PSYCHOBIOLOGICAL FUNCTIONING IN MID-ADOLESCENT GIRLS AND BOYS: LINKAGES TO STRESS, SELF-ESTEEM AND RECURRENT PAIN

Lisa Folkesson Hellstadius
Psychobiological functioning in mid-adolescent girls and boys
Linkages to self reported stress, self-esteem and recurrent pain

Lisa Folkesson Hellstadius
To young people everywhere:

“What can be explained is not poetry.”

W.B. Yeats
Abstract

Among adolescents, the day-to-day functioning of the hypothalamo-pituitary-adrenal-axis (HPA-axis) and of the autonomic nervous system (ANS) and their relationships with stress, subjective health complaints and psychological factors such as self-esteem, studied in naturalistic settings, have been largely unexplored. This thesis aimed to investigate the diurnal activity of the HPA-axis (Studies I & II) in terms of salivary cortisol and the ANS/SNS system (Study III) in terms of salivary alpha-amylase (sAA) in mid-adolescent girls and boys. Additionally, linkages between self-reported stress, self-esteem, recurrent pain and biomarkers were investigated. A further aim was to describe potential differences between girls and boys respectively. Study I showed that both girls and boys exhibited the typical diurnal cortisol profile with high levels in the morning that decreased throughout the day. Girls had higher total cortisol levels, while no differences emerged for measures of the cortisol increase. Study II showed no significant linkages between self-ratings of stress and cortisol. However, stress was associated with recurrent pain in girls. Study III showed that, for girls, both self-esteem and self-reported stress were related to morning levels of both cortisol and sAA, to the diurnal sAA output and to a conjoint measure of amylase over cortisol, AOC. To conclude, the findings suggest that both stress and self-esteem may be linked to different measures of ANS and HPA-axis activity, but also to measures of ANS and HPA-axis dysregulation, particularly among mid-adolescent girls.

Keywords: Adolescents, Cortisol, Amylase, Stress, Self-esteem, Recurrent pain.
Sammanfattning


Ett ytterligare syfte var att undersöka och beskriva eventuella skillnader mellan pojkar och flickor avseende psykobiologi och sambanden med självskattningar. I Studie I visade sig både flickor och pojkar visade sig ha en typisk kortisolprofil med förhöjda nivåer i samband med uppvaknad, om sedan sjunker till låga nivåer framåt kvällen. Flickorna hade högre salivkortisolnivåer under första timmen efter uppvaknadet, CAR_G, högre area under kurvan AUC_G och slope_{awake to last}, medan inga skillnader framkom för de mått som inkluderade den dynamiska ökningen av salivkortisol i de aggregerade måtten. I Studie II fanns inga signifikanta kopplingar mellan självskattningar av stress i form av press och aktivering i förhållande till kortisol. Båda dessa stressdimensioner var dock kopplade till återkommande smårt bland flickor. I Studie III visade sig flickornas självkänsla och självrapporterade stress vara kopplad till morgonnivåer av kortisol och sAA, samt till den totala dygnsnivån av sAA, till en integrativ kvot av sAA över kortisol, AOC. Sammantaget visar resultaten från avhandlingen på ett mön-
ster där skillnader mellan pojkar och flickor framkommer för kortisol, men inte för sAA. Avslutningsvis visar resultaten också att både stress och självkänsla kan vara kopplade till olika mått avseende ANS- och HPA-axel aktivitet, samt till mått som speglar problem i regleringen mellan ANS- och HPA-axel aktivitet, bland tonårsflickor.

Nyckelord: Tonåringar, Kortisol, Alfa-Amylas, Stress, Självkänsla, Smärta.
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List of Studies


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# Abbreviations

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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AAR</td>
<td>The $\alpha$-amylase awakening response as measured by area under the curve ground</td>
</tr>
<tr>
<td>ANS</td>
<td>The autonomic nervous system</td>
</tr>
<tr>
<td>AOC</td>
<td>$\alpha$-amylase over cortisol as measured by area under the curve ground for $\alpha$-amylase, over area under the curve ground for cortisol</td>
</tr>
<tr>
<td>AUC</td>
<td>The area under the curve</td>
</tr>
<tr>
<td>AUC$_G$</td>
<td>The area under the curve ground (overall level)</td>
</tr>
<tr>
<td>AUC$_I$</td>
<td>The area under the curve increase (level of increase)</td>
</tr>
<tr>
<td>CAR</td>
<td>The cortisol awakening response</td>
</tr>
<tr>
<td>CAR$_G$</td>
<td>The cortisol awakening response as measured by area under the curve ground</td>
</tr>
<tr>
<td>CAR$_I$</td>
<td>The cortisol awakening response as measured by area under the curve increase</td>
</tr>
<tr>
<td>COA</td>
<td>Cortisol over $\alpha$-amylase as measured by area under the curve ground for cortisol, over area under the curve ground for $\alpha$-amylase</td>
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<tr>
<td>HPA-axis</td>
<td>Hypothalamo-pituitary-adrenal axis</td>
</tr>
<tr>
<td>sAA</td>
<td>Salivary $\alpha$-amylase</td>
</tr>
<tr>
<td>SNS</td>
<td>The sympathetic nervous system</td>
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Stress among youth in Western societies is becoming an issue of increasing concern. Although young people in general are doing well in terms of living conditions and overall well-being, many adolescents also report high levels of stress and stress-related health problems. Among these adolescents a majority of them are girls (Ahrén 2010; Lindgren & Lindblad, 2010; Schraml, Perski, Grossi & Simonsson-Sarnecki, 2011). Experiencing a certain level of stress is to be expected during adolescence, which is a developmental period of major physiological, psychological and social changes and challenges (Arnett, 1999) such as puberty, cognitive development, school transitions that entail new self-perceptions, social behaviors and ambitions (Eccles et al., 1993; Steinberg & Morris, 2001). However, the reporting of stress and mental health problems among adolescents has increased during the last decades (Collishaw, Maughan, Natarajan, & Pickles, 2010; Hagquist, 2011), and they have also been found to have reciprocal effects (McEwen, 2004). Diseases connected with basic somatic functions such as diabetes type II are on the rise in recent years, as well as adolescents diabetes type I (Patterson, Dahlquist, Gyürüs, Green & Soltész, 2009). Because diseases of the immune and metabolic systems have been shown to have a high degree of comorbidity with psychiatric illness and stress (Agid, Kohn & Lehrer, 2000; O'Mahony, Marchesi, Scully, Codling, Ceolho, Quigley, Cryan & Di-
Taken together, the health and disease-patterns among adolescents calls for further in depth study of potential psychobiological mechanisms that are involved in adolescent stress and health development.

Research on the psychobiology of adolescent stress has mainly come to focus on biomarkers of stress in experimental contexts and clinical or other non-normative groups of adolescents, which means that research on a general population level is limited. While the research literature is limited in this area, prior relevant studies have drawn on study groups with wide age spans (Rotenberg, McGrath, Roy-Gangnon & Tu, 2012) or large groups with fewer measures, and no waking sample (Kelly, Young, Sweeting, Fischer, & West, 2008). Thus, day-to-day functioning of the hypothalamo-pituitary-adrenal-axis (HPA-axis) and of the autonomic nervous system (ANS) in a naturalistic setting, and their relationships to stress and psychological factors such as self-esteem and subjective health complaints in non-clinical groups of well-functioning mid-adolescents, are largely unexplored areas.

Aim
The rationale behind this thesis is to further what is known about basic psychobiological self-regulatory functioning such as normal variations of stress-related biomarkers, for a clearly defined age group of well-functioning adolescents. Specifically, this study examines linkages between HPA-axis and ANS activity and the experiences of stress, individual factors and subjective health complaints for this group. And
so the aim of this thesis is to examine psychobiological functioning in a group of healthy and overall well-functioning students in the midst of their adolescent years.

The means by which this aim is attained, is an investigation into the day-to-day activity of the HPA-axis and the ANS/SNS in mid-adolescent girls and boys, and their linkages to various health related covariates, self-reports of stress, self-esteem and recurrent pain. An additional aim of this thesis is to examine psychobiological functioning and its associations to self-reports in girls and boys separately, in order to explore gender specific associations that might deepen our understanding of the currently reported differences in psychobiological functioning (Rotenberg et al., 2012; Vigil, Geary, Granger & Flinn, 2010) as well as mental and physical health (Schraml et al., 2011; Östberg, Alfvén & Hjern, 2006) between adolescent girls and boys.

Specifically, girls and boys were expected to have diurnal rhythms similar to that of adults and girls were expected to have similar sAA levels, and higher cortisol levels, than boys (Adam, Till, Hoyt & Granger, 2011; Nater, Rohleder, Schlotz, Ehlert & Kirschbaum, 2007; Rotenberg et al., 2012). Regarding associations with self-reports, drawing on data from the adult population (Kristenson, Garvin & Lundberg, 2012), along with the few studies of adolescent groups pursuing similar research questions, sAA and multiple system measures of dysregulation between sAA and cortisol were expected to be more strongly related to the health related self-report measures reflecting
different aspects of health that were investigated (Ali & Preussner, 2012; Vigil et al., 2010).
Life of the mid-adolescent

Biopsychosocial development
Adolescents generally live at home with their caregivers, and spend a good portion of their day in school. Both at home and in school adolescents engage in social interactions through which they create their own sense of self, including social identity (Eccles & Roeser, 2011). Early adolescence has been described as a time of "storm and stress" brought on by puberty and changes in self-perception and identity (Coleman, 1978). This concept of adolescence as a period of storm and stress has been under scrutiny, in part because the original idea was developed in the early 1900s, which in itself warrants the need for updating. Also, adolescents seem to enjoy and be satisfied with most of their lives (Arnett, 1999; 2006) and given the complexity of the developmental period it has been suggested that a more systemic approach is necessary (Hollenstein, & Lougheed, 2013). Nevertheless, adolescence does involve changes at many levels including physiological and cognitive development, social role negotiations and school transitions (Eccles et al., 1993; Steinberg & Morris, 2001). While early years of life are associated with the most rapid period of brain development, adolescence too is a phase of important brain development, which is related to changes in cognitive functioning (Rutter, 2007).
When children move into the early years of puberty, this development has implications for a wide range of cognitive changes. Adolescents increasingly deepen their capacity for viewing, and thus judging themselves from an outside perspective (Blakemoore, 2008; Eccles et al., 1993). Recently studies of adolescent brain development have begun to show how adolescence represents a period of marked social development and psychological changes that interact with identity development and changes in relationships. Adolescents become more sociable, form more complex and hierarchical peer relationships and are sensitive to acceptance and rejection by their peers (Blakemoore, 2008). Adolescents’ self-perceptions begin to be more strongly influenced by the appraisals of others. For example, positive self-evaluations based on the feedback of teachers, parents, and peers has been found to predict lower levels of self-reported depressive symptoms, while negative self-evaluations were associated with increases in depressive symptoms over time (Cole, Jacquez, & Maschman, 2001).

**Gender differences**

Biological differences between many girls and boys become increasingly evident during adolescence including menarche for girls and production of sperm for boys. In Europe, this development normally starts at an age of 12-13 among girls and 14 among boys (Lee & Styne, 2013). It is sometimes supposed that only female sex hormones change in girls and male sex hormones change in males, although this is actually not the case. Sex hormones are produced by the adrenal glands as well as by the sex glands and testosterone levels in girls ap-
pear to rise substantially with puberty, even though the rise is much smaller than in boys (Rutter, 2007).

Adolescent girls in general report stress, emotional and stress-related problems such as headaches, stomach aches, anxiety, and sleeping disorders more often than boys, and some studies have found a larger increase in stress related problems for girls than for boys during recent years (Ahrén, 2010; Collishaw et al., 2010; Modin & Östberg, 2009; Östberg, Alfven & Hjern, 2006). While girls have been found to be more likely to report peer-related stressors, boys have been found to be more reluctant to admit to interpersonal stress or to express emotion (Pole-Lynch et al., 2000; Washburn-Ormachea, Hillman & Sawilowsky, 2004). However, in addition to the biological factors associated with sex and puberty, gender roles and psychological factors may account for a part of the gender differences in stress responses, (Lundberg, 2005).
Stress and arousal

Psychobiology of stress and arousal
The study of stress and psychophysiological arousal has over the years come to include a number of definitions, each emphasizing different aspects of the stress concept. Selye (1976) stated that a stressor is an agent that produces stress at any time and describes the response to prolonged stress as the general adaptation syndrome (GAS). According to the GAS, the psychophysiological response to stress is divided into three phases starting with an alarm reaction, which is followed by resistance and may result in the final stage, which is exhaustion. Selye’s (1976) concept of stress as a non-specific response of the body to any demand implies the idea of the human body as a system striving for balance, or homeostasis, and has a clearly defined physiological focus.

Lazarus (1966) instead focused on a psychological perspective, and described the relationship between cognitions and emotions. Stress, according to Lazarus, begins with a psychological appraisal involved in stress reactions, which occur when individuals attempt to adapt to perceived demands and find themselves unable to successfully manage those demands. Here, the focus lies on individual appraisals of demands as stressful, based on the capacity for coping with those external or internal demands.
In later years, there has been a focus on how different stress responses are accompanied by physiological arousal and activation of the cardiovascular system, such as heart palpitations, and the neuroendocrine system, including for instance the release of cortisol into the blood stream. There has also been extensive research into the health consequences of prolonged physiological arousal (McEwen, 1998; Seeman, McEwen, Rowe & Singer, 2001).

The autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenocortical (HPA) axis, are fundamental regulatory systems involved in regulating stress responses. The adaptation to stressful experiences can cause the cardiovascular system to increase heart rate and blood pressure (McEwen & Seeman, 1999). The initial alarm reaction is characterized by the secretion of adrenocorticotropic hormone (ACTH), corticoids and cathecolamines. Secretion of ACTH from the anterior pituitary is controlled by the hypothalamus, and this process stimulates the secretion by the adrenal cortex of glucocorticoid hormones cortisol. (Selye, 1976; Tsigos & Chrousos, 2002).

The body's ability to activate different physiological systems is crucial to acute survival and to facilitate the immediate adaptation to new situations (Korte, Koolhaas, Wingfield & McEwen, 2005; Seeman et al., 2001; Tsigos & Chrousos, 2002). However, a state of chronic stress in the individual is suggested to follow from constant adaptation and the lack of rest and restoration will contribute to an over- or under activation of multiple bodily systems that together form what can be
described as an allostatic load (allostasis meaning the maintaining of stability through change).

Allostatic load is not beneficial for the individual, unlike the early stages of adaptation. Instead allostatic load is considered to be involved in the creation of various physical and psychological health problems (Evans, Kim, Ting, Tesher, & Shannis, 2007). The reactions to stress involve a number of bodily systems, such as the endocrine, cardiovascular, metabolic and immune systems. Allostatic load in the brain that results from constant activation in the HPA-axis can cause cognitive dysfunction and atrophy of the hippocampus, which leads to a more prolonged HPA response to psychological stressors (McEwen, 2007).

Within the allostatic load framework, McEwen and Seeman (1999) described the physiological reactions as including actual diseases like coronary heart disease (CHD), diabetes and arthritis. In the long run, the increase in heart rate and blood pressure together with changes in metabolism also caused by the release of stress hormones, can lead to the development of atherosclerosis, hypertension, abdominal obesity and Type II diabetes (McEwen & Seeman, 1999). Hyper-activation of the HPA-axis has been associated with obsessive-compulsive disorder (OCD), panic disorder and diabetes mellitus, while hypo-activation has been associated with fibromyalgia, chronic fatigue syndrome, hypothyroidism (Tsigos & Chrousos, 2002), hypocortisolism following adverse life circumstances (Gunnar & Vazquez, 2001) and ADHD (Isaksson, Nilsson, Nyberg, Hogmark & Lindblad, 2012).
The cognitive activation theory of stress, CATS (Ursin & Eriksen, 2004), represents recent attempts to synthesize psychological and physiological stress theories, as it includes a wide definition of stress. The psychological expectancy of the outcome is an essential element of CATS, which also view stress both as a stimulus, the experience of stress, a general neurophysiological stress response and the psychological experience of the stress response (Ursin & Eriksen, 2010; 2004). The CATS model describes stress as an alarm reaction in a homeostatic system, as a response to a homeostatic imbalance causing various degrees of arousal (which if prolonged can lead to allostatic load). The CATS model also suggests sensitization of brain networks as an important process of the pathophysiology of prolonged activation. Sensitization of neural circuits due to repeated use will, according to CATS, cause increased sensitivity to stimuli. The process of sensitization is suggested to affect cognitive networks and cause an attentional bias, which promote further sustained cognitive activation and prolonged stress responses (Ursin & Eriksen, 2010). Taken together, the different models provide a theoretical foundation for studying stress and arousal by using biomarkers indicative of activity in various psychophysiological systems, two of which are the HPA-axis and the ANS.
Biomarkers of stress and arousal: salivary cortisol and α-amylase

Measuring stress and arousal can be done in various ways, including HPA-axis markers such as cortisol and biomarkers of other peripheral systems such as catecholamines, cardiovascular indicators, body temperature or skin conductance for the ANS or immune system markers such as cytokines (Chatterton, Vogelsong, Lu, Ellman & Hudgens, 1996; Chrousos & Gold, 1992; Cohen, Kessler & Gordon, 1995; De Bellis et al., 1999). Also, different markers can be assessed in blood or plasma, but also in saliva. Saliva is an easily accessible body fluid and salivary cortisol is widely used as a biomarker of stress and has been positively related to several psychological and physical health problems (Kirschbaum & Hellhammer, 1994; Mc Ewen, 2007).

Recently salivary α-amylase (sAA) has been included in several studies as a marker of autonomic and sympathetic nervous system (SNS) (Nater & Rohleder, 2009). The enzyme sAA is produced in the salivary glands and shows a response pattern to physical as well as psychological stress that makes it useful as a non-invasive biomarker of acute and chronic stress. As such sAA has been found to predict plasma catecholamine levels and to be associated with increases in heart rate, and decreases in heart rate variability (HRV). The HR increases are indicative of sympathetic activation, while the HRV decreases are indicative of parasympathetic activation (Bosch, de Geus,
Veerman, Hoogstraten, & Amerongen, 2003; Chatterton et al, 1996). Increases in sAA have been observed in several studies in relation to physical exercise, psychosocial stress-tests and state anxiety, whereas chronic conditions like asthma and atopic dermatitis (chronically relapsing eczema) have been linked to decreases in sAA output (Crespi et al., 1982; Noto, Sato, Kudo, Kurata & Hirota, 2005; Takai et al, 2004; Wolf, Nicholls & Chen, 2008). It has been concluded that sAA, might be a useful biomarker, and also suggested that sAA may be more sensitive to subtle psychological stress than other markers such as blood pressure or heart rate (Nater & Rohleder, 2009; van Stegeren, Rohleder, Everaerd & Wolf, 2006).

Secretion and diurnal variations
As an established marker of HPA-axis activity (Preussner et al., 1997; Kristenson, et al., 2012), salivary cortisol is known to follow a diurnal rhythm which is characterized by maximum excretion 30 minutes after awakening which produces a sharp increase shortly after awakening. This is followed by a steadily decreasing curve, which reaches its lowest point around midnight. Intra-individual values tend to be relatively stable, however there is room for comparatively large differences between individuals within the limits of what can be considered a normal day curve (Wüst, Wolf, Hellhammer, Federenko, Schommer & Kirschbaum, 2000) Cortisol has also been found to have state like characteristics, with as much as 50% of its variation due to short term fluctuations (Ross, Murphy, Adam, Chen & Miller, 2014).
Lately, focus has shifted from assessing salivary cortisol secretion by the use of single measures, to including measures of aggregate cortisol measures. Frequently used aggregate measures of salivary cortisol include the cortisol awakening response, CAR and area under the curve, AUC, that take into account the diurnal cortisol variations (Pruessner, Kirschbaum, Meinlschmid & Hellhammer, 2003; Rottenberg et al., 2012).

The diurnal sAA rhythm has an inverted pattern, compared to that of cortisol, which involves showing the lowest levels immediately after awakening, and then staying low for a continuation of approximately sixty minutes. This is followed by a marked increase in sAA, which then continues to rise throughout the day (Nater, Rohleder, Schlotz, Ehlert & Kirschbaum; 2007). Although sAA has been associated with other sympathetic markers it has also been shown not to reliably predict catecholamine levels (Nater et al., 2006). Therefore, it is probable that sAA is a complement rather than a replacement for catecholamines and cardiac markers (DeCaro & Worthman, 2008).

Recently, studies have found that measures that make use of a combination of biomarkers cortisol and sAA may be indicative of dysregulation of the HPA-axis and the SNS. This type of psychobiological asymmetry has shown linkages to stress, aggression and depression, and may be overlooked when cortisol and sAA are used as separate measures (Ali & Preussner, 2012; Gordis, Granger, Susman & Trickett, 2006). Attempts to standardize measurements of HPA-axis and SNS asymmetries have been made and currently, existing research
suggests a ratio-measure of sAA over cortisol, or vice versa (Ali & Pruessner, 2012). As for the connection between salivary cortisol and sAA they both reflect activity of psychological and physiological stressors, although as biomarkers of the HPA-axis and the SNS respectively they do not necessarily relate when measured at the same time (Afifi et al., 2011; Susman et al., 2010; van Stegeren et al., 2008; Wolf et al. 2008).

Measurement procedures
Both cortisol and α-amylase can be measured in saliva (Kirschbaum & Hellhammer, 1994; Nater, Rohleder, Schlotz, Ehlert, & Kirschbaum, 2007). In laboratory studies, assessments of salivary cortisol provide a neuroendocrine measure of acute stress, with an increase in salivary cortisol that lasts for over 60 minutes, although it reaches its peak between 20-30 minutes after the initial stressor is presented (Dickerson & Kemeny, 2004). With sAA on the other hand, the response of the SNS is quicker and stress related peaks in sAA levels are measurable at five minutes after stressor onset (Nater et al., 2006). The time frame is similar to that of catecholamines, although sAA is assumed to reflect changes in other parameter of SNS activity (such as heart rate). However, it is still unclear to what degree salivary alpha-amylase reflects changes in adrenaline and noradrenaline secretion (Chatterton et al., 1996; Nater et al., 2006).

In field studies, taking place in naturalistic environments, salivary measurements are highly suitable because of the non-invasive nature
of the sampling procedure, which is relatively easy and can be performed without assistance of medical staff. Also, due to its non-invasiveness, it causes a comparative minimum of psychophysiological stress (Kirschbaum & Hellhammer, 1994; Schumacher, Kirschbaum, Fydrisch & Ströhle, 2013). The most common device for obtaining measures of salivary measures of cortisol and sAA is the ‘Salivette’ (Sarstedt Inc., Rommelsdorf), which is a plastic test-tube, with a lid that holds a pocket containing a sterilized cotton swab. The swab is used when sampling by chewing it for 30-60 seconds, or keeping it for a minimum of 30 seconds under the tongue, after which it is once again placed in the test-tube (Kirschbaum & Hellhammer, 1994; Rotenberg et al., 2012).

Test tubes containing saliva samples that are to be analyzed for cortisol can be stored differently. Although saliva samples preferably should be stored frozen at a minimum of -20°C after the samples are collected, saliva samples can be stored at 20°C for up to four weeks without any significant reduction in cortisol levels (Kirschbaum & Hellhammer, 1989; Nater et al., 2005; Rohleder & Nater, 2009). For sAA, studies have found the enzyme to be stable at room temperature as well as 37°C for up to three weeks, and sAA is also not affected by repeated freeze-thaw cycles (DeCaro, 2008; Granger et al., 2006). This enables sampling outside of the laboratory, as in the naturalistic conditions of field studies.

Instructions for obtaining the most reliable measure of cortisol vary from sampling multiple samples during one day to sampling for up to
a week (Rotenberg, 2012). Although it has been thought that it is necessary to take measurements at the same time point for more than one day, using the same baseline (Kirschbaum & Hellhammer, 1994), there are also indications that stability varies between types of measurement. Aggregate measures of total amount of cortisol, and the maximum cortisol value for single measures which are measured over three to four days have also been found to reach an optimum of stability (Rotenberg et al., 2012). Levels of sAA are not as well studied with regard to day-to-day stability, which makes a case for treating sAA with similar caution in terms of stability. In all studies using saliva, participants are generally instructed to avoid smoking, eating or drinking caffeinated beverages or sodas for an hour before sampling. Participants are also often instructed to rinse their mouth with water in order not to contaminate their samples with remnants of food or blood (Granger et al., 2012; Nater & Rohleder, 2009; Kirschbaum & Hellhammer, 1994).

Measuring stress and arousal in saliva normally involves repeated measures, meaning that study participants sample saliva at different times during one day, to capture a diurnal rhythm (Wüst, Hellhammer, Federenko, Schommer & Kirschbaum, 2000). It is generally preferable to use electronic devices as reminders, to ensure adherence to sampling protocol, although when this is not possible participants can keep a diary where they note the exact time points of when they take their samples to increase stability of the data (Granger et al., 2012; Rotenberg & McGrath, 2014). Also, assessments of cortisol and sAA need
to include important covariates identified in previous research (Rohleder & Nater, 2009).

While the concentration of cortisol in saliva has been found not to vary as a result of salivary flow rate (Kirschbaum & Hellhammer, 1989) concentrations of sAA in saliva have been declared safe with regard to it being unrelated to salivary flow rate (Bosch et al., 1996; Rohleder, Wolf, Maldonado & Kirschbaum, 2006) only to be associated with salivary flow rate in later studies (Beltzer et al., 2010; Bosch, Veerman, de Geus & Proctor, 2011). Unlike the gender differences reported for HPA-axis activity as measured by salivary cortisol (Kudielka & Kirschbaum, 2005), findings suggest no differences between women and men in average sAA levels, in the diurnal rhythm or in acute responses to stress or exercise (Granger et al., 2006; Kirschbaum, 1999; Nater et al., 2007).

Aggregate measures
Curve values for the whole day are often calculated using area under the curve (AUC), for both cortisol and sAA (Nater et al., 2005; Preussner et al., 2003). Cortisol and sAA values can vary quite substantially between individuals, but the curve pattern should not appear abnormal, lacking for example the characteristic drop in the evening, for cortisol, or the opposite pattern for sAA, which would indicate a deviation from the norm. Cortisol curves are usually relatively stable between two ordinary days, unless study participants are subjected to temporary and fairly grave sources of stress or arousal. This also applies to the increased cortisol secretion that normally occurs during the
process of awakening, the “cortisol awakening response” (CAR), which is often used instead of (or in addition to) the area under the curve for the whole day, which is referred to simply as the area under the curve or AUC.

The CAR is considered to have several practical advantages and minimal loss in precision compared with the entire day measurements (Fries et al., 2008), even if using CAR means that one cannot detect abnormalities in diurnal rhythm such as elevated evening values, which have been associated with depression (Goodyer, Herbert, Moor, & Altham, 1991). Diurnal slopes have also been used as aggregate measures of cortisol. The slope calculation have different anchoring points, for example time of awakening or the peak sample (Rotenberg et al., 2012). To standardize procedures for a multiple system approach, where the associations between HPA-axis and the SNS-system functioning are measured conjointly, recent research suggests using a ratio-measure of cortisol over amylase (COA) or amylase over cortisol (AOC) (Ali & Pruessner, 2012).

Table 1. Formulas used to compute the aggregate measures.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Abbreviation</th>
<th>Formulae used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area under the curve, ground. Overall level</td>
<td>AUC&lt;sub&gt;G&lt;/sub&gt;</td>
<td>[ \sum_{i=1}^{n-1} \frac{(m_{i+1}+m_i) \cdot t_i}{2} ]</td>
</tr>
<tr>
<td>Area under the curve, increase. Dynamic increase</td>
<td>AUC&lt;sub&gt;1&lt;/sub&gt;</td>
<td>[ \left( \sum_{i=1}^{n-1} \frac{(m_{i+1}+m_i) \cdot t_i}{2} \right) - \left( m_1 \cdot \sum_{i=1}^{n-1} t_i \right) ]</td>
</tr>
<tr>
<td>Area under the curve, ground. Overall level for sample 1-3</td>
<td>CAR, AAR</td>
<td>[ \sum_{i=1}^{n-1} \frac{(m_{i+1}+m_i) \cdot t_i}{2} ]</td>
</tr>
<tr>
<td>Formula</td>
<td>Description</td>
<td>Formula</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>--------------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Diurnal slope rise over run awake to last(^b)</td>
<td>$\text{Slope}_{\text{awake to last}}$</td>
<td>$\frac{S_1 - S_0}{t_1 - t_0}$</td>
</tr>
<tr>
<td>Diurnal slope rise over run max to last(^b)</td>
<td>$\text{Slope}_{\text{max to last}}$</td>
<td>$\frac{S_1 - S_{\text{max}}}{t_1 - t_{\text{max}}}$</td>
</tr>
<tr>
<td>Ratio of sAA over cortisol(^c)</td>
<td>$\text{AOC}_G$</td>
<td>$\frac{AUC_GsAA}{AUC_Gcortisol}$</td>
</tr>
<tr>
<td>Ratio of cortisol over sAA(^c)</td>
<td>$\text{COA}_G$</td>
<td>$\frac{AUC_Gcortisol}{AUC_GsAA}$</td>
</tr>
</tbody>
</table>

*Note.* Formulas based on \(^a\)Pruessner et al. (2003) and \(^b\)Rotenberg (2012) \(^c\)Ali & Preussner (2012)
Salivary cortisol and α-amylase in research on adolescents

Normal variations
Studies carried out with healthy children and adolescents, studying the cortisol awakening response (CAR) and the diurnal curve during everyday circumstances show relatively similar functioning among adolescents and adults, with the same diurnal curve found for both groups (Adam, 2006; McCarthy, Hanrahan, Kleiber, Zimmerman, Lutgendorf & Tsalkian, 2009; Rosmalen, Oldehinkel, Ormel, de Winter, Buitelaar & Verhulst, 2005).

In a study by Adam (2006) a 64% increase in cortisol was measured 40 min post time of awakening and an overall average reduction of 11% / per hour after awakening. Also, 3000 Scottish students aged 15 years old, had a median value at school arrival of 6.11 nmol/l (girls) and 10.5 nmol/l (boys), which thirty minutes later had dropped to 8.1 (girls) and 8.2 (boys) nmol/l, a decrease of around 10% (Kelly et al., 2008). In a recent Swedish study a healthy control group of children aged 6 to 18 years old, a median of 8.8 nmol/l was measured in girls and 8.3 nmol/l among boys at 8:00. At 13.00 hours, the median value was 5.5 nmol/l in girls and 5.3 nmol/l among boys. Finally, at 20.00, 2.1 nmol/l was found in girls and 2.3 nmol/l among boys (Törnhage & Alfvén, 2006).
In general, alpha-amylase is found in relatively high concentrations in saliva. The primary function of alpha-amylase is to digest macromolecules such as carbohydrates and starch (Granger et al., 2006). Circulating levels of salivary amylase have been found to be very low (mean 20 U/l) during ages of zero to four months old. While pancreatic levels have been found to increase gradually with age (reaching adult levels with a mean of 74 U/l by eight years of age), salivary amylase levels have instead shown a pronounced rise between the ages of 0.9–1.9 years (reaching maximum levels with a mean of mean 99 U/l by years 5–6) (O’Donell & Miller, 1980).

Covarying factors
The years between childhood and adulthood are marked by changes, physiological as well as social and psychological. Measuring and interpreting cortisol secretion among individuals in the midst of this period of significant change, requires a variety of theoretical and methodological considerations. To begin with, adolescents have been found capable to follow sampling protocol. Specifically 72% of self-reported wake-times were within five min and 90% were within 15 min of objective wake times as determined using actigraphy (DeSantis, Adam, Mendelsohn & Doane, 2009). This is in accordance with later reports of adolescents having 88.1% of the awakening samples collected within 15 min of accelerometer-verified waking (Rotenberg & McGrath, 2014).

In general, salivary cortisol levels seem to increase with age, and the
onset of puberty, so that total cortisol concentrations increase from childhood to adolescence (Gunnar, Wéverka, Frenn, Long & Griggs, 2009; Törnhage, 2002). There is some uncertainty about the extent of the effect of puberty on the young individual's cortisol production. However, mid- and post pubertal girls have been shown to have higher salivary cortisol concentrations than boys (Törnhage & Alfvén, 2006; Netherton et al., 2004). The increases in salivary cortisol levels from childhood to adolescence have been linked to developmental changes during adolescence, including pubertal maturation, and their influence on HPA-axis functioning. Research suggests that pubertal maturation is a better predictor of diurnal cortisol patterns than is age (Adam, 2006; Matchock, Dorn, & Susman, 2007; Oskis, Loveday, Hucklebridge, Thorn & Clow, 2009). Typically, pre-menarche girls (Oskis et al., 2009; Rotenberg et al., 2012) and boys (Rotenberg et al., 2012) have a cortisol peak 30 min post-awakening which is similar to that of adult men (Pruessner et al., 1997). In contrast, menarche and reproductive maturation in girls seems to drive a cortisol pattern characterized by a sustained increase in cortisol until 45 minutes post-awakening which is typical for that in adult women (Netherton et al., 2004; Oskis et al., 2009).

However, cortisol levels have also been shown to decrease with increasing maturity stages, and linked to whether first menstruation has started or not (Oskis, et al., 2009). There are also examples of studies showing different phases of puberty as unrelated to salivary cortisol secretion (Rosmalen et al., 2005). The associations between pubertal development and sAA levels are less well studied, although there is
some evidence that sAA levels increase during the course of pubertal development (Adam, Till Hoyt & Granger, 2011; Granger, Kivlighan, El-Sheikh, Gordis & Stroud, 2007) and that antisocial adolescents exhibit lower sAA levels and with earlier puberty (Susman et al., 2010).

Apart from developmental factors and related differences between girls and boys, additional covariates of salivary cortisol concentrations in adolescents include the time of waking, body mass index, caffeine intake and sleep have been associated with salivary cortisol concentrations in adolescents (Oskis et al., 2009; Rotenberg et al., 2012). Research on adolescents has also included other covariates such as physical exercise, life events, alcohol and nicotine consumption. However, recent research suggests that covariates account for less than 10% of the variation in cortisol and the use of covariates across studies is inconsistent (Kelly et al., 2008; Rotenberg et al., 2012). Several studies have shown that neither regular smoking or caffeine intake affects CAR, however, there are also studies which indicate the opposite, although the effects presented are relatively small for both adolescents (Adam et al., 2006) and adults (Wüst et al., 2000, Fries et al., 2008). Coffee intake close to the sampling has been studied with no significant effects. However, a relatively small percentage of coffee drinkers are normally found in adolescent based studies, which may affect the ability to draw conclusions from these studies (Kelly et al., 2008).

How these factors affect the daily cortisol secretion is unclear, however, the results are not conclusive and habitual smoking have, for ex-
ample shown both significant and non-significant associations with CAR (Fries, Dettenborn & Kirschbaum, 2009). During what is referred to as normal or "natural" conditions, both significant (Kelly et al., 2008) and insignificant (Adam, 2006, McCarthy et al., 2009) relations between sex and cortisol secretion has been discerned among adolescents. In a school based study, boys were found to react more strongly than girls to just having eaten or smoked, and also to age and maturity (Kelly et al., 2008).

Today, most studies of sAA reactivity have been performed on adult research participants and do not report on covariates such as smoking or physical exercise, but mainly sampling techniques and equipment (e.g., Beltzer et al., 2010; Nater et al., 2005) This pattern is evident also for studies on children and adolescents sAA reactivity (Gordis, Granger, Susman & Trickett, 2008; Spinrad et al., 2009) although it is considered beneficial for adolescents to, during field studies, keep notes on emotional or physical factors that may interfere with sampling and control for health issues and medication (Granger et al., 2012).
Self-reported stress and health indicators in adolescents

Self-reported stress
The number of ways to measure self-reported stress among adolescents include focusing on stress related health problems, emotional states, resources such as social support, cognitive aspects of the stress experience or one or more perceived stressors in home, school or peer environments (Osika, Friberg, & Währborg, 2007; Byrne, Davenport & Mazanov, 2007). In studies of adolescents, stress is conceptualized in a variety of ways. This heterogeneity of measurement procedures may partly be enriching the field of stress research, but also it is in part problematic (Lindblad et al. 2008; Östberg et al., 2014).

While well-established measures of stress such as the Perceived Stress Scale (PSS; Cohen, Kamarck, Mermelstein, 1983) largely focus on whether or not an individual has a sense of control over life, and are not specifically developed for use on adolescents. Those scales that are for use on adolescents have a number of ways to measure self-reported stress among adolescents. Such scales include focusing on stress related health problems, emotional states, resources such as social support, cognitive aspects of the stress experience or one or more perceived stressors in home, school or peer environments (Osika et al., 2007; Byrne et al., 2007).
Lindblad, Backman and Åkerstedt (2008) have developed a stress scale, the Pressure and Activation Stress scale (PAS), that is mainly intended for use in studies of children and adolescents. The PAS scale focuses on perceived arousal levels and also of the psychological experience of external and internal demands and restraints. The objective behind developing the PAS scale was to keep the measurement of stress close to its conceptual origin, in addition to it being developed specifically for children and adolescents (Lindblad et al., 2008). For these purposes, the PAS scale address respondents with simple and direct questions of common and non-complex everyday experiences.

**Subjective health complaints**

Every day stressors in the school environment have been associated with pain and psychological complaints in school children (Hjern, Alfvén & Östberg, 2006). Recurrent pain is a common symptom of stress and involves the co-occurrence of two or more types of pain (Petersen, Brulin & Bergström, 2006; Alfvén, Östberg & Hjern, 2008). Hjern et al. (2008) found that 29.1% of the adolescent respondents had headaches every week while 19.9% suffered from recurrent abdominal pain (RAP) (girls experienced more of both headache and RAP than boys). Stress related health problems like depressive feelings and sleeping problems have been associated with pain in girls specifically (El-Metwally, Salminen, Auvinen, Kautiainen & Mikkelssohn, 2004). Also, gender specific associations between somatic complaints and psychological problems have been found, where while musculoske-
letal pains were associated with depression in both girls and boys, for
girls, stomach aches, headaches and musculoskeletal pains were asso-
ciated with anxiety disorders, while stomach aches were associated
with oppositional defiant disorder and attention-deficit hyperactivity
disorder for boys (Egger, Costello, Erkanli & Angold; 1999). For self-
reported health indicators, girls generally report poorer self-esteem,
higher levels of stress and higher levels of psychosomatic health prob-
lems than boys. Differences in reporting, sensitivity and exposure to
specific stressors and different developmental stages of girls and boys
are among the factors used to explain these different patterns (Rutter,
2007; Sweeting, West & Der, 2007; West & Sweeting, 2003). It ap-
ppears that in research on adolescents’ self-reports of stress as well as
psychological and physiological functioning, gender specific patterns
should be considered, so as not to obscure important linkages between
perceived stress and health related factors in girls and boys psycho-
physiological development.

Individual factors; global self-esteem
Studies of self-esteem in adolescents have considered low self-esteem
as a major factor behind deviant behavior such as substance abuse and
sexual risk behavior in, although these results are inconclusive
(McGee & Williams, 2000; Schrier, Harris, Sternberg, & Beardslee,
2001). However, there is a relative stability in findings which link low
self-esteem to self-harm and suicidal thoughts (McGee & Williams,
2000). The role of self-esteem in associations between stress and
health in adolescents’ everyday lives, however, is less studied. The
Rosenberg self-esteem scale (RGSES) (Rosenberg, 1965) is an estab-
lished global self-esteem measure, which is designed to capture an individual’s overall (global) sense of self-worth. The RGSES show a similar factor structure across nations. It is also a cross-national pattern to score above midpoint of the RSES, which indicates a culturally universal tendency towards positive self-evaluation (Schmitt & Allik, 2005) Global self-esteem has, in a recent study of stress in Swedish adolescents, been linked to lower levels of stress symptoms and chronic stress (Schraml et al., 2011).
Salivary cortisol, α-amylase and self-reports in adolescents.

How self-reports and biomarkers of stress and health indicators relate to one another is not well studied in the adolescent populations. A recent review on the role of cortisol as a measure of health and disease has shown that findings are often ambiguous and that the quality of studies varies greatly (De Vrient, et al., 2011; Kristenson et al., 2012). For adults perceived stress and cortisol, among high quality studies there seem to be relatively few findings of significant association between self-reports of psychosocial work stress and cortisol (Karlsson, Lindfors, Riva, Mellner, Theorell, & Lundberg; 2012). Existing studies of adolescents that include cortisol and alpha-amylase are mostly based on groups with clinical or behavioral problems (e.g., Adam, Zinbarg, Mineka, Craske & Griffith, 2010; Susman et al., 2010) or include stress tests in laboratory settings (e.g., Takai et al., 2004).

Self-esteem has long been regarded as having a protective function in the development of health and disease (Baumeister, Campbell, Krueger, & Vohs, 2003). Reactivity of psychophysiological systems involved with stress responses have been linked to psychological factors such as self-esteem and locus of control (Preussner, Baldwin, Dedovic, Renwick, Mahani, Lord, Meaney & Lupien, 2005). Self-esteem has been associated with a higher and more frequent cortisol output in response to psychosocial stress, but also to a smaller hippo-
campal volume. The association between lower self-esteem and smaller hippocampal volume has been hypothesized to be mediated by cortisol reactivity (Preussner et al., 2005).

Recent developments in developmental psychobiology have shown, with a multi system approach, that individual differences in HPA-axis activity and sensitivity of the SNS have linkages to psychological functioning. Or, more specifically, that individuals with high sAA activity and low cortisol may be more withdrawn, or at greater risk for developing aggression or low self-esteem, whereas high sAA combined with high cortisol may be associated with higher psychological resilience. (Fortunato, Dribin, Granger & Buss, 2008; Gordis, et al., 2006; Vigil et al., 2010).

Adolescents who suffer from recurrent abdominal pain and anxiety had been found to react with higher heart rate and systolic blood pressure to a social stressor than healthy controls, while no such differences appeared for cortisol (Dorn, Campo, Thato, Dahl, Lewin, Chandra & Di Lorenzo, 2003) It has been suggested that pain and anxiety may be more influential on the ANS than on the HPA-axis level which calls for further studies on biomarkers such as amylase.

Recent studies of salivary cortisol activity in natural settings (school) have shown both differences in girls and boys levels of salivary cortisol, with girls producing a higher cortisol awakening response (Rotenberg, 2012; Kelly et al., 2008) as well as no sex differences (Adam, 2006; McCarthy et al., 2009). In studies of sAA men have shown higher baseline sAA level than women in an experimental setting alt-
hough these differences did not appear in the measures of reactivity to the stressors (van Stegeren et al., 2008). A recent study of self-reported stress and cortisol linkages in adolescents found that, only for boys, less than half of the included sub-scales measuring perceived stress were associated with cortisol levels at awakening (De Vriendt et al., 2011). For the everyday environment, there are no reports of sex differences in average sAA levels or in slopes of the diurnal rhythm, or to reactivity to stressors (Nater et al., 2007). It is not yet clear, under which circumstances sAA levels differ for men and women, and even more so for adolescent boys and girls.

Stress in adolescence is a complex phenomenon, on the one hand expected, as part of life, and on the other hand stress levels are reportedly increasing and causing psychological and somatic health problems for a many adolescents, a majority of which are girls (Lindgren & Lindblad, 2010). In this thesis, the aim is to advance the understanding of psychobiological functioning and every day activity of stress systems in adolescent girls and boys, and their associations with self-reported stress, self-esteem and recurrent pain in a group of healthy mid-adolescents. These studies will contribute to furthering the understanding of psychobiological pathways involved in the self-regulation of arousal and relaxation, and thus stress related health issues, among adolescents.
Methods

Setting and study participants.
The data were collected within the larger research project entitled ‘Stress and support in school’ (TriSSS), which was carried out in two schools in the Stockholm city area during the spring of 2010. All studies draw on questionnaire and biomarker data from adolescents aged 14 to 16, years old, who at the time of the study were in grades 8 and 9 of their compulsory education.

The two schools who participated in the “Stress and Support in School” data collection is based, consist of one high performing (statistics from the database SIRIS, www.siris.skolverket.se), inner city school with a music profile and a broad catchment area (with many students travelling from out of the city to school every morning) that contained a total of 12 8th and 9th grade classes. In addition to this, the study also contained a second high performing (although slightly less so than the city school) suburban school with a local catchment area that held a total of seven 8th and 9th grade classes.

The city school also had higher education levels in the parent group, than the suburban school. As for ethnicity, the amount of students with who were born abroad or had two foreign-born parents was similarly low in both schools, compared with the national averages (statis-
tics from the database SIRIS, www.siris.skolverket.se). In the case of both of the schools included in the TriSSS study, members of the research team had previous professional contacts with school staff, through which the schools were both deemed suitable for the purpose of studying stress and health in adolescents. Both of the approached schools granted permission to conduct the study during scheduled class time, and provided the practical support needed for the study to materialize.

In total there were 545 pupils attending the 8th and 9th grade in the study schools, all of which were contacted, first through mail and later by telephone. These contacts involved obtaining parental consent to approach each student to participate in the TriSSS study. Consent could be given or withheld from the various separate parts of the study. The separate parts of the wider study that this thesis deals with involve questionnaire data and salivary samples.

The need for parental consent was evident due to the students’ age as well as the time consuming nature of the study, the sensitive nature of the self-reports on health and due to the included “stress measurements” which included collection of biological data. Of the 545 pupils attending the 8th and 9th grade, 413 (76%) pupils agreed to participate in the survey, which was carried out in the spring of 2010, of these participants, 167 (~40%) were boys and 246 (~60%) were girls. All of the students who filled out the questionnaire, and for whom there was a registered parental consent to participate in the saliva sampling, were asked to participate in the saliva sampling, of these, 277 students
joined the saliva sampling, referred to as the “stress measurements” that was carried out over the two weeks following each class room visit.

Table 2. Participation in the different parts of the studies.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Question</th>
<th>Saliva day 1</th>
<th>Saliva day 2</th>
<th>Study I</th>
<th>Study II</th>
<th>Study III</th>
</tr>
</thead>
<tbody>
<tr>
<td>School A</td>
<td>365</td>
<td>290</td>
<td>144</td>
<td>128</td>
<td>98</td>
<td>128</td>
<td>59</td>
</tr>
<tr>
<td>School B</td>
<td>180</td>
<td>123</td>
<td>46</td>
<td>38</td>
<td>23</td>
<td>47</td>
<td>11</td>
</tr>
<tr>
<td>8th grade</td>
<td>261</td>
<td>213</td>
<td>108</td>
<td>100</td>
<td>72</td>
<td>104</td>
<td>39</td>
</tr>
<tr>
<td>9th grade</td>
<td>284</td>
<td>200</td>
<td>82</td>
<td>66</td>
<td>49</td>
<td>71</td>
<td>31</td>
</tr>
<tr>
<td>Girls</td>
<td>314</td>
<td>246</td>
<td>129</td>
<td>115</td>
<td>79</td>
<td>119</td>
<td>47</td>
</tr>
<tr>
<td>Boys</td>
<td>231</td>
<td>167</td>
<td>61</td>
<td>51</td>
<td>42</td>
<td>56</td>
<td>23</td>
</tr>
<tr>
<td>Total</td>
<td>545</td>
<td>413</td>
<td>190</td>
<td>166</td>
<td>121</td>
<td>175</td>
<td>70</td>
</tr>
</tbody>
</table>

*Note.* Participants in study I were those with complete data on both sampling days, while participants in study II draw on data from day one only, and study III is based on data from participants with complete cortisol and sAA samples from day two.

Procedure

After having implemented the project at the schools and asked parents to provide ethical approval during 2009, the data collection was carried out at the schools during three weeks in March 2010. During one hour of class, students received information concerning the project and completed a questionnaire covering their living conditions, health functioning along with a wide variety of psychosocial factors. Having completed the questionnaire, all students who also volunteered to participate in the stress study were given a kit including Salivette® tubes and sampling diaries along with a written sampling protocol.
Saliva sampling was administered by the students themselves, and took place both at home and in school five times each day (from time of awakening to 8 pm.), during two voluntary weekdays within the two weeks following the questionnaire. All saliva samples were returned to the researchers, at the schools, on the day after sampling and stored in the laboratory freezers for later biochemical analysis of cortisol and alpha-amylase. Taken together, the data collection produced a cross sectional dataset with repeated measures of salivary biomarkers. For each day that the students returned saliva samples, they received a voucher (worth about 15 US dollars). This research was ethically approved by the Regional Ethics Committee in Stockholm (Ref. no: 2009/857-31/4).

Measures

Questionnaire data

Self-esteem
Self-esteem was measured with the 10-item Rosenberg (1965) Global Self-Esteem Scale (RGSES), using a 5 point Likert scale ranging from 1 (strongly agree) to 5 (strongly disagree). The Rosenberg Global Self-Esteem Scale (RGSES; Rosenberg, 1965) is a well-established, measure of general self-esteem, developed for use with adolescents. Global self-esteem is a concept that reflects one’s overall (and thus
non area specific) sense of self-worth. All scores are coded after the test so that a higher score represents a higher self-esteem.

**Stress**

Perceived stress was measured with an instrument called the pressure-activation-stress (PAS) measure (Lindblad, Backman & Åkerstedt, 2008) that includes the subscales of activation and pressure. This validated measure has been used among adolescents and includes in total 11 items that was answered on a frequency scale ranging from 1 (never) to 5 (always). The two dimensions, pressure and activity have been found to be correlated with one another, and it is yet unknown to what degree these dimensions manage to capture two aspects of stress. One hypothesis about the PAS scale has been that the activation dimension would show a stronger connection to HPA-axis activity, because the activation items ought to reflect psychophysiological activation. In addition to this, it has been suggested that the pressure dimension may have physiological correlates that differ from those of activation with regard to cortisol responses (Lindblad et al., 2008). For the questionnaire sample (n=413), the internal consistency was measured with Cronbach’s alpha, which showed the following coefficients for the activation scale score, alpha = .84; pressure scale score, alpha = .77.

**Recurrent pain**

Recurrent pain was computed by four items from the Swedish welfare surveys of 2000-2003, also known as the Child-LNU (the Level of Living Survey) and Child-ULF (the survey of Living Conditions) in which different aspects of health functioning are assessed using sin-
gle-items. For the purpose of this study these items have been used to create an index of recurrent pain (headaches, stomach ache, back ache and pain in neck and shoulders). These are rated along a 5-point frequency scale (1=every, day 2= once a week, 3= several times a week, 4=sometimes every month, 5= rarely or never) or a 4-point Likert scale (1=not at all, 2=badly, 3=approximately 4 =precisely) was used. Ratings are coded so that high values correspond to greater levels of recurrent pain. Ratings of each item were categorized to show weekly pain. Categorizations across items were then combined into a continuous measure of recurrent pain. Such recurrent pain has previously been linked to stress, particularly among girls (Alfvén et al., 2008).

**Sampling diary**

Participants were instructed to complete a structured sampling diary, for both days of sampling. The two diaries were to be returned with the saliva samples. All participants were instructed to report on actual time of sampling. Adolescents were asked if, during both days, they had consumed tea, coffee or other caffeinated beverages, used nicotine or exercised. The diary also included questions on chronic disease, medications, whether they had started menstruating and number of life events they had experienced during the last year. For the reporting of life events, adolescents were presented with a list of twelve potentially stressful events such as parent divorcing, serious illness in close relations, break up with boyfriend/girlfriend or serious disputes with friends (Kelly et al., 2008). For the break up with girlfriend/boyfriend and having a serious dispute with someone the time frame was during the last month. For all other events the time frame was the past year.
The total number of life events was computed so that a higher score reflected more stressful events.

Biomarkers

**Salivary cortisol and sAA**

*Salivary cortisol* and sAA was obtained using the Salivette® sampling device (Sarstedt, 51582 Numbrecht, Germany); a centrifuge tube with a suspended insert containing a sterile neutral cotton wool swab. Pupils who had signed up for a two-day stress measuring trial were given a sampling kit and those who participated administered their own samples, at five time points, during two normal school days, with less than two weeks apart. Those pupils who had parental consent to participate in the stress study had been given two test-kits with their questionnaire, one fore each day of sampling, containing five pre labelled Salivettes® for day one, and 10 prelabelled Salivettes for day two, when additional samples of sAA were to be collected. The test kits also contained instructions and sampling protocol inside a diary, which also consisted of questions regarding smoking, menstrual-phase, life-events and other known confounders. Before starting the 40 min questionnaire, the whole group was informed about all parts of the TriSSS study, and also instructed on how to remove and chew on the cotton wool for approximately 2 min, or alternatively to spit into the test tube. The class was also instructed on procedures for, after having completed samples for one day, they would be returned into a bin located at the school nurse facilities where they were collected.
every day by the research team. Saliva samples were stored within the stress hormone laboratory at the Department of Psychology at Stockholm University, for biochemical analysis. When they had completed the questionnaire, those who had decided to participate in the stress study were handed a check list, containing useful tips and important information regarding the sampling procedure and urged to contact the research team with questions at any time, by telephone, or to visit at the school nurse station where a member of the research team was to be situated every day, throughout the study. The saliva samples were self-administered and the first sample was taken at the time of awakening, the second sample at 30 min after awakening, the third sample at 60 min after awakening, the fourth sample was taken when the participant arrived at school and the fifth sample was taken at 8pm. During each day of sampling students were asked to complete a diary to get an exact time point for every sample, and also to inquire after any daily events or life event that might interfere with an individual’s normal level of salivary cortisol, so that any deviation from protocol could later be taken into account in the process of analysis.

**Statistical measures**

For calculation of the diurnal curve, the area under the curve ground (AUC\(_G\)) and the area under the curve increase (AUC\(_I\)) were used as measures of overall levels and dynamic increase, respectively. The AUC\(_G\) was also used for the cortisol awakening response (CAR), as well as the amylase awakening response (AAR) (Pruessner et al., 2003). The area under the curve was calculated from the sample collected at waking up, 30 min post-awakening and 60 min post-
awakening. The diurnal curve also included an evening sample, collected at 20:00h. To obtain a second type of aggregate measure of cortisol output, rise over run was calculated as \( \text{slope}_{\text{awake to last}} \) and \( \text{slope}_{\text{max to last}} \), in order to provide a second measure of the total cortisol level and the increase (cf. Rotenberg et al., 2012). Calculation of ratio of amylase over cortisol (AOC) was based on AUC for sAA and cortisol respectively, as was ratio of cortisol over amylase (COA) (Ali & Preussner, 2012).

Data analyses

Biochemical analyses

Cortisol
Saliva samples were collected at five points in time respectively during two ordinary school days within two weeks after completing the initial questionnaire. To maximize the sample size, this study analysis involved four samples collected: 1) immediately at waking up, 2) at 30 minutes post-awakening, 3) at 60 minutes post-awakening, and 4) at 20.00h.

Saliva samples were collected using the Salivette® sampling device (Sarstedt Inc., Rommelsdorf, Germany), a plastic tube with a suspended insert containing a sterile neutral cotton wool swab. Adolescents were instructed to chew on the swab for two minutes before putting it
back into the tube and sealing it, or to actively spit saliva (no passive
drooling) directly into the tube, if preferred. Adolescents were in-
structed not to eat, smoke, drink coffee/tea (or other beverage contain-
ing caffeine), or brush their teeth 10 minutes before sampling saliva
(Hanrahan et al., 2006). All samples were stored in plastic-bags in
room temperature before returned to the research team on the next
school day. Then saliva samples were transported to the laboratory
where they were stored in a freezer (-20°C) until analyzed. Cortisol
was determined using competitive radioimmunoassay (Spectria Corti-
sol RIA, Orion Diagnostica, Espoo, Finland; intraassay precision <
5%, 1.7-4.1% and inter-assay precision < 10%, 4.3-9.0%). Each sam-
ple was analyzed twice and in randomized order.

**Alpha-amylase.**

Samples obtained in Salivette tubes were stored in a freezer and trans-
ported to the Department of Laboratory Medicine section for Clinical
Chemistry, Örebro University Hospital where they were placed in -20°C
freezer until the biochemical analysis. When analyzed all sam-
ple were thawed in room temperature and then centrifuged for seven
minutes at 2000 x g to separate saliva from the synthetic swab. The
amylase analysis (ref 120 2670, Ortho Clinical Diagnostics, Johnsson
& Johnsson, Raritan, NJ, USA) was performed on a VITROS® Chem-
istry System 5,1 FS system (Ortho Clinical Diagnostics, Johnsson &
Johnsson, Raritan, NJ, USA) with the VITROS® MicroSlide™ tech-
ology. All samples were diluted 40 times with FS Diluent Pack 3 (ref
680 1754, Ortho Clinical Diagnostics, Johnsson & Johnsson, Raritan,
NJ, USA) and analyzed in duplicates. External controls were analyzed to ensure MicroSlide and instrumental stability.

Statistical analyses
Salivary cortisol and sAA showed a skewed distribution and apart from descriptive statistics of untransformed data and non-parametric partial correlations of single measures, all analysis (containing aggregate measures of cortisol and sAA) used log-transformed data. For calculation of the diurnal curve the area under the curve ground (AUC_G) was used to establish overall level of diurnal cortisol, and the area under the curve increase (AUC_I) was used a measure of diurnal cortisol reactivity (Pruessner et al., 2003). The diurnal curve included a waking up sample, a sample taken at 30 min post awakening, one at 60 min post awakening and an evening sample, collected at 8:00 p.m. For the cortisol awakening response, CAR, and the amylase awakening response (AAR), the AUC was calculated based on the three first morning samples.

In study I, a second measure of the total cortisol level and the increase calculations of slope measures rise over run was included and calculated as slope_{awake to last} and slope_{max to last} (Rotenberg et al., 2012). Partial Pearson’s product moment correlation coefficients (partial r_p) were used to examine how covariates relate to all single and aggregate measures (AUC, CAR, and slopes) included in the study, while controlling for time of awakening and time between awakening and the first sample in the full sample. To examine differences between the two sampling days, and differences between girls and boys correlation...
coefficients ($r_p$) were used, and separate ANCOVAs were performed to investigate association between a number of relevant covariates, and the six aggregate measures included in the study.

In study II, calculation of differences in self-report measures between girls and boys were performed with ANOVA and MANOVA. Pearson correlation coefficients were used to examine associations between self-reported measures and aggregate measures of cortisol. Hierarchical regression analyses were used to calculate the relation between self-reported stress and the aggregate cortisol measures and recurrent pain, while statistically controlling for school year and menarche.

For Study III, Partial Pearson’s product moment correlation coefficients (partial $r_p$) were used to explore associations between single measures while controlling for time of awakening and between awakening and each sample. Study II also includes calculations of ratio of amylase over cortisol (AOC) and cortisol over amylase (COA), which were based on AUC for sAA and cortisol respectively (Ali & Preussner, 2012). Also, hierarchical regression analyses (including covariates time of awakening, time between awakening and first sample, age, sex and life events) were performed for girls and boys together, as well as separately, to examine linkages between self-report measures of stress, self-esteem and aggregate cortisol and sAA measures.
Study I – Single and aggregate salivary cortisol measures during two schooldays in midadolescent girls and boys

Background and aim
Salivary cortisol has been identified as an important biomarker involved in stress reactions and stress related health problems. Cortisol marks activity of the hypothalamic pituitary adrenal (HPA) axis, and its diurnal regulation of wakefulness and self-regulatory response to stress. Most studies of HPA-axis activity are based on adult groups or clinical groups of children and adolescents (e.g., van den Bergh & van Calster, 2009), with antisocial behavior (e.g., Susman et al., 2010) or in experimental settings (e.g., Kelly et al., 2008; Kudielka et al., 2004). Thus there are few studies of day-to-day cortisol levels in healthy adolescents, sampled in natural settings (Adam, 2006; Rotenberg et al., 2012). Also, the types of measures utilized differ and include single measures and aggregate measures of morning levels as well as diurnal variations. This calls for in depth study into multiple aspects of HPA-axis activity in healthy adolescent girls and boys.
Methods
This study is based on salivary samples from 91 girls and 44 boys collected at four points in time during two ordinary school days, who sampled saliva themselves in their home and school: 1) immediately at awakening, 2) 30 minutes after waking up, 3) 60 minutes after waking up, and 4) at 8 p.m. Also, the study made use of diary data on time of awakening, actual sampling times and covariates such as smoking and drinking coffee close to sampling.

Main findings and conclusions
For both girls and boys, the typical diurnal cortisol profile was high levels in the morning that decreased throughout the day. Girls were found to have higher cortisol levels during the first hour after awakening than the boys. For the aggregate measures girls had a larger total level of cortisol as measured by the cortisol awakening response, CAR_G, area under the curve AUC_G and rise over run, slopeawake to last, while no differences emerged for reactivity measures taking into account the cortisol increase. These findings suggest a consistent pattern of gender differences for single measures of the morning, and aggregate cortisol measures based on total level of cortisol for both morning and the full diurnal curve.
Study II - Perceived stress, recurrent pain and salivary cortisol in mid-adolescent girls and boys

Background and Aim
This study was part of a research project involving the study of stress and health in adolescent students (Östberg, Almquist, Folkesson, Brolin Låftman, Modin & Lindfors, 2014). Knowledge of stress related biomarkers and their association with self-report measures of stress in adolescents is limited and this study aims to investigate the associations between self-reported stress and salivary cortisol in mid-adolescent girls and boys. Somatic pains such as headaches and stomachache have been associated with stress (Alfvén et al., 2008), and this study also investigated the associations between self-reported stress and recurrent pain in a group of otherwise healthy adolescents. Two dimensions of stress, namely activation and pressure, were measured using the Pressure Activation Stress scale (Lindblad et al., 2008) and the associations between self-reported stress, recurrent pain and diurnal HPA-axis activity were investigated in both girls and boys.

Methods
This study is based on questionnaire data on activation and pressure stress and recurrent pain (headache, stomachache, neck/shoulder and back pain), and salivary cortisol from 119 girls and 56 boys. The study participants sampled saliva during an ordinary school day: 1) at the time of waking, 2) 30 minutes after waking, 3) 60 minutes after waking, and 4) at 8 p.m. Girls and boys were also analyzed separately in
order to explore gender specific patterns of psychobiological functioning.

Man findings and conclusions
There were no significant associations between self-ratings of stress as measured by the PAS scale, and cortisol for the girls, or the boys in this study. However, both stress dimensions activation and pressure were significantly associated with recurrent pain in girls. The lack of association between subjective and objective measures of stress that were included in this study may be due to self-reports of stress and biomarker cortisol reflecting specific and unrelated aspects of functioning. It is possible that this group of healthy mid-adolescents have bodily systems yet unaffected by the wear and tear of daily activation and pressure stress.
Study III - How do self-reported stress and self-esteem relate to diurnal profiles of salivary alpha-amylase and cortisol in mid-adolescent girls and boys?

Background and aim
Perceived stress and self-esteem are psychological factors that are viewed as important for individual health development (McEwen, 2007; Southhall & Roberts, 2002). Still, studies of how psychological measures such as stress and self-esteem relate to biological markers are scarce, and in this case for adolescents in particular (Vigil, Geary, Granger & Flinn, 2010) and even more so for normative groups of adolescents in their daily environment (Adam, 2006; Rotenberg, McGrath, Roy-Gagnon, & Thanh Tu, 2012). In addition to this, there is a new interest in understanding of how the diurnal hypothalamo-pituitary-adrenal (HPA) axis activity and autonomic/sympathetic nervous ANS/SNS system, and their internal associations and dysregulation, relate to psychological functioning. This study investigates diurnal HPA and SNS activity separately as well as using integrative multiple system measures in the day-to-day activity of mid-adolescent girls and boys, and also examine how perceived stress and self-esteem relate to diurnal HPA and SNS functioning.

Methods
This study draws on data from questionnaires and self-administered salivary samples collected from 47 girls and 23 boys during four time points during one school day, and had complete data on bot salivary cortisol and salivary alpha-amylase. The student sampled saliva 1)
immediately at awakening, 2) 30 minutes after waking up, 3) 60 minutes after waking up, and 4) at 8 p.m.

Main findings and conclusions
While there were no differences in sAA, as was expected (Nater et al., 2007), girls were found to have higher levels of morning cortisol than the boys, which also replicates previous findings (Rotenberg et al., 2012). Additionally, among girls self-esteem and stress were associated with cortisol and sAA measures, both measured separately and conjointly. These findings suggest that both stress and self-esteem are linked to separate measures of ANS and HPA-axis activity, as well as measures of ANS and HPA-axis dysregulation among mid-adolescent girls.
Discussion

The present thesis aims to advance understanding of the complex relationship between adolescence, stress and subjective health factors by exploring important psychobiological pathways involved in self-regulating the interplay between arousal and relaxation, in a group of healthy mid-adolescents. Adolescents are a group for which there is limited knowledge of day-to-day activity of the HPA-axis and the autonomous/sympathetic nervous system (ANS/SNS). This thesis explores and reports on diurnal levels of cortisol and sAA. In addition to this, it examines whether cortisol and sAA are biomarkers of physiological mechanisms involved with subjective experiences of stress, self-esteem and recurrent pain.

Also, this thesis explores gender specific psychobiological patterns by looking at girls and boys separately, in addition to a group of mid-adolescents which is otherwise fairly homogenous with regard to age, social background, school performance and environment. After summarizing the main findings of this thesis, a general discussion of these findings, and their implications, will follow. Then there will be a discussion on methodological issues and limitations of the included studies. Finally, there will be a short summary of concluding remarks and discussion of the implications of this thesis.
Main findings

Investigating the diurnal patterns of cortisol and sAA, the expected diurnal patterns were found showing an inverse patterns with peak levels after waking for cortisol and sAA levels peaking in the evening. Although diurnal HPA-axis activity has been described in earlier studies (Rotenberg, 2012). An important contribution of this thesis was to provide information on sAA levels at four time points in the diurnal curve in parallel to salivary cortisol, for adolescents, collected in a natural setting. As for the diurnal rhythms of cortisol and sAA, the expected gender differences in cortisol levels, and the expected absence of gender differences for sAA levels or multiple system measures (based on previous research on sAA among adults) were found (Ali & Preussner, 2012; Nater et al., 2007; Rotenberg, 2012).

As for the self-reports, the overall findings showed that girls reported significantly higher levels of stress (for both activation and pressure), lower self-esteem and more recurrent pain than the boys. These findings are consistent with previous research on gender differences in stress and self-esteem among Swedish adolescents (Alfvén et al., 2008; Schraml et al., 2011). Also, the present findings (Study II) follow previous research (Alfvén et al., 2008) showing linkages between perceived stress and recurrent pain, particularly among girls. The two dimensions of stress, activation and pressure, were significantly associated with recurrent pain among the girls. Results for boys, although pointing in the same direction, did not reach statistical significance. It is likely that this was due to the small sample size.
In study II there were no significant relations between self-reported stress and aggregate cortisol measures, which is in line with previous studies showing no significant associations between the PAS and cortisol in healthy participants and other self-report inventories of stress as related to single measures of salivary cortisol (De Vrient et al., 2011; Isaksson et al., 2012). However, in study III the girls showed an association between the cortisol awakening response and the two stress dimensions. The larger sample size in study II, compared with study III that was based on data from another day, suggests that linkages between self-reports of stress and CAR found for the girls in study III must be viewed as uncertain and need replication.

Statistical analyses of the total group of adolescents which provided complete sAA data (n=70) examining associations between self-reported stress, self-esteem and biomarkers for the HPA-axis and ANS system showed that for this group of adolescents self-reported stress measured as pressure (and not activation) and morning sAA (AAR) were significantly associated. Self-esteem was, for the total group, significantly associated with overall diurnal level of sAA (AUC) and multiple system measure AOC.

Both activation, pressure and global self-esteem were significantly associated with one or more of AOC, sAA for the morning and the full diurnal curve (AAR and AUC) among the girls. Previous research has also shown relations between psychosocial measures and AOC (Ali & Preussner, 2011) and other measures of sAA (Vigil et al., 2010; Fortunado et al., 2008). The gender separate analyses showed that both
dimensions of self-reported stress were also associated with cortisol, (pressure and activation with CAR and activation with COA) among the girls. This might indicate that self-regulation of physiological arousal in relation to cognitive emotional appraisals of day-to-day stressors and perceptions of the self are perceived as more arousing for girls than for boys, and the stress responses to these types of stressors may be less effectively down regulated than for boys.

When analyzing the boys separately, the number of life events experienced during the last year was associated with physiological activation of the ANS, measured as sAA AUC. No significant associations between self-reports of stress and self-esteem were found for the boys. Due to the small sample sizes, particularly for boys, these findings should be interpreted cautiously and need to be replicated in a larger group of boys. The suggestion, nevertheless, is that possibly boys, unlike the girls in this sample, have a pronounced sympathetic stress response for life event-related challenges, rather than for day-to-day hassles. And if so, is the effect of life-events mixed with the daily stressors for the girls or, are girls not as troubled with major life changes as boys? These questions require additional examination in future research studies.

Usage of aggregate measures for psychobiological system markers
Salivary cortisol measures over two days, sampled within a time frame of two weeks, were found to be significantly correlated. Stability of the aggregate measures appear to benefit from being based on a larger number of single measures (and also similar time points to a
larger extent), as measures that include all four cortisol values were associated with all of the other aggregate measures across the two days of sampling (unlike the slope measures which were not associated with the CAR measures across the two sampling days). The aggregate cortisol measures examined in study I were all correlated between the two days, unlike the single measures where a variation was found for the awakening and evening levels between the two sampling days when looking at girls and boys separately. Thus, overall levels of AUC and CAR, as opposed to measures of increase, are the aggregate measures that appear to be more stable. This result is in line with a recent study of 233 children and adolescents aged 9-18 (Rotenberg, 2012).

The present study also investigated sAA, as well as HPA-axis and ANS system dysregulation, using (guided by results of study I) only ground measures (Preussner et al., 2003) and the two ratio-measures of sAA and cortisol suggested by Ali and Preussner (2012). Because sAA was only sampled one day it is not possible to examine stability of single and aggregate measures of cortisol. Salivary cortisol and sAA is not often correlated with each other (Afifi et al., 2011; Susman et al., 2010) and there were no linkages between sAA and cortisol except for the girls +30 min sample.

**Stress, self-esteem, and different aspects of HPA-axis and SNS-activity**

Findings for the measures of biological functioning in the morning (CAR and AAR) and for those including the full diurnal measures (AUC) came out slightly different (Study III). Self-esteem was nega-
tively associated with sAA, as expected. This, while activation (measures fast paced behavior such as eating rapidly, rushing and having difficulties relaxing) was negatively associated with the cortisol awakening response and a higher COA, and negatively associated to all measures including sAA for the girls. This was less expected and suggests different psychobiological pathways of different aspects of stress and arousal. For while activation was linked to lower levels of both cortisol (apart from the COA) and sAA, pressure (measuring perceived external as well as internal demands) was associated with higher morning levels of cortisol and sAA. These pathways needs examination with a larger data set, to study for example whether activation, like self-esteem, can somehow be a protective factor for this age group, or if the result is due to inactive adolescents having higher levels of sAA as a result of being too passive. Possibly, an active lifestyle is less stressful and more beneficial in this age group, that may have different needs of recovery in comparison to older individuals (Kristenson et al., 2012). These findings of linkages between self-reported stress, self-esteem and SNS/HPA-axis functioning among adolescent girls (as well as the finding of linkages between stressful life-events and overall levels of sAA) adds to the existing body of research regarding psychobiological functioning in adolescents (Rotenberg et al., 2012), or more specifically mid-adolescents.

**Gender differences in self-reports, cortisol and sAA**

A stable finding of the studies included in this thesis is that girls reported significantly higher levels of stress (for both activation and pressure), lower self-esteem and more recurrent pain than the boys. The girls also had a more pronounced cortisol awakening response
during both sampling days. However, there were no associations between the self-reported measures of stress and cortisol in study II, while study III, despite its smaller sample size (albeit different covariates) with samples collected on another day than in study II found an association between activation, pressure and CAR. For the adult population results regarding associations between self-reports of stress and cortisol reactivity are ambiguous (Kristenson et al., 2012) and while there are not many existing studies of self-reported stress and cortisol levels in adolescent girls and boys, De Vrient et al. (2011) found some support for an association between self-reports of stress and higher levels of salivary cortisol at the time of awakening for boys in a large scale cross national study.

Even though some of the girls of this study had reported that they had their first menarches (and there were no differences between those who had and those who had not had their first menarche, for the included variables), girls have generally progressed further into puberty at the ages of 14–16, than boys, and this may well be a main factor behind the higher levels of morning cortisol in girls. This explanation is supported by previously found increases in salivary cortisol for mid- or post-pubertal individuals (Adam et al., 2006; Törnhage & Alfvén, 2006) with higher levels for girls than boys of that developmental stage (Netherton et al., 2004). However, adult men also have lower morning cortisol than adult women. Another possible factor behind the gender difference in cortisol is that women have shown a higher sensitivity to the ACTH release occurring during and after the time of awakening, here measured as CAR (Kudielka, 2004), while no signifi-
cant differences between girls and boys were found for the sample taken at 8:00 pm (while controlling for time elapsed since waking and time difference between awakening and the first sample).

As the cognitive activation theory of stress goes, a stress response is activated in the limbic structures of the brain, as a result of cognitive appraisal. From a CATS standpoint (Ursin, 2009), one might want to investigate further whether for the boys, this cognitive appraisal is not measurable as self-reported stress, psychological problems or self-rated health, for which there are no associations with life events among the boys in this sample (results not shown). Girls on the other hand, have no physiological trace of stress from life events, while there is a connection between stress and a lower self-esteem, both of which are concepts with a clearly expressed cognitive-emotional basis. The core issue here is whether or not girls and boys have the same expectations of their life circumstances, or of themselves, and the same tolerance for deviances from these expectations. This study suggests a reinforced stress response at life event-related challenges for boys, and a prolonged stress response in association with cognitive emotional appraisals regarding the self, and bodily feedback in the form of arousal and feelings of stress, particularly among the girls.

Limitations and methodological issues
A possible problem with the data collection is that the adolescents were administering the saliva samples and keeping notes of exact sampling times themselves. And even though the differences between
the self-reported wake up time and the first sample was small, and statistically controlled for, it has been suggested that small delays in the sampling protocol, especially for the cortisol awakening response, can be problematic (Smyth, Clow, Thorn, Hucklebridge & Evans, 2013). Despite not using electronic reminders most adolescents were taking their samples within the first minute after waking up, although the lack of control over sampling procedures and compliance, effects of noncompliance cannot be ruled out. However, there was no difference in the included self-ratings of stress, self-esteem, pain, or of SRH, between the group of adolescents with complete data and those who failed to provide sample saliva in accordance with the study protocol (results not shown). Regarding the group of adolescents who declined to participate altogether or who were missing from school during the questionnaire, this information is missing and there is a possibility that they might have differed from the study group on important, health related, parameters.

During the data collection all participants were instructed to leave samples at five time points each day, the first at awakening, the second at +30 min, the third at +60 min, the fourth at school arrival and the fifth at 20.00 pm. Since the sample taken at school arrival often coincided and sometimes preceded the third sample at +60, participants who left out or had insufficient or contaminated saliva in their fourth test tube were not excluded from the study, but instead the fourth sample was excluded from the three studies of this thesis, in order to allow for a larger number of participants to qualify for inclusion in the analyses.
There is a methodological tradeoff between letting the study participants follow their ordinary week schedule in terms of wake up times and weekly activities, and instructing the participants to wake up at a specific time point. This may have secured a greater stability of measurement between the participating individuals and over the two sampling days, although the study setting would have lost in naturalistic properties. Still, this may be a factor behind the associations between school and salivary cortisol that was found for two of the aggregate measures for girls during day one and weekday which was found for two aggregate measures of cortisol for girls during day one, and a majority of the aggregate measures for both of the sampling days among the boys. Regarding the effect of weekday for salivary cortisol levels, apart from different week day related activities it is likely that these results are due to a very few students sampling on other days than Tuesdays (day one) and Wednesdays (day two). As a precaution all participants who had failed to note the exact time of each sample were excluded from the analyses. In addition, all participants who had 15 minutes or more (study II and II) or on one occasion more than five minutes (study II), were also excluded from further analyses. This is in line with recommendations for when the use of electronic devices is limited, in order to increase stability of the data (Rotenberg & McGrath, 2014).

An important factor to consider when using biomarkers of stress in a sample of healthy adolescents, is that this group is probably character-
ized by highly functioning self-regulatory systems, which means a low risk of the psychophysiological wear and tear seen in adults or adolescents with early life adversities or depression (Goldman-Mellor, Hamer & Steptoe, 2012; Jonsdottir, Halford & Eek, 2012; Van den Bergh & van Calster, 2009; Gonzalez, Jenkins, Steiner, & Fleming; 2009). Effects of health behaviors and other covariates have in general been small but some studies suggest an effect of smoking and drinking caffeinated beverages close to sampling, (Fries et al., 2009; Adam, 2006). In this study sample, only three adolescents reported that they smoked regularly. And, while a few adolescents reported that they normally drank coffee and exercised approximately three times per week, they appear to have been adhering to the study protocol which instructed them not to smoke, drink coffee or exercise close to sampling. This means none of these health behaviors could be expected to have any larger influence on the psychobiological data. A limitation regarding covariates is that no information on sleep in association with sampling was included, because sleep has been found to be of importance for adolescents’ cortisol levels (Rotenberg, 2012). Furthermore, the cross sectional study design for the student self-reported data, limited the ability to make causal attributions between measured self-reported study constructs, and to examine longe term development in these constructs over time.

Guided by previous research (Kelly et al., 2008), life events over the past year were included as a covariate. While the number of life events was not associated with any of the cortisol measures, for boys there is an association between sAA and number of life events during the past
year. This may indicate that compared with the reported life events there may be other daily life stressors that have greater importance for HPA axis functioning. There was no association for AOC or COA for life events among the boys, so there appear not to be any dysregulation of HPA-axis and SNS functioning for the boys in association with having experienced stressful life events. This will have to be studied further, as previous research has shown altered cortisol responses, and a greater cortisol-sAA asymmetry are related to early life stressors (Hunter, Minnis & Wilson, 2011; Gordis et al., 2008).

Also, a multiple informant approach would have been useful for the self-reported constructs because parental and structural factors may be of importance for adolescents psychobiological development. Low maternal responsiveness has been associated with an increased AL in youth who are subjected to psychosocial risk factors (Evans et al., 2007) and parental depression, socioeconomic status (SES) and maltreatment in youth are other psychosocial factors associated with salivary cortisol and sAA levels, and possibly asymmetry between HPA and ANS systems, that may be taken into account more frequently (Gordis et al., 2008; Lupien, King, Meaney & McEwen, 2001; Miller et al., 2009; Lupien et al., 2000). The study initially included a questionnaire for parents but response rates for parental SES were low, and parental stress and health was not included, which would have been of particular interest because a parallel increase in adult mental health problems has been suggested as a possible explanation for the increasing mental health problems in adolescents (Schepman et al., 2011).
Conclusions
To conclude, the included studies showed the expected diurnal cortisol rhythms in a fairly homogeneous group of well-functioning adolescents and also presented consistent differences between girls and boys that follow previous research (Rotenberg et al., 2012). Investigating relations between activation and pressure stress, aggregate salivary cortisol measures and recurrent pain in mid-adolescent girls and boys, the present study showed no associations between any of the stress dimensions and HPA-axis functioning, neither for girls nor for boys. However, activation and pressure stress were significantly related to recurrent pain, but only in girls. The expected diurnal rhythm of sAA was also found, and for sAA levels there were no differences between the girls and boys of this study. As for the investigation into linkages between cortisol, sAA and self-reports of stress, self-esteem and recurrent pain, cortisol was not a relevant biomarker when included individually, while sAA and ratio measures of both cortisol and sAA appear to be more suitable for use in this particular group of well-functioning mid-adolescents.

These findings also show that while there are no differences in cortisol increases between the groups of girls and boys, there are some differences in total levels of cortisol. Considering that the study protocol did not include electronic surveillance or prompters, the overall findings suggest that adolescents were capable of following the study protocol and sample saliva themselves in their daily life settings. As for the single and aggregate cortisol measures, having a total of eight cortisol measures distributed across two ordinary school days, AUC for the
CAR or the full diurnal curve seem to be the more useful index in both adolescent girls and boys.

Also, the present study investigated a fairly homogeneous group of well-functioning mid-adolescents and replicated previously found differences in stress and recurrent pain between girls and boys (Rotenberg et al., 2012). However, there were no significant linkages between activation and pressure stress and aggregate salivary cortisol measures, neither among girls nor among boys. Yet, among girls, activation and pressure stress were associated with recurrent pain. These differences between subjective and objective measures may relate to these measures reflecting distinct and unrelated aspects of functioning or from the mid-adolescents still having bodily systems that are unaffected by activation and pressure stress.

Although additional research is needed to delineate clearly the linkages between stress, self-esteem and biological markers, the present study underscores the value of investigating aggregate markers reflecting different bodily systems and their combinations. In suggesting linkages between self-esteem and ANS/HPA-axis ratios among mid-adolescents, the study expands on previous findings and suggests a trace of psychobiological dysregulation, and subjective experiences of stress and self-esteem. Additionally, the findings for girls and boys respectively may point up areas for future research and, if replicated, perhaps guide the development of educational health promotion programs for adolescent girls and boys.


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