variables, in addition to processes and variables not measured before the selected time points in the current studies could have determined and accounted for aspects of the current results. The question of when to begin measuring and examining a process that may have already began is an important one to pose because of the possibility of incomplete (and possibly inaccurate) conclusions about directions and sequence of effects over time.

Future Directions

From the current studies, there are several future avenues of research to be considered and pursued. Although the current studies controlled for children's depressive symptoms in all analyses, it may be warranted to examine in more detail the associations and sequential relationship between anxiety and depressive symptoms, within a psychobiological model or framework in future studies. The "cost" of controlling for depressive symptoms in the current analyses included effectively neutralizing the shared variance between depression and anxiety. Future studies may incorporate a combined measure of internalizing symptoms, or examine specifically what is shared and unique to anxiety and depressive symptoms in the associations with cortisol and sIgA. This is important to consider given that numerous studies have already shown robust relations between HPA axis functioning, cortisol, and depressive symptoms (Carroll et al., 2007). Other studies have also shown associations between major depression and immune functioning, notably inflammation in adults (Dantzer, 2012).

Future study designs may benefit from combining anxiety and depressive symptoms within the same model or design in order to examine their joint and differential associations with aspects of the underlying biological systems in children and adolescents. This may help to

disentangle antecedents, correlates, and divergent points in the biological correlates and etiology of anxiety and depression symptoms. Given the associations between depressive and anxiety symptoms, their comorbidity as well as the theory that anxiety symptoms tend to precede later onset of depression by possibly sensitising the HPA-axis, future studies may best to examine both facets in more detail in a larger sample of children and adolescents.

The dissertation studies focused on diurnal cortisol as an important biomarker of the stress response system. Future studies may want to include daily hassles and/or stressful life events as measures of objective and identifiable stressors within the models to examine and tease out the impact of stressors on both anxiety symptoms and underlying biological functioning. Although chronic anxiety symptoms can be argued to be stressful and strain the underlying immuno-endocrine systems, examining the same questions with identifiable objective stressors may have been a valuable addition to the studies.

Future studies might also consider incorporating multiple biomarkers, not only restricted to cortisol and sIgA in more complex models. Only a few studies to date have examined the synergistic associations between multiple biological systems in children and adolescents (Marceau, Ruttle, et al., 2013). By incorporating more than one biological markers, it may better capture the complex associations and possible "coupling" between multiple systems (e.g., endocrine and immune system functioning). In addition to examining diurnal cortisol and sIgA, other relevant biological correlates such as alpha-amylase, DHEA, C-reactive proteins, amongst other may also be relevant biomarkers to examine further in their relations with anxiety symptoms based on existing literature.

To extend the current findings, one possible future study design is to incorporate dimensions of anxiety symptoms, cortisol and sIgA, and physical health outcomes (i.e., illness diagnoses) all within one longitudinal model and study design (please see Future Studies Conceptualization for details). This will better integrate and clarify joint mechanisms and processes, in addition to understanding the sequence of the relationship between these related variables over time. Research questions of moderation and mediation between these variables of interest could also be examined utilizing a larger sample than was available in the current studies. Furthermore, the sequence of cascading events and interactions on a psychobiological level (i.e., anxiety symptoms → endocrine system → immune system → physical health) may be explored further to extend and expand upon the current results, which clarified independently the

associations between anxiety and aspects of the endocrine and immune systems separately as a starting point.

Finally, biological sex was included as a control variable in all the analyses. Due to the limited sample size, we were unable to conclude the existence of any sex differences in the associations between anxiety symptoms and both diurnal cortisol and sIgA. Future studies with larger samples should test for differences in the association between anxiety and endorine and immune systems in both boys and girls. On a related note, a more exact measure of pubertal status should be included given important interactions between the sex hormones, cortisol and immune markers. Although children's age was controlled for as a time-varying covariate in all the analyses, and has been found as an adequate indicator of pubertal status (L. R. Stroud et al., 2009), a more valid measure of puberty may have been of value.

Theoretical and Practical Implications

The current findings helped to clarify the long-term associations between cognitive-emotional versus physiological anxiety symptoms in relation to specific aspects of the endocrine and immune system, which are implicated in stress (threat) response. The results from the current dissertation studies are novel in demonstrating a mutual regulation between anxiety symptoms and 1) diurnal cortisol; and 2) sIgA, over several years from childhood to adolescence. This is an addition to the current literature that has predominantly elucidated concurrent associations in adult samples and focused on disorders, rather than symptom dimensions of anxiety. At the core of these findings may be a shared biological vulnerability to stress or threat response, most likely genetically transmitted, which may manifest across numerous psychobiological systems (Degroot & Treit, 2004).

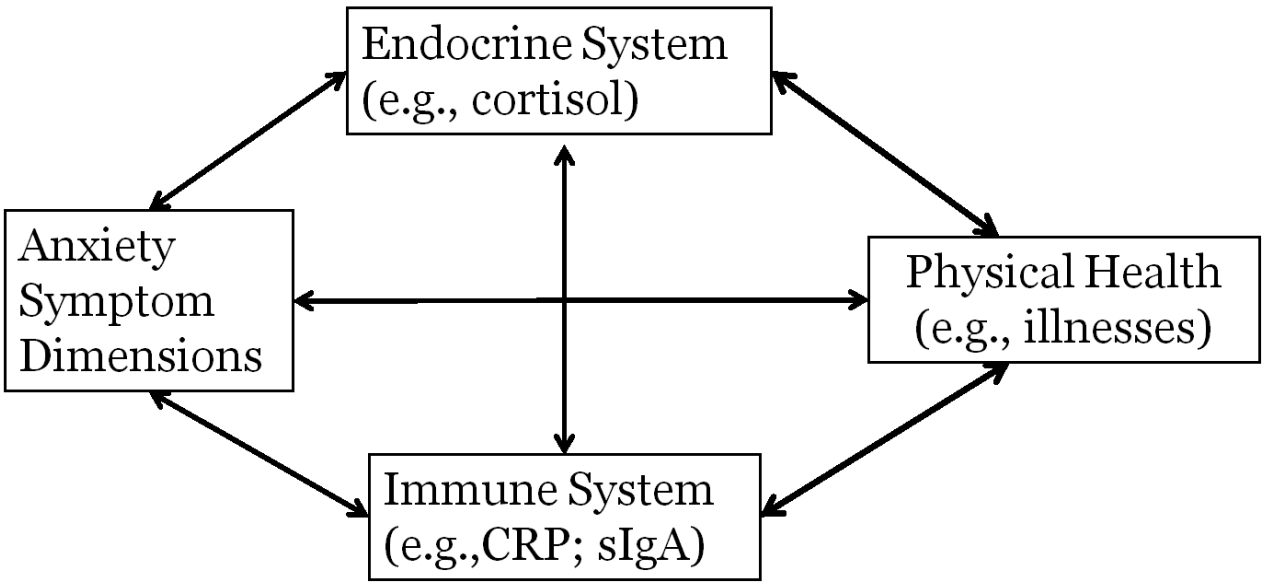
Given the differential associations between physiological versus cognitive-emotional anxiety symptoms, future prevention and intervention efforts may best assess anxiety from a dimensional approach especially in young children. A diagnosis of anxiety disorder is important also, but in younger children, examining the symptoms that comprise anxiety may be more relevant. A more integrated medical health care approach may be best implemented at the level of primary care. Given the associations between the stress response system, mucosal immunity, and anxiety symptoms, Children's anxiety symptoms are often associated with medically unexplained physical symptoms, or diagnosed as functional somatic symptoms (FSS) by primary care physicians and pediatricians (Campo, 2012). Therefore, an accurate understanding of anxiety

symptoms, both physiological land cognitive-emotional symptoms and their associations with underlying physiological systems, will allow for appropriate interventions and follow-up services in primary care health care settings.

Given that anxiety symptoms are especially prevalent in young children and is a precursor to later negative psychological and physical health outcomes in adulthood, preventions and interventions should be aimed at decreasing or managing these symptoms early on. Interventions, including optimizing social support and relaxation training, which have been shown to have positive effects in reducing both anxiety symptoms and elevating immune functioning should be incorporated into the prevention, treatment, and management strategies of anxiety in children and adolescents (Pawlow & Jones, 2005; Uchino, 2006).

In conclusion, the results from the current set of studies show important longitudinal and transactional associations between anxiety symptoms in children and adolescents and underlying immuno-endocrine functioning. Evidence of dysregulation in diurnal cortisol rhythms and slopes, in addition to possible mutual regulation of anxiety symptoms and sIgA levels in the manner of an augmenting feedback cycle were found. Taken together, these results are the beginnings in our understanding of the developmental psychobiology of anxiety in children and adolescents. The current findings provided a better understanding of how anxiety symptoms and immune functioning reciprocally interact and mutually regulate each other from childhood through to adolescence, adding to our current knowledge-base of the inter-relations between psychological and physical health.

Future Study Conceptualization



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