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Sex differences in cortisol response to corticotropin releasing hormone challenge over puberty: Pittsburgh Pediatric Neurobehavioral Studies☆

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Summary
Objective: Consistent sex differences in regulation of the hypothalamic pituitary adrenocortical (HPA) axis have been shown in animal models and emerge over puberty. However, parallel work in humans is lacking despite implications for elucidating the emergence of sex differences in depression over puberty. We investigated sex differences in HPA response to corticotropin releasing hormone (CRH) challenge over puberty in a carefully screened normative sample.
Methods: Participants were 68 healthy children (41% girls), ages 6—16, with no personal or family history of psychiatric disorder. Pubertal maturation was determined by Tanner staging. Following 24 h of adaptation, 9—10 plasma cortisol samples were collected over 30—40 min pre-infusion baseline, 1 μg/kg CRH infusion, and 90—180 min post-infusion recovery. Thirty-seven participants completed 2+ CRH challenges allowing inclusion of cross-sectional and longitudinal data in all analyses. The influence of gender and pubertal maturation on parameters of cortisol response to CRH challenge was investigated using nonlinear mixed model methodology.

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Results: Girls showed increasing total cortisol output following CRH challenge over puberty, while boys showed little change in total cortisol output over puberty. Increased cortisol output in girls was explained by slower reactivity and recovery rates leading to prolonged time to reach peak cortisol and delayed return to baseline over puberty. Girls also showed increasing baseline cortisol over puberty, while boys showed declining baseline over puberty.
Conclusion: Results reveal subtle normative sex differences in the influence of pubertal maturation on HPA regulation at the pituitary level. This normative shift may tip the balance towards stress response dysregulation in girls at high risk for depression, and may represent one potential mechanism underlying elevated rates of depression among pubescent girls.

1. Introduction

Across nations and cultures, women of reproductive ages are approximately two times as likely to suffer from depressive symptoms and syndromes as men (Nolen-Hoeksema, 1987, 1990; Weissman et al., 1993; Kessler et al., 2003). Yet, rates of depression in preadolescent children have been shown to be approximately equal or slightly higher in boys (Kashani et al., 1982, 1983; Fleming et al., 1989; McGee et al., 1992; Kessler et al., 2001). Girls' greater rates of both depressive symptoms and depressive disorders appear to emerge during adolescence, with the majority of studies showing sex differences to emerge around age 13–14 (McGee et al., 1992; Nolen-Hoeksema and Girgus, 1994; Hankin et al., 1998; Oldehinkel et al., 1999; Twenge and Nolen-Hoeksema, 2002; Wade et al., 2002; Kessler et al., 2005).

That this age coincides approximately with the mid-point of pubertal development has led to a growing body of theory and research supporting the role of pubertal processes in the emergence of sex differences in depression (Patton et al., 1996; Angold et al., 1998, 1999, 2003; Cyranowski et al., 2000; Born et al., 2002; Parker and Brotchie, 2004). Although some studies have highlighted links between pubertal timing (i.e., girls' early onset of puberty relative to peers) (Hayward et al., 1997; Ge et al., 2001) and depression, more recent research suggests that pubertal status may be a better marker for the emergence of gender differences in depression than age or pubertal timing (Patton et al., 1996; Angold et al., 1998; Ge et al., 2001; Conley and Rudolph, 2009). Specifically, Angold et al. (1998) found that reaching the middle stage of puberty (Tanner stage III) was associated with a greater prevalence of major depressive disorder in girls. Further, effects of Tanner stage in girls were mediated by rising levels of testosterone and estradiol in girls (Angold et al., 1999). Similarly, in a large, representative, population-based sample, Patton et al. (1996) found that recency of menarche, independent of chronological age, was the strongest predictor of increased rates of depression and anxiety in girls. Finally, Conley and Rudolph (2009) revealed a potentiating effect of life stress on links between puberty and depression in girls, highlighting the importance of understanding interactions between puberty and response to stress for elucidating sex differences in depression.

In adults, depression has been associated with multiple abnormalities along the hypothalamic pituitary adrenal (HPA) axis, including increased basal cortisol levels, altered circadian rhythms, higher cortisol–dehydroepiandrosterone (DHEA) ratios, dexamethasone and dexamethasone–corticotropin releasing hormone (DEX–CRH) nonsuppression, impaired recovery from psychological stressors, and increased central corticotropin releasing hormone (CRH) drive (Carroll, 1982; Halbreich et al., 1985; Rubin et al., 1987; Ribeiro et al., 1993; Nemeroff, 1996; Arborelius et al., 1999; Young et al., 2000; Parker et al., 2003; Burke et al., 2005; Nemeroff and Vale, 2005). Further, normalization of HPA dysregulation has paralleled clinical response to antidepressant therapies (Ising et al., 2007; Schule, 2007). Although associations are less consistent, depression in adolescents has also been associated with alterations along the HPA axis, particularly increased basal cortisol prior to sleep onset dexamethasone nonsuppression, higher cortisol–DHEA ratios, alterations in free cortisol, and altered response to psychological challenge (Dahl et al., 1992a; Goodyer et al., 1998, 2003; Forbes et al., 2006; Rao et al., 2008; Lopez-Duran et al., 2009). A large body of research has also revealed relationships between regulation and dysregulation of the HPA and hypothalamic pituitary gonadal (HPG) axes, with alterations in HPG functioning influencing (dys)regulation of the HPA axis and vice versa (Chrousos et al., 1998; Viau, 2002; Kalantaridou et al., 2004; Mastorakos et al., 2006). Given links between depression and alterations along the HPA axis, connections between the HPA and the HPG axes, and associations between pubertal stage and the emergence of sex differences in depression, we propose that sex differences in HPA regulation emerge over puberty and help to explain sex differences in depression.

In support of this hypothesis, preclinical research reveals consistent sex differences in HPA regulation, with adult females showing higher basal levels (Kitay, 1961; Chritchlow et al., 1963; Hiroshige and Wada-Okada, 1973; Griffin and Whitacre, 1991; Chisari et al., 1995; Atkinson and Waddell, 1997), greater and faster responses to acute stressors (Hiroshige and Wada-Okada, 1973; Le Mevel et al., 1979; Kant et al., 1983; Patchev et al., 1995; Young, 1996) and failure to adapt to chronic and repeated stress, an animal analogue of human depression (Haleem et al., 1988; Galea et al., 1997). Sex differences in HPA responses in preclinical models may be mediated by both activational and organizational effects of sex hormones (Viau and Meaney, 1991; Burgess and Handa, 1992; Handa et al., 1994; Patchev et al., 1995; Patchev and Almeida, 1996; Viau and Meaney, 1996; Lund et al., 2004). Further, sex differences in basal and stress-reactive HPA responses in animals appear to emerge over puberty. Females' greater basal levels of corticosterone, CRH, and corticosteroid binding globulin (CBG) are evident in post but not prepubertal rats (Critchlow et al., 1963; Honma and Hiroshige, 1977; Mataradze et al., 1992; Viau et al., 2005). Males' attenuated and females' increased HPA responses to stress also appear to emerge over puberty, with postpubertal males showing decreased responses to physical and psychological challenge

compared to prepubertal males and postpubertal females showing increased responses to stress compared to prepubertal rats (Ramaley and Olson, 1974; Gomez et al., 2002, 2004; Hodes and Shors, 2005; but see Romeo et al., 2005; Viau et al., 2005). Thus, paralleling the emergence of sex differences in depression, sex differences in HPA responses to stress in animals appear to emerge over puberty.

Likewise, a growing number of human studies have shown influences of gender and/or pubertal stage (Kiess et al., 1995; Klimes-Dougan et al., 2001; Legro et al., 2003; Rosmalen et al., 2005; Scheifelbein and Susman, 2006) on *basal* cortisol levels, with higher levels in girls and in the later stages of puberty. In the study most consistent with preclinical literature, Netherton et al. (2004) found 20–30% greater morning cortisol levels in postpubertal (Tanner $>$ 2) 2) girls compared to postpubertal boys, with no sex differences in prepubertal children. However, several human studies have shown no effect of pubertal stage or gender on basal cortisol regulation (Gomez et al., 1991; Kerrigan et al., 1993; Knutsson et al., 1997). Thus, although effects are inconsistent, there is some evidence for sex differences in basal HPA regulation that emerge over puberty. However, we know of few human studies examining sex differences in HPA response to *challenge* over puberty. Our group previously showed sex differences (males $>$ females) in cortisol response to CRH challenge in analyses which included approximately one third of participants from the present sample (Dahl et al., 1992b). However, the sample was small (n = 25), most children were in the early stages of puberty (Tanner I–II), and statistical analyses were based on peak-post CRH levels only. In the present study, we had the opportunity to extend these analyses by examining sex differences in cortisol responses to CRH challenge across all pubertal stages (Tanner I–V) in a larger sample (n = 68) who completed 182 CRH challenge protocols) using a statistical model designed to allow detailed characterization of a number of cortisol response parameters. Participants were carefully screened controls pooled from all three phases of the Pittsburgh Pediatric Neurobehavioral Studies. We hypothesized that paralleling the emergence of sex differences in depression rates, girls would show increased response to challenge over pubertal stages, while males would show little change in response to CRH challenge over puberty.

2. Methods and materials

2.1. Participants

Participants were 68 carefully screened controls (41% girls, 59% boys) recruited over three phases of a multi-project study of neurobehavioral characteristics of pediatric affective disorders who completed at least one baseline CRH challenge with available cortisol and Tanner stage data. Across participants, 182 CRH infusion protocols were completed with available cortisol and Tanner stage data. Of 68 participants, 31 completed the CRH protocol once, while 37 participated twice or more (Sessions per participant: *Med* = 2; range = 1–6). Repeated CRH assessments spaced about a year apart (Months between sessions: *Med* = 12, range = 1–26) contributed within-subject data on the effects of pubertal maturation (Changes in Tanner stage between sessions: *Med* = 1, range = 0–2) that complemented between-subject data provided by participants assessed only once. Participant ages were 7.1–14.2 years old at baseline (M = 10.5, SD = 1.7), with age range 7.1–16.5 (M = 11.6, SD = 1.9) including longitudinal data. Table 1 shows the number of CRH protocols completed and age range for boys and girls in each Tanner stage.

Participants were recruited through printed advertisements, health fairs, direct mailings, and personal contacts. Racial breakdown included 90% Caucasian, 4% African Americans, and 6% other races. The sample was primarily middle/upper class, with Hollingshead socioeconomic status ranging from 27 to 66 (M = 50, SD = 12). All participants were physically healthy with no current or personal history of psychiatric disorder with low familial risk for depression. Medical history, physical examination, laboratory tests (including electrolytes, liver function, thyroid function, renal function, urinalysis, complete blood count, and ECG) were collected as part of the larger protocol. Personal history of psychiatric disorders was assessed using School Age Schedule for Schizophrenia and Affective Disorders, Epidemiologic version (K-SADS-E) (Orvashel and Puig-Antich, 1987) with both the participant and parent(s) or guardian(s) as informants. Family history of psychopathology was assessed through interviews with first- and second-degree relatives using the K-SADS-E for relatives ages 6–18, and the Schedule for

Table 1 Sample and age distribution by gender and pubertal stage.

Tanner stage	Girls			Boys		
	CRH runs	*N*	Age median (range)	CRH runs	*N*	Age median (range)
I	23	16	9.3 (7.8–12.1)	16	12	9.6 (7.1–13.4)
II	17	14	11.3 (9.9–12.7)	54	27	11.2 (8.3–14.4)
III	7	7	11.8 (10.2–12.7)	17	13	12.4 (9.5–14.5)
IV	15	12	12.2 (11.2–13.1)	14	10	13.8 (11.7–16.0)
V	10	8	13.8 (12.4–15.3)	9	8	14.9 (13.7–16.5)

Note: CRH runs = number of CRH challenge protocols completed across participants per Tanner (pubertal) stage; *N* = number of participants per Tanner stage; Tanner stage = mean of nurse/physician determined Tanner breast/gonad (B/G) and pubic hair (PH) measurements.

Schizophrenia and Affective Disorders-Lifetime (SADS-L) (Endicott and Spitzer, 1978) for adult relatives. Unavailable adult first- and second-degree relatives were assessed using the Family History-Research Diagnostic Criteria (RDC) technique with the child's parent(s) serving as the informant(s) (Spitzer et al., 1978). Low familial risk for depression was defined as having *no* first-degree relative with a lifetime episode of any affective disorder, having no first or second-degree relative with a lifetime episode of mania, schizoaffective disorder or schizophrenia, and having no more than 20% of second-degree relatives with a lifetime episode of major depression. Additional exclusion criteria were: (a) use of medications except acetominophen within two weeks of the protocol, (b) obesity, (c) height or weight below 3rd percentile, and (d) IQ below 70 or learning disability.

2.2. Procedure

The CRH infusion protocol was included in all three phases of the Pittsburgh Psychobiologic Studies, a series of investigations focused on elucidating the neurobiological correlates of affective disorders in childhood and adolescence (Ryan et al., 1992). Procedures were approved by the University of Pittsburgh Institutional Review Board. Informed consent was obtained from all parents/guardians. Participants who were 14–16 years old also provided consent; participants younger than 14 years old were present when procedures were described to parents, and all provided verbal assent to participate. Participants were admitted to the Child and Adolescent Sleep and Neuroendocrine Laboratory at Western Psychiatric Institute and Clinic for three consecutive days and nights of neurobiological assessment. All procedures took place in a comfortable setting with free access to a large recreational area including age-appropriate books, art supplies, board games, and entertainment materials. Unlimited family visitation access was allowed. Upon entry into the study, participants were situated in the laboratory, and an intravenous catheter was placed in an antecubital vein; a slow drip of heparinized saline allowed the vein to remain patent. The catheter was attached to a mobile system allowing for a free range of activities. Participants then completed 24 h of adaptation to the laboratory and mobile system. The CRH infusion protocol began at 4:00 pm (Phases 2 and 3) or 5:00 pm (Phase 1) on the second laboratory day. These times were chosen as points when the HPA axis is believed to be relatively quiescent (Puig-Antich et al., 1989). The CRH challenge protocol included 30–40 min pre-infusion baseline, followed by 1 μg/kg human CRH (hCRH) administered as an intravenous infusion over two minutes, then 90–180 min of recovery. Nine to ten plasma cortisol samples were collected over the course of the CRH challenge protocol. For phase one of the study, basal samples were collected at −30, −15, and 0 min, with 0 as the time of CRH infusion. After hCRH infusion, samples were obtained at 15, 30, 60, 90, 120, 150, and 180 min. Time points for phases two and three were: −40, −20, and 0 min for the basal sampling, and 5, 10, 15, 30, 60, 90 min after hCRH infusion. Blood samples were immediately centrifuged under refrigeration, and plasma was carefully separated and stored at −80 °C until assayed.

2.3. Cortisol

Cortisol levels were determined from 25 μL samples assayed in duplicate by solid phase ^{125}I radioimmunoassay (Diagnostic Products Corporation; Los Angeles, CA). Sensitivity of this assay is 13.79 nmol/L (0.5 μg/dL). Duplicates exceeding a 5.0% coefficient of variation were reassayed. All samples from each CRH challenge protocol from each participant were determined in the same assay run. Intra-assay coefficients of variation ranged from 1.3% to 2.7% (*M* = 1.9%). Inter-assay coefficients of variation ranged from 11.7% at 103.7 nmol/L (3.76 μg/dL) to 7.0% at 839.0 nmol/L (30.4 μg/dL).

2.4. Assessment of pubertal development

Pubertal development was determined by physical examination conducted by a physician or nurse practitioner trained in the assessment of pubertal development. Sexual maturity staging criteria and definitions representing five stages of breast/genital (B/G), and pubic hair (PH) development (Tanner I–V) were determined according to the criteria described by Marshall and Tanner (1969, 1970). Percentage agreement for Tanner stage classification in the longitudinal study has been greater than 90%. Final Tanner stage was determined as the mean of B/G (I–V) and PH (I–V) ratings. Ratings were rounded to the nearest integer for presentation in Tables 1–3.

2.5. Statistical analyses

Overview. The key aim of this study was to characterize the joint effects of gender (*G*) and pubertal stage (*Tanner*) on cortisol response to CRH challenge. We initially examined associations between gender, pubertal stage, and key potential confounders (body mass index, age, race, and socioeconomic status) using polyserial correlations (r_{PS}) for continuous confounders, and polychoric correlations (r_{PC}) for categorical confounders. We then developed a nonlinear mixed model to characterize numerous parameters of cortisol response to challenge. Finally, we assessed the influence of *G*, *Tanner*, and significant confounding factors on model parameters.

Model development. To characterize various parameters of the cortisol response to CRH challenge (e.g., diurnal rhythm, reactivity, recovery, area under the curve), we developed a nonlinear mixed model with mean cortisol response given by:

$$\mu = \theta_0 + \theta_1 \times (Time + Start) + \theta_2 \times T \exp(-\theta_3 \times T)$$

Time was standardized, so that CRH infusion occurred consistently at *Time* = 0, while simultaneously ensuring that the nonlinear departure from baseline trend only operated in the post-infusion phase by setting *T* = *max* (*0*, *Time*). In this model, θ_0 represents cortisol levels at *Time* = 0 (**Baseline Cortisol**); θ_1, the slope of the linear approximation to the underlying diurnal rhythm over the study period (**Baseline Slope**); θ_2, a proportionality constant that modulates the magnitude of peak change from baseline (**Modulating Constant**); and θ_3, the rate of both response to and recovery from the CRH challenge (**Reactivity/Recovery Rate**). Baseline

Table 2 Point and interval estimates for model parameters across pubertal stage and gender.

	Point estimate (95% confidence interval)		
	Tanner I/II	Tanner III	Tanner IV/V
Baseline Cortisol (μg/dL)			
Girls	5.31 (4.89, 5.74)	5.78 (5.34, 6.21)	6.28 (5.56, 7.00)
Boys	5.96 (5.54, 6.38)	5.35 (4.94, 5.76)	4.80 (4.16, 5.44)
Modulating Constant			
Girls	.794 (.711, .878)	.661 (.593, .730)	.552 (.456, .648)
Boys	.811 (.743, .881)	.848 (.784, .912)	.886 (.768, 1.004)
Reactivity rate (μg/dL/min)			
Girls	.033 (.031, .036)	.028 (.026, .031)	.024 (.021, .027)
Boys	.030 (.028, .032)	.031 (.029, .032)	.032 (.029, .035)
Area under the curve (μg/dL)			
Girls	709 (642, 776)	819 (734, 903)	945 (779, 1110)
Boys	898 (820, 975)	881 (816, 946)	865 (758, 972)
Time to Peak (min)			
Girls	29.9 (27.8, 31.9)	35.2 (32.5, 37.8)	41.4 (36.1, 46.7)
Boys	33.3 (31.2, 35.3)	32.2 (30.5, 34.0)	31.2 (28.4, 34.1)
Peak Change (μg/dL)			
Girls	8.73 (8.10, 9.36)	8.57 (7.96, 9.17)	8.40 (7.41, 9.39)
Boys	9.93 (9.34, 10.51)	10.05 (9.53, 10.58)	10.18 (9.26, 11.11)

Table 3 Change in model parameters across pubertal stage by gender.

	Point estimate (95% confidence interval)		
	Tanner III vs. I/II	Tanner IV/V vs. I/II	Tanner IV/V vs. III
Baseline Cortisol (μg/dL)			
Girls	**.47 (.10, .82)**	**.97 (.18, 1.75)**	**.50 (.08, .93)**
Boys	**−.61 (−1.03, −.20)**	**−1.16 (−1.91, −.41)**	**−.55 (−.88, −.22)**
Modulating Constant			
Girls	**−.133 (−.206, −.058)**	**−.242 (−.367, −.118)**	**−.109 (−.161, −.060)**
Boys	.037 (−.031, .104)	.075 (−.067, .217)	.038 (−.036, .112)
Reactivity rate (μg/dL/min)			
Girls	**−.005 (−.007, −.003)**	**−.009 (−.013, −.006)**	**−.004 (−.006, −.003)**
Boys	.001 (−.001, .003)	.002 (−.002, .006)	.001 (−.001, .003)
Area under the curve (μg/dL)			
Girls	**110 (35, 184)**	**236 (63, 408)**	**126 (28, 224)**
Boys	−17 (−85, 52)	−33 (−168, 102)	−16 (−82, 50)
Time to Peak (min)			
Girls	**5.3 (2.9, 7.6)**	**11.5 (6.0, 17.0)**	**6.2 (3.0, 9.4)**
Boys	−1.1 (−2.9, .8)	−2.1 (−5.6, 1.6)	−1.0 (−2.7, .7)
Peak Change (μg/dL)			
Girls	−.16 (−.76, .42)	−.33 (−1.49, .83)	−.17 (−.73, .70)
Boys	.12 (−.43, .69)	.25 (−.88, 1.39)	.13 (−.45, 3.12)

Note: Point estimates and 95% confidence intervals highlighted in bold indicate significant differences between pubertal stages at the $p < .05$ level.

cortisol levels were adjusted for start time (4:00 vs. 5:00 pm) to account for differences in diurnal rhythm at baseline (*Start* = 0 for sessions commencing at 4:00 pm; *Start* = 60 min for sessions commencing at 5:00 pm). Baseline cortisol, the modulating constant, and the reactivity/recovery rate were all modeled in the logarithmic scale.

To allow detailed characterization of cortisol response, model findings are presented in terms of the four basic model parameters defined above, as well as three derived parameters given by: (a) $\theta_4 = \theta_2/\theta_3^2$, total cortisol response to CRH challenge as measured by the area under the curve **(AUC)** from time of CRH infusion until return to the underlying diurnal rhythm pattern; (b) $\theta_5 = 1/\theta_3$, the time point at which peak cortisol is reached (**Time to Peak**); and (c) $\theta_6 = (\theta_2/\theta_3)\exp(-1)$, peak change in cortisol calculated relative to a linearly declining baseline (**Peak Change from Baseline**). Changes in the **Reactivity/Recovery Rate** alter the overall shape of the cortisol response curve. Specifically, increases in **Reactivity/Recovery Rate** accelerate **Time to Peak** cortisol levels, with a swift rise to peak cortisol levels followed by quick return to baseline (spike shape); decreases in **Reactivity/Recovery Rate** flatten the cortisol response curve around

the peak, prolonging **Time to Peak** and return to baseline (bell-shape). Introduction of the **Modulating Constant** offers additional modeling flexibility, allowing for separation between changes in peak cortisol (**Peak Change from Baseline**) and changes in the overall shape of the cortisol response curve resulting from variations in **Reactivity/Recovery Rate**.

To accommodate heteroscedasticity, within-run variation was modeled by a quadratic function of the mean, given by $\sigma_1^2(\mu) = \sigma_1^2 \times \mu^2$. Between-session variability in cortisol response was modeled through session-level random effects on baseline cortisol level, θ_0, assumed to be normal variables with zero mean and constant variance given by σ_0^2. Although this model specification accounts for associations among cortisol values within the same CRH session (session-level random effects), it assumes that cortisol values are uncorrelated across runs for the subset of 37 participants completing more than one CRH challenge (no subject-level random effects). Model findings were also replicated by assuming subject-level, rather than session-level, random effects. However, as patterns and significance of primary findings did not differ, we present results for session-level random effects.

3. Results

3.1. Testing potential confounding variables

Polychoric and polyserial correlation coefficients were utilized to test associations between *G*, *Tanner* and key potential confounders: body mass index (*BMI; weight/height*^{2}), age, race, and socio-economic status (*SES*, determined by the Hollingshead Index; Hollingshead, 1975). Race and SES showed no significant associations with either *G* or *Tanner*. However, we found significant associations between *BMI* and *Tanner* (r_{PS} = .28, $p < .05$), and highly significant associations between age and *Tanner* (r_{PS} = .71, $p < .0001$).

3.2. Model refinement

All model parameters were estimated using SAS/STAT PROC NLMIXED 9.2 (SAS Institute Inc., 2010), with point estimates supplemented by 95% confidence intervals. Due to the high collinearity between age and pubertal maturation, initial attempts to separate their effects in the nonlinear mixed model were not successful. Since our theoretical models are primarily focused on the effects of pubertal maturation, age was dropped from the model to avoid multicollinearity concerns.

Given evidence of moderate associations between *BMI* and *Tanner*, we included *BMI* as an independent variable in our model by parameterizing $\eta_0 = \log(\theta_0)$, $\eta_1 = \theta_1$, $\eta_2 = \log(\theta_2)$, $\eta_3 = \log(\theta_3)$ as gender-specific linear functions of *Tanner*, *BMI* and their interaction. Thus, we set $\eta_i^G = \alpha_i^G + \beta_i^G \times Tanner + \gamma_i^G \times BMI + \delta_i^G \times Tanner \times BMI$ for $i = 0, \ldots, 3$, and G = male (*M*) or female (*F*). There was no evidence of *BMI* main effects or of *BMI* interactions with pubertal stage on any of the parameters in either males or females. Thus, *BMI* was eliminated from the model, and parameters were re-expressed as gender-specific linear functions of pubertal stage alone, i.e., $\eta_i^G = \alpha_i^G + \beta_i^G \times Tanner$ for $i = 0, \ldots, 3$, and $G = M, F$.

This simplified model revealed no significant *G* main effects ($\alpha_1^M = \alpha_1^F$, p = .44) or *G* by *Tanner* interactions ($\beta_1^M = \beta_1^F$, p = .61) on **Baseline Slope**. Upon re-estimating the model with η_1 assumed to be a linear function of pubertal stage alone ($\eta_1 = \alpha_1 + \beta_1 \times Tanner$), no maturation effects on the linear slope of the diurnal rhythm emerged either ($\beta_1 = 0$, p = .19). In contrast, very highly significant *G* by *Tanner* interactions were obtained for all remaining model parameters: **Baseline Cortisol** ($\beta_0^F = \beta_0^M$, $p < .0001$), **Modulating Constant** ($\beta_2^F = \beta_2^M$, p = .001), and **Reactivity/Recovery Rate** ($\beta_3^F = \beta_3^M$, $p < .0001$). Hence, our final model was obtained by treating η_1 as constant across gender and pubertal stage, while allowing (η_0, η_2, η_3) to depend on both *G* and *Tanner*.

3.3. Influence of gender and pubertal stage of cortisol response to CRH challenge

Although all analyses were conducted with Tanner stage as the mean of B/G and PH ratings, results in Tables 2 and 3 and Fig. 1 are shown with Tanner stage divided as Tanner I/II, III, IV/V for ease of presentation. In estimating model parameters for each pubertal stage grouping, Tanner score was set to the midpoint of the corresponding interval, i.e., 1.5 and 4.5 respectively for the Tanner I/II and Tanner IV/V groups. Girls' and boys' cortisol response to CRH challenge over pubertal stages are shown in Fig. 1. Gender-specific estimates of model parameters by pubertal stage are shown in Table 2, with the nature of *G* by *Tanner* interactions elucidated in Table 3 in terms of pairwise comparisons across pubertal stage groupings (*Tanner III* vs. *I/II*, *Tanner IV/V* vs. *I/II*, *Tanner IV/V* vs. *III*), stratified by gender. All point estimates are supplemented by 95% confidence intervals. Confidence

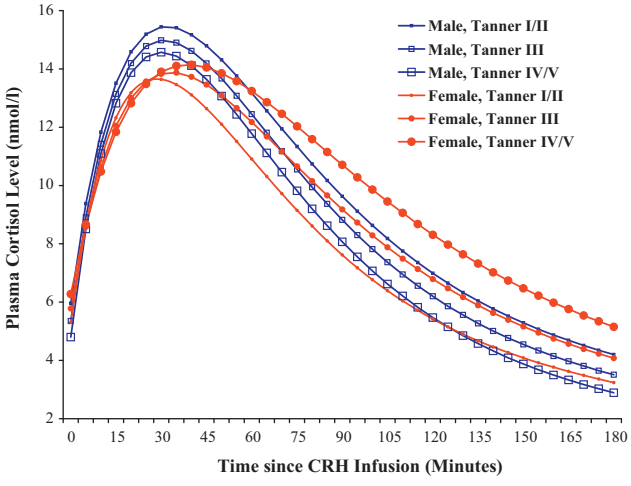

Figure 1 Modeled cortisol response to CRH infusion by gender across pubertal stage based on a nonlinear mixed model with mean cortisol response given by: $\mu = \theta_0 + \theta_1 \times$ (*Time* + *Start*) + $\theta_2 \times T\exp(-\theta_3 \times T)$. CRH infusion occurs at *Time* = 0; ensuring that nonlinear departure from baseline operates only in the post-infusion phase, $T = max(0, Time)$. θ_0 (Baseline Cortisol) represents baseline cortisol at *Time* = 0; θ_1 (**Baseline Slope**) is the slope of the underlying diurnal rhythm; θ_2 (**Modulating Constant**) is a constant that modulates magnitude of the peak change from baseline; and θ_3 (**Reactivity/Recovery Rate**) is the rate of reactivity and recovery from CRH challenge.

intervals in Table 3 that do not contain zero suggest that the corresponding parameters differ across pubertal stages at the 5% level of significance. As **Baseline Slope** was taken as constant across *G* by *Tanner* combinations, it was omitted from the tables.

We found highly significant *G* by *Tanner* interactions for **Baseline Cortisol**, **Modulating Constant** and **Reactivity/Recovery Rate** (p's $< .0001$). Specifically, girls showed significant increases in **Baseline Cortisol** with pubertal maturation (p's $< .02$ for all three pairwise differences between pubertal stage groups), while boys showed highly significant declines of comparable magnitude (all p's $< .01$); when subject-level rather than session-level random effects were included, the magnitude of the changes was attenuated for boys, but not for girls. Girls also showed very highly significant decreases in the **Modulating Constant** with increasing pubertal stage (all p's < 001), while no significant differences emerged for boys (p's $> .29$). Finally, very highly significant decreases in **Reactivity/Recovery Rates** were observed in girls with increasing pubertal stage (all p's $< .0001$), while boys showed little change in **Reactivity/Recovery rates** over puberty (all p's $> .27$).

Significant *G* by *Tanner* interactions in **Reactivity/Recovery** Rates led to significant *G by Tanner* interactions for two of the derived parameters more closely related to the shape rather than the overall level of the cortisol response curve: **AUC** ($p < .05$), and **Time to Peak** ($p < .0001$). Specifically, girls showed significant increases in total cortisol response to CRH challenge with pubertal maturation (p's $< .01$ for all three pairwise differences in **AUC** between pubertal groups), while boys showed no significant changes in total response with increasing pubertal stage (all p's $> .63$). Additionally, girls took longer to reach peak cortisol levels with pubertal maturation (all p's $< .001$), while boys showed no significant influence of pubertal stage (all p's $> .27$) on time to peak cortisol response. In contrast, with changes in **Reactivity/Recovery Rate** neutralized by compensatory changes in the **Modulating Constant** in girls, we found no significant *G by Tanner* interactions for the derived parameter most closely related to cortisol level rather than shape of the cortisol response curve: **Peak Change from Baseline** ($p = .77$). Pubertal stage main effects on this parameter did not appear significant for either girls (all p's $> .57$) or boys (all p's $> .66$). However, gender effects were significant across pubertal stage with boys showing larger **Peak Change** than girls for all Tanner stages (all p's $< .001$).

4. Discussion

To our knowledge, our group is the first to investigate sex differences in HPA response to biological challenge over puberty. In the present analyses, we found subtle changes in cortisol responses to CRH infusion challenge over puberty in girls but not boys. Girls showed increases in total cortisol response to CRH challenge (area under the curve; AUC) over puberty, while boys showed little change in total cortisol response to challenge. Increased total cortisol response to CRH challenge in girls was explained by decreasing rates of reactivity and recovery (**Reactivity/Recovery Rate**) leading to prolonged time to reach peak cortisol level (**Time to Peak**) and flattening of the overall cortisol response curve across pubertal stages. Despite these changes in the *shape* of the cortisol response curves among girls, no changes in peak cortisol *level* (**Peak Change from Baseline**) were observed. This was due to girls' compensatory decreases in a proportionality constant (**Modulating Constant**), which controls the magnitude of both peak and total cortisol response given a fixed rate of reactivity/recovery. Girls' decreasing rates of reactivity/recovery combined with increasing magnitude of the modulating constant over puberty resulted in a prolonged cortisol response and increased area under the curve. Girls also showed increasing baseline cortisol over pubertal stages while boys showed small decreases in baseline cortisol across pubertal stages. Boys showed increased peak cortisol (**Peak Change from Baseline**) relative to girls at all pubertal stages, but no changes in additional cortisol response parameters across pubertal stages.

Although preclinical studies suggest that sex differences in HPA regulation emerge over puberty (McCormick and Mathews, 2007), human studies of sex differences in HPA regulation over puberty have been lacking despite implications for understanding the emergence of sex differences in depression. Our group and others have recently revealed overall increased HPA response to *psychological* challenge over adolescence/puberty with postpubertal adolescents showing increased cortisol (and autonomic) response to developmentally relevant psychological challenges relative to prepubertal children (Gunnar et al., 2009; Stroud et al., 2009; Westenberg et al., 2009; Sumter et al., 2010). However, sex differences were not a primary focus of these studies. The proposed study extends this work to investigate response to biological challenge with an explicit focus on sex differences.

Results from the present study suggest that sex differences in cortisol response to biological challenge over the pubertal transition are evident even in carefully screened children with no personal or family history of psychiatric disorder. Results highlight the possibility of sex specificity in the unfolding of pubertal processes. Given known potent programming effects of glucocorticoids and increased plasticity of the adolescent brain, even subtle increases in cortisol output may have implications for influencing girls' future brain, behavioral, and endocrine response to challenge (McCormick and Mathews, 2007). Further, given links between depression and dysregulation of the HPA axis, it is possible that subtle changes in HPA regulation in girls may tip the balance towards stress response dysregulation in girls at high risk for depression. Thus, normative sex differences in HPA regulation, which may be amplified in high-risk girls, may be one potential mechanism underlying the emergence of girls' greater rates of depression during this time. Future research should investigate whether more or less pronounced sex differences emerge in depressed or high-risk adolescents, and the influence of other stressor types (social and performance stressors, repeated stress) on HPA response to more fully characterize sex differences in stress response over the pubertal transition.

There are several notable strengths of the present study. First, to our knowledge, results represent the first example of translational research aimed at characterizing the intricacies of sex differences in HPA response to biological challenge over the adolescent transition. Our novel statistical model

for characterizing cortisol response to CRH challenge allowed us to capture sex differences in numerous characteristics of cortisol response curves. We applied nonlinear mixed model methodology to characterize baseline cortisol as well as circadian rhythmicity, rate of reactivity and recovery, peak cortisol change from baseline, time to peak cortisol response, and integrated cortisol output over the CRH challenge (area under the curve). Second is the careful selection of the ''normative'' sample, which included only physically healthy boys and girls with no personal or family history of psychiatric conditions as determined by gold-standard interview. Third is the high level of acclimatization of the children to the laboratory environment prior to administration of the CRH infusion—the challenge procedure followed 24 h of adaptation to a laboratory designed to be as comfortable as possible for child participants. Fourth is the large number of CRH challenge sessions (182) available for analyses with more than half of the sample contributing both longitudinal and cross-sectional data to the analyses.

We also acknowledge key limitations of this study with respect to cortisol response. First, although glucocorticoids clearly influence numerous physiologic and neural pathways (Dallman et al., 1987; McEwen, 2000) and may offer a window into upstream responses including ACTH, and central pathways influencing the HPA axis, the use of solely this peripheral measure represents a study limitation and precludes assessment of links between cortisol response and additional HPA hormone and higher level brain response. Second, we did not measure cortisol binding globulin (CBG) or plasma free cortisol, which would have allowed investigation of differences in levels of CBG in relation to peak cortisol, and in relation gender and pubertal stage (Romeo et al., 2006; Romeo and McEwen, 2006). Future studies of sex differences in additional HPA hormones, CBG/free cortisol and higher level brain response to challenge over puberty are needed.

Our results with respect to increased cortisol output and more prolonged response/delayed time to peak cortisol in girls in later puberty are generally consistent with a small group of related human studies. Results with respect to overall cortisol output are complemented by a study of sex differences in HPA response to ovine CRH challenge in adults by Gallucci et al. (1993), who found increased ACTH response to ovine CRH challenge in females relative to males, although they found no sex differences in *cortisol* response to the challenge. In another relevant study, Gunnar et al. (2009) showed increased cortisol response to a laboratory psychological challenge (Trier Social Stress Test-Child Version; TSST-C) in older (13 and 15 years) versus younger (9 and 11 years) adolescents. In the 13-year-old age group only, they found greater cortisol response to the TSST-C in girls versus boys, potentially suggestive of sex differences in response to psychological challenge over the adolescent transition. Finally, results with respect to a more prolonged time to peak cortisol are consistent with Oskis et al. (2009) who studied circadian cortisol rhythms in 13—19-year-old girls. They found increased cortisol awakening response in post-menarcheal versus pre-menarcheal girls was primarily due to delayed time to peak cortisol after awakening (M = 45 vs. 30 min) in post-menarcheal versus pre-menarcheal girls. Although Oskis et al. focused on basal cortisol levels, it is notable that similar to the present study, increased response to awakening was also due to prolonged time to peak cortisol.

Results are inconsistent with those of Dorn et al. (1996) who found no sex differences in cortisol but greater ACTH response to CRH challenge in adolescent males relative to females; however, effects of pubertal stage were not investigated, and the sample was small and included both depressed and non-depressed children. Results also contrast with those of Klimes-Dougan et al. (2001), who investigated cortisol response to psychological stressors: a family conflict task (conflictual discussion with mother) and social performance tasks (interaction with shy confederate, public speaking) in girls and boys ages 11—13 and 14—16. In response to the social performance tasks, older boys showed greatest cortisol reactivity, relative to younger boys and older and younger girls. Future studies should extend results to investigate sex differences in response to ecologically valid and developmentally relevant challenges over puberty. In particular, a growing body of research has highlighted females' greater neurobiological sensitivity to social stressors, and the importance of peer influences over the adolescent transition (Nelson et al., 2005). For example, Guyer et al. (2009) showed greater activation of brain affective processing regions in response to the anticipation of peer evaluation with increasing age in girls but not boys across the adolescent transition. Additional studies of sex differences in regional brain activation in response to biological and ecologically valid psychological challenges over puberty are needed.

Our results also complement some aspects of preclinical studies of HPA response to challenge. Postpubertal/adult female rats have shown increased corticosterone in response to acute stress relative to prepubertal rats (Hodes and Shors, 2005; Viau et al., 2005). Similarly, in the present study, postpubertal girls showed increasing integrated cortisol response to challenge over pubertal stages; however, girls' peak levels showed no significant changes over puberty. Additionally, results corroborate some aspects of a body of preclinical studies revealing sex differences in corticosterone response to acute and chronic stressors, with adult females showing increased and prolonged corticosterone response relative to adult males (Kant et al., 1983; Haleem et al., 1988; Burgess and Handa, 1992; Handa et al., 1994; Armario et al., 1995; Rivier, 1999). Although postpubertal girls in the present study showed lower peak cortisol relative to boys, paralleling preclinical studies, girls showed more prolonged cortisol response leading to increased total cortisol output (AUC) relative to postpubertal boys. Results are not consistent with preclinical studies of male rats, in which *prepubertal* males have shown more prolonged and delayed rise in corticosterone following several types of stress relative to postpubertal males (McCormick and Mathews, 2007). Boys in our study showed no significant changes in either total cortisol output or peak cortisol over pubertal stages, but showed greater peak cortisol across all pubertal stages. Our results are also inconsistent with those of Romeo et al. (2005), who investigated pre and postpubertal female rats and found no differences in basal or peak corticosterone response to acute challenge, but more prolonged stress response in *prepubertal* relative to postpubertal/adult females. Thus, although preclinical studies of sex differences in HPA stress response in adults are consistent, future preclinical studies of the *emergence* of sex differences in HPA response over various stages of puberty are needed to resolve inconsistencies and complement human studies.

We can speculate on potential mechanisms to explain increased cortisol output and more prolonged response/delayed time to peak cortisol in girls over puberty. One possibility is that negative feedback inhibition in girls becomes more delayed over puberty. Preclinical studies of adult females have revealed relative resistance to glucocorticoid negative feedback systems relative to adult males, effects that appear to be mediated by gonadal hormones (Young, 1996; Altemus et al., 1997; Weiser and Handa, 2009). Central glucocorticoid and mineralocortoid receptors (GR, MR), which mediate negative feedback, have also been shown to be modulated by gonadal hormones in adult animals (Burgess and Handa, 1992; Patchev et al., 1995; Patchev and Almeida, 1996). Effects of pubertal changes on central GR and MR in females and males are needed (Romeo, 2010). Another possibility is altered sensitivity of the adrenal or pituitary over the pubertal transition. Finally, alterations in corticotropin releasing hormone (CRH) expression may also influence the peripheral stress response. Viau et al. (2005) found increased CRH expression in the parvocellular paraventricular nucleus (PVN) in post versus prepubertal rats at baseline, but no changes in response to stress. Gonadal hormones may also influence the PVN or higher brain structures which regulate the stress response (McCormick and Mathews, 2007). Future studies are needed to understand mechanisms underlying pubertal alterations in HPA (and neural) response to stress in humans and animals.

Results with respect to baseline cortisol complement human and preclinical studies examining basal cortisol/corticosterone levels. We found increasing baseline cortisol levels over pubertal stage in girls, and decreasing baseline cortisol level in boys. Similarly, in a longitudinal study of 24-h urinary free cortisol levels over pubertal stages in girls only, Legro et al. (2003) showed increasing cortisol levels over pubertal stages (especially Tanner stages III—V). Oskis et al. (2009) also showed increased cortisol awakening response and daytime cortisol secretory activity in post-menarcheal girls. Likewise, Scheifelbein and Susman (2006) found increasing basal cortisol levels in girls and decreasing cortisol in boys in a one year longitudinal study of links between morning cortisol and anxiety symptoms in 9—14 years olds; Netherton et al. (2004) found increased morning but not evening cortisol levels in mid/post pubertal girls relative to mid/post-pubertal boys who showed similar levels to pre/early pubertal girls and boys. Other human studies have also shown increased cortisol levels in adolescent girls (Jonetz-Mentzel and Wiedemann, 1993; Halligan et al., 2004; Rosmalen et al., 2005); however it is notable that several human studies have not shown gender differences (Kiess et al., 1995; Walker et al., 2001; Elmlinger et al., 2002; Adam, 2006), although none were designed explicitly to investigate gender differences. Future longitudinal studies of circadian cortisol variation assessed at multiple times of day over multiple time points across puberty/adolescence are needed to reveal a detailed picture of sex differences in cortisol regulation over puberty. Our findings also complement preclinical studies revealing the emergence over adolescence/puberty of increases in female basal corticosterone levels, with corresponding decreases observed among males, as well as of increases in adrenal weight and adrenal corticosterone content among females alone (Critchlow et al., 1963; Sencar-Cupovic and Milkovic, 1976; Honma and Hiroshige, 1977; Mataradze et al., 1992; Viau et al., 2005; McCormick and Mathews, 2007).

We also acknowledge additional limitations of the study that point to new directions for future research. First, although the carefully selected healthy sample represents a strength of the study in terms of defining a ''normative'' sample, the preponderance of Caucasian and middle/upper socio-economic status families represents a limitation in terms of generalizability of study findings. Future studies of ''normative'' samples with greater racial/ethnic and socio-economic diversity are needed, especially given evidence for differential links between menarche and depression in non-Caucasian girls (Hayward et al., 1999). Second, the smaller number of participants and CRH runs in the later relative to earlier stages of puberty represents a study limitation. However, because analyses were based on pubertal stage modeled as a continuous variable, power remained high for differentiating differences between early, mid, and later pubertal stages. Finally, although the novel modeling of cortisol response parameters is a strength of the study, our model did not accommodate both subject and session-level random effects. To overcome this limitation, however, we ran models with both subject or session level random effects and found few differences in patterns or significance of effects. Thus, we do not believe that the inclusion of both subject and session-level random effects would change results significantly from those presented here. Relatedly, due to high levels of multicollinearity, we were not able to simultaneously investigate the influence of age and pubertal stage in our models. We focused on pubertal stage based on prior research and our theoretical models highlighting puberty as a critical process in influencing sex differences in stress response and depression (Patton et al., 1996; Angold et al., 1998; Ge et al., 2001). Future studies of age-matched adolescents differing in pubertal stage would help to resolve the primacy of age versus pubertal stage in explaining developmental increases in response to challenge.

In sum, there is a remarkable paucity of human studies of sex differences in stress response over the adolescent transition despite potential implications for understanding the emergence of sex differences in depression over puberty. Results from the present study suggest that even in carefully screened children with no history of psychiatric disorder, subtle sex differences in cortisol response to biological challenge over the pubertal transition are evident; girls show a delayed and more prolonged cortisol response over puberty with little change in response to challenge across puberty in boys. Given links between depression and dysregulation of the HPA axis, it is possible that subtle changes in HPA stress response in girls may tip the balance towards programming of brain and peripheral stress response dysregulation in girls at heightened risk for depression. Normative sex differences in HPA stress response, then, may be one potential mechanism underlying the emergence of girls' greater rates of depression during this time. Future research should investigate sex differences in HPA stress response over puberty in clinical and high-risk populations and in response to ecologically and developmentally relevant psychological challenges.

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Conflict of interest

None of the authors have any conflicts of interest.

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