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Validation of a stress reactivity assessment protocol for children aged 4–5 years: Exploring the influence of sex, emotional responses, and crying

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ABSTRACT

Background: Early childhood is an important period for the development of stress regulation systems, yet validated protocols to assess stress reactivity in preschoolers remain scarce. The current study aimed to validate a stress reactivity laboratory protocol based on the matching task in a Spanish sample of 4–5-year-old children, while exploring the influence of sex, emotional responses, and potential confounding variables.
Methods: Fifty-eight preschoolers participated in the Stress Reactivity Task for Preschoolers (SRTP), which included six salivary samples for the measurement of cortisol (as a marker of hypothalamic-pituitary-adrenal [HPA] axis activity) and alpha-amylase (as a marker of sympathetic nervous system [SNS] activity). Behavioral and emotional responses were also coded. Statistical analyses included repeated-measures GLMs, paired t-tests, and correlation analyses to evaluate biomarker patterns and confounders.
Results: The SRTP effectively elicited a stress response: 77.6 % of children were classified as alpha-amylase responders, and 64.9 % as cortisol responders. Alpha-amylase levels increased sharply post-task and gradually returned to baseline within 40 min. In contrast, cortisol levels peaked later and remained elevated for a longer period. No correlation was found between the two biomarkers. Emotional and observational data supported the presence of stress, with significant increases in anger, sadness, and nervousness during the task. Notably, girls exhibited faster cortisol reactivity and greater sadness than boys. Among all examined variables, crying emerged as the most influential confounder, being strongly associated with heightened cortisol reactivity.
Conclusions: The SRTP is a valid and sensitive protocol for assessing stress reactivity in preschool-aged children. It enables simultaneous assessment of SNS and HPA axis activity and captures meaningful interindividual differences. These findings contribute to a more nuanced understanding of early stress physiology and may inform future longitudinal studies and preventive interventions.

1. Introduction

Children’s stress reactivity is commonly assessed through hypothalamic–pituitary–adrenal (HPA) axis and Autonomic Nervous System (ANS) activity (Bleker et al., 2020; Engel and Gunnar, 2020; Filetti et al., 2024), yet the coordination between these systems is increasingly recognized as essential for understanding the complexity of stress responses and their links to behavior and cognition (Allwood et al., 2011; Bauer et al., 2002; de Weerth et al., 2013; Del Giudice et al., 2011; Gordis et al., 2006; Roos et al., 2017; Spinrad et al., 2009; Tolep and Dougherty, 2014). Salivary measures provide a non-invasive, painless and reliable way to assess both systems, with cortisol indexing HPA activity and salivary alpha-amylase (sAA) reflecting sympathetic activation (Allwood et al., 2011; Angeli et al., 2018; Gordis et al., 2006; Spinrad et al., 2009). Although SNS activity unfolds rapidly and can be faster captured by other physiological methods (e.g., Roos et al., 2017), integrating cortisol and sAA allows simultaneous assessment of distinct stress systems through a single, young-children–appropriate procedure

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(Spinrad et al., 2009).

Beyond methodological advantages, assessing both HPA and SNS responses is key to identifying early markers of developmental risk. Dysregulation of these systems has been associated with heightened vulnerability to psychopathology, poorer physiological adaptation, and adverse cognitive and behavioral outcomes (Aguilar et al., 2014; Filetti et al., 2024; Maldonado et al., 2019; Selvaraju et al., 2020; Spinrad et al., 2009). In addition, asymmetries between HPA and SNS activity have been linked to internalizing and externalizing problems, with associations between cortisol and behavioral symptoms emerging only under conditions of low sAA reactivity (Allwood et al., 2011). Similarly, Gordis et al. (2008) biomarkerst maltreated children displayed such asymmetry, reflected in the absence of correlations between cortisol and sAA responses across indices including AUC (Area Under the Curve) and baseline levels, whereas coordinated responses have been related to more adaptive attention processes (Ursache and Blair, 2015).

Given the importance of assessing both systems, recent research has focused on developing protocols capable of eliciting such responses in preschoolers. Although several studies have successfully induced cardiovascular ANS activation through executive function, cognitive, fine motor, or matching tasks in children aged 3–5 years (Blair and Peters, 2003; Boyce et al., 1995) and 4–6 years (Roos et al., 2017), to our knowledge only one study—Spinrad et al. (2009)—attempted to elicit sAA responses in children aged 4–5 years. However, their protocol did not yield a detectable sAA response, likely because the biomarker was not integrated into an existing effective stress reactivity protocol.

Furthermore, inducing reliable HPA axis responses in preschoolers has proven particularly challenging. Gunnar et al. (2009) reviewed studies across multiple developmental periods and reported that only 9 % of the eleven studies available at that time showed a mean cortisol increase in preschoolers (2–5 years), despite the use of diverse tasks such as fear, separation, conflict, peer stress, not-sharing, novelty, or frustration paradigms. Failures were attributed to adult support, poor sampling timing, anticipatory stress, and—most importantly—the lack of an age-appropriate social-evaluative threat. Cortisol increases in preschoolers and older children occurred mainly during anger or frustration tasks, specifically at age 4 (Lewis and Ramsay, 2002) and 8–11 years (Van Goozen et al., 1998). Later meta-analysis in adults confirmed that uncontrollability and social-evaluative threat are the key elements required to elicit cortisol reactivity (Dickerson and Kemeny, 2004), posing a major challenge for preschool paradigms—how to provoke shame and uncontrollability in a preschool-age child.

In this context, Kryski et al. (2011) achieved a major breakthrough by adapting Lewis and Ramsay's (2002) "matching task," an anger/-frustration paradigm, to meet the conditions outlined by Gunnar et al. (2009) and Dickerson and Kemeny (2004). Their version incorporated unpredictability, uncontrollability, and explicit social evaluation to enhance cortisol reactivity and was the first to elicit a clear cortisol response in preschoolers (3 years old). In this task, children matched animal figures under time pressure to win a prize, while being told the game was easy and that "little kids" could complete it. A traffic-light timer indicated the time remaining on each attempt, and the experimenter remotely controlled it so that every child failed three times, embedding uncontrollability, unpredictability, and social-evaluative threat.

Subsequent studies refined this paradigm. Roos et al. (2017) included children from 4.2 to 6.7 years old, incorporated a control group and validated the task as a laboratory stressor eliciting both HPA (cortisol) and ANS (respiratory sinus arrhythmia, pre-ejection period) responses; attributing its effectiveness to the presence of an unfamiliar, affectively neutral assessor and non-reinforcing language. Later, Send et al. (2019) confirmed its robustness in a large sample (N = 339) of children aged 45 months, reporting responder rates of about 60 %. As noted by the authors, this is close to the responder rates typically observed in the TSST—generally above 70 % (Kudielka et al., 2007). It is also close to other paradigms in for adults like the Socially Evaluated Cold Pressor Test with 60 % (Schwabe and Schächinger, 2018).

Therefore, the matching task has been validated as an effective stressor for preschoolers' cortisol responses. However, differences across studies in sampling schedules—and some variation in the timing of observed cortisol peaks—highlight the need for more frequent sampling and further investigation of response dynamics. Kryski et al. (2011) collected six samples (pre-task, and 10, 20, 30, 40, and 50 min post-task), while Roos et al. (2017) used four samples (post-task, and 20, 40, and 50 min post-task). Both studies observed increases from baseline to 20 min post-stressor, followed by declines from approximately 20–30 min to 50 min. In contrast, Send et al. (2019), who collected four samples (pre-task, and 10, 30, and 40 min post-task), found a delayed response with significant increases from 10 to 30 min and sustained elevations up to 40 min.

Although other valuable approaches, such as the CREST protocol (Children's Reactions to Evaluation Stress Test)—a laboratory stress paradigm involving task performance in front of an evaluative judge that reliably elicits cortisol responses in children aged 5–6 years (de Weerth et al., 2013)— have also induced HPA activation, we adapted the matching task for the development and validation of our protocol. This paradigm remains the only one to reliably induce cortisol responses in 4–5-year-olds, supported by three studies (N = 612), and has proven sensitive to both HPA and ANS activation (Kryski et al., 2011; Roos et al., 2017; Send et al., 2019).

Previous studies have described children's stress reactivity patterns, yet the influence of potential confounders has not been examined in depth and may partly account for the heterogeneous findings across studies. Several variables warrant attention. Napping produces acute cortisol fluctuations, with decreases during sleep and rebounds upon awakening, including a post-nap cortisol awakening response in children 0–5 years (Mesas et al., 2022). Crying has been associated with elevated salivary cortisol in infants (8-week-old) and toddlers (15-month-old) (Ahnert et al., 2004; Bruinhof et al., 2025). Evidence on sex differences remains mixed: studies conducted in preschool- and school-aged children—including samples aged 7–12 years (Allwood et al., 2011), 5–6 years (de Weerth et al., 2013), and 3 years (Kryski et al., 2011)—have generally reported no robust main effects. However, Send et al. (2019), working with children aged 45 months, found a marginally stronger cortisol increase in girls. In addition, a broader meta-analysis including samples from 6 to 18 years (Seel et al., 2025) showed that studies with a higher proportion of girls tended to exhibit greater cortisol reactivity, although categorical sex differences—based on only-male vs. mixed vs. only-female samples—were not significant. Seasonality can also affect cortisol in children aged 10–12 years, with higher levels in winter and spring than in summer or autumn (Feneberg et al., 2025). Finally, socioeconomic factors may shape stress physiology: although some studies from children 3–6 years found no associations with parental education (de Weerth et al., 2013; Kryski et al., 2011; Send et al., 2019), lower socioeconomic status has been linked to elevated basal cortisol and greater exposure to adversity in samples aged 6–10 years (Lupien et al., 2001), at age 3 (Send et al., 2019), and in adults (Anneser et al., 2025).

Collectively, these findings highlight napping, crying, sex, season, and socioeconomic status as key factors that may modulate children's stress responses and should be considered.

In conclusion, the matching task effectively elicits cortisol responses in preschoolers, but further research is needed to expand its scope. Integrating salivary alpha-amylase (sAA) into this paradigm would represent a major advance, enabling assessment of both HPA and SNS activation and their coordination or asymmetry—thus providing a more comprehensive understanding of stress physiology and its developmental implications (Allwood et al., 2011; Spinrad et al., 2009). Refining sampling strategies and systematically considering relevant confounders are also essential to optimize the detection of these responses and clarify cortisol secretion patterns in early childhood. Moreover, validation beyond Northern Europe and the United States is warranted, as testing schedules may differ across cultural contexts—for instance, late-afternoon assessments may better fit Spanish children's routines.

The present study aimed (1) to confirm that the task developed by Kryski et al. (2011) elicits stress responses in both salivary cortisol and alpha-amylase in a Spanish sample of 4- to 5-year-olds, (2) to characterize typical SNS and HPA reactivity patterns, and (3) to examine the influence of potential confounders. We hypothesize our Stress Reactivity Task for Preschoolers (SRTP) to activate both systems, with earlier and stronger SNS responses, and that the following factors would be associated with higher stress reactivity: previous napping, crying during the task, being female, being assessed in winter/autumn, lower parental education, and lower socioeconomic status.

2. Materials and methods

2.1. Participants

The participants were preschool-aged children, mainly from the city of Granada (Spain), and part of the "GestaStress-ChildStress" cohort. Their mothers were contacted via phone message. Of the 85 mothers informed, 23 declined participation in the stress reactivity task, and four were excluded based on exclusion criteria. The inclusion criterion was being 4 or 5 years old. Exclusion criteria included using glucocorticoids or medication known to alter glucocorticoid metabolism (for example, antibiotics), using medication known to alter salivary alpha-amylase, and the presence of any disability or diagnosed developmental disorder. None of the children were acutely ill at the time of assessment, as parents postponed participation if their child was sick, and no parent reported recent antibiotic use. Finally, a sample of 58 children aged 4–5 years (M = 53.71 months; SD = 4.95 months; 53.4 % females and 46.6 % males) was included in the study.

The Human Studies Ethics Committee of the University of Granada (Spain) approved the study (968/CEIH/2019), which was conducted in conformity with the American Psychological Association's (APA) Ethical Principles of Psychologists and Code of Conduct, and the Good Clinical Practice Directive (2005/28/CE) of the European Union. The sample was collected in accordance with the 1975 Helsinki Declaration and its subsequent revisions. After reading the information file, each mother signed the consent form for their offspring to participate in the study.

2.2. Instruments

2.2.1. Sociodemographic and daily routine questionnaire

Caregivers completed a sociodemographic and daily routine questionnaire specifically designed for this study. The sociodemographic section included the child's sex, age, and school starting age, as well as parental educational attainment (primary; secondary; university) and family socioeconomic status (monthly household income: < €1000; €1000–2000; €2000–3000; €3000–4000; > €4000). The daily routine section consisted of both closed- and open-ended questions. Closed-ended items (yes/no) assessed medication intake in the last 48 h, recent routine changes within the last 24 h, substantial behavioral changes within the last 24 h, stressful events in the last 24 h, napping before the session, and physical activity prior to evaluation. For each affirmative response, an open-ended follow-up item allowed caregivers to provide further details. Affirmative responses were recorded and included as confounders for subsequent analyses.

2.2.2. Stress reactivity measurement

Stress reactivity was assessed non-invasively using two salivary biomarkers: alpha-amylase and cortisol. Six saliva samples were collected from each participant using the Salimetrics SalivaBio Infant Swab, designed for children aged 6 months to 6 years. The cotton swab was placed in the child's mouth for 60–90 s, then stored in a Salimetrics Storage Tube and frozen at −20ºC. Samples were thawed and centrifuged at 1500 rpm for 15 min, yielding a low-viscosity supernatant for analysis. Alpha-amylase activity was measured with the Salimetrics® α-Amylase Kinetic Enzyme Assay Kit, and cortisol levels with a salivary ELISA kit (ALPCO Diagnostics). Both assays followed manufacturer instructions. According to the manufacturers, intra- and inter-assay coefficients of variation (CVs) ranged between 5.0–9.4 % and 3.8–8.1 %, respectively, for cortisol, and between 3–7 % and 4–6 %, respectively, for alpha-amylase.

Saliva samples were collected in the afternoon, beginning within 30 min of arrival at the laboratory and concluding approximately 1 h and 40 min later.

2.2.3. Observational subjective stress and activity scales

Both caregivers and the experimenter completed brief subjective stress and activity rating scales before, during, and after the stress task. The stress scale was adapted from Send et al. (2019), and the activity scale from Kryski et al. (2011). These single-item measures served as global behavioral indicators of the child's state. Notably, the stress scale assesses subjective and observable signs of psychological stress, defined as the observable degree of emotional distress or discomfort, reflected in indicators such as physical tension, snorting, or nervousness. It was rated on a 4-point scale (1 = very slightly stressed, 2 = slightly stressed, 3 = stressed, 4 = very stressed). Activity was defined as the child's level of motor activation, ranging from low to high activity. It was rated on a 3-point scale (1 = low activity: stays mostly seated and plays quietly, 2 = medium activity: stands up and moves around frequently, 3 = high activity: attempts to stand and play actively most of the time).

2.2.4. Emotion coding

The stress task was videotaped for subsequent coding of emotions. Anger, sadness, nervousness, neutral emotions, and happiness were scored on a 4-point scale (0 = not at all, 1 = slight, 2 = moderate, 3 = strong), so that higher values reflected stronger expressions. Coding was conducted in 30-second intervals, yielding between 7 and 28 observations per child depending on task duration. Two psychologists were trained in emotion recognition using the NimStim set of facial expressions (Tottenham et al., 2009). Only images depicting sadness, happiness, anger, and neutral/calm expressions were included. They were introduced to the defining characteristics of each emotion, supported by the classic facial expression features described by Ekman and Friesen (1971), and then practiced independently—receiving feedback until reaching ≥ 90 % accuracy in three consecutive sets of 40 images, with Interclass Correlation Coefficients (ICC) ≥ .80. Then, they then independently coded the videos. Anger, sadness, and happiness were identified using Ekman and Friesen's (1971) facial expressions. Neutral emotion was defined as the absence of emotional cues; nervousness was indicated by behaviors such as hand-shaking, hesitation, frequent glances at the traffic light, behavioral activation, and snorting. Interrater reliability was assessed on the total scores of each emotion (anger, sadness, nervousness, neutral, and happiness) independently coded by the two raters. ICCs were calculated in SPSS using a two-way mixed model with absolute agreement. The ICCs for average measures ranged from.79 to.82 (95 % CI), indicating good interrater reliability. For subsequent analyses, the mean scores of both raters were used.

2.2.5. The laboratory visit with the standard stress paradigm

The Stress Reactivity Task for Preschoolers (SRTP) protocol presented in this study was based on the recommendations of Gunnar et al. (2009), Kryski et al. (2011), Tolep and Dougherty (2014), Roos et al. (2017), and Send et al. (2019) for reliably eliciting and measuring a stress response in preschoolers.

As illustrated in Fig. 1, the protocol began with 30 min of "quiet play" to allow for laboratory acclimatization. Various materials (e.g., toys, puzzles, books, drawings) were available during this period. Saliva sample 1 (S1) was then collected, followed by the stress-inducing task. After completing the task, sample 2 (S2) was collected, and a 40-minute

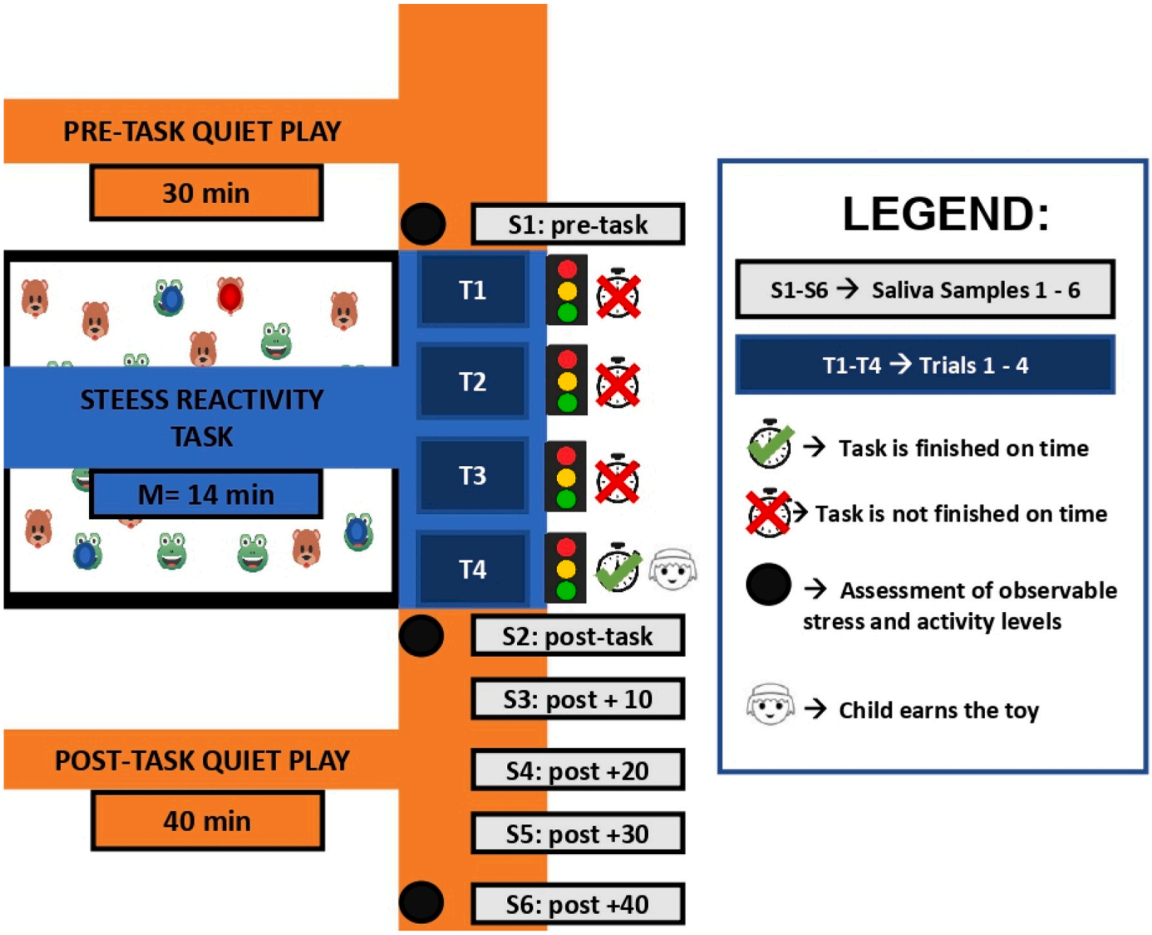

Fig. 1. SRTP Protocol Design.

post-task quiet play period began. During this time, four additional saliva samples were collected within 10-minute intervals (S3–S6). Caregivers were allowed to interact with their children during quiet play to promote a calm and natural environment, but were instructed to avoid high-activity play and any behaviors that could provoke anger or frustration in the child, including scolding or reminders of embarrassing situations. S1 served as a baseline measure, capturing pre-task levels of alpha-amylase (which reflects within 5–10 min) and cortisol (typically reflecting 10–20 min prior) (Gordis et al., 2006; Kryski et al., 2011; Roos et al., 2017; Send et al., 2019; Spinrad et al., 2009).

The stress reactivity task represents the stress-inducing stimulus of the protocol. It is a matching task where preschoolers must sort a total of 40 blue and red magnets with pictures of frogs and bears, respectively, within a time limit indicated by a traffic light. A preferred toy was awarded only if the task was completed on time. However, the traffic light was covertly manipulated so that preschoolers could not finish the task on time until Trial 4, when the traffic light was revealed to be "broken" and replaced with a "new and properly working" one. In Trials 1–3 (the unfair part of the task), the traffic light lasted for a maximum of 1 min 30 sec (1 min of green light and 30 sec of yellow light), which was the maximum time available for active sorting. However, the total duration of each trial could extend to approximately 3 min due to feedback, corresponding verbal instructions (see Supplementary Material), and the time required to collect the magnets. In Trial 4, the traffic light had no maximum duration and remained on until the child finished sorting and earned the toy.

Throughout the task, the experimenter maintained a flat affect and provided negative feedback for failures. The preschooler and the experimenter sat facing each other across a table. On the table, a horizontal magnetic board displaying pictures of frogs and bears was placed in front of the child. To the left of the board was a box containing the magnets, and to the right a screen displayed the traffic light with both image and sound. Children faced the board at a comfortable distance, ensured by an adjustable-height chair, so that all items and the traffic-light timer were simultaneously visible within the child's field of view. The caregiver was seated behind the child, outside the child's field of vision, instructed to appear occupied with questionnaires and to refrain from interacting with the child during the stress task. If a child refused to continue the task after any trial, the examiner proceeded directly to the "broken traffic light" theater. In this way, each child completed the task independently and earned the toy.

Additionally, the examiner recorded the timings of sample collection, number of sorting mistakes, the presence of crying (yes/no), and any changes in strategy (yes/no). A strategy change was defined as any shift from an identifiable sorting approach to a different one, whether sustained or momentary. Task duration was measured from the start of Trial 1 to the end of the final trial.

2.3. Procedure

The stress paradigm was conducted in a laboratory setting at the Mind, Brain and Behaviour Center (CIMCYC) in Granada, Spain. The laboratory task took place between 4:00 and 8:00 p.m., corresponding to the period of greatest cortisol stability in Spain (Ruiz et al., 2010), allowing for two evaluation shifts given children's school schedules and aligning with later metabolic cycles in Spain compared to other European countries. Sessions were scheduled either at 4:00 or 6:00 p.m. In the Spanish context, where children typically engage in afternoon activities and maintain later bedtimes, evening sessions were not perceived by parents as a disruption to the children's daily routine. Additionally, providing two time slots enhanced participation feasibility by allowing families to select the schedule most compatible with their daily commitments. Comparisons between the 4:00 and 6:00 p.m. shifts were performed to examine potential differences. All assessments were conducted by the same trained evaluator, who was responsible for administering the task, collecting the saliva samples, and overseeing the entire procedure to ensure consistency. Children were accompanied by their primary caregiver, who remained present throughout the procedure.

Prior to scheduling, mothers were asked about their child's medication intake. To control for potential biomarker confounders, children were instructed to refrain from eating, drinking (except water), or exercising for at least 90 min before and during the assessment. Upon arrival, caregivers read the information sheet, signed informed consent, and completed the sociodemographic and daily routine questionnaire. Informed consent was obtained by the same trained evaluator, who then conducted the entire SRTP protocol.

2.4. Statistical analysis

Descriptive statistics included means (M) and standard deviations (SD) for continuous variables and relative frequencies for categorical variables. Stress reactivity was assessed using the Area Under the Curve with respect to ground (AUCg) and increase (AUCi) for both biomarkers. AUCg estimated total secretion across the six saliva samples, while AUCi reflected change from baseline to final sample (de Veld et al., 2014). Children were classified as 'responders' to the stress task when their peak biomarker value exceeded baseline levels by at least twice the average intra- and inter-assay coefficients of variation (Send et al., 2019; Tolep and Dougherty, 2014), corresponding to an increase of 19 % for Cortisol Responders (CR) and 14 % for Alpha-Amylase Responders (AAR).

To evaluate the protocol's effectiveness in eliciting a biological stress response, repeated-measures General Linear Models (GLMs; equivalent to repeated-measures ANOVAs) were computed across the six saliva samples, complemented by paired *t*-tests comparing all time points. Because these exploratory comparisons involved multiple tests, the full set of p-values from the original analyses was corrected using Bonferroni. GLM and paired *t*-tests were also performed on observational activity, subjective stress, and emotion scores.

Potential confounders for alpha-amylase and cortisol responses included: sex, age, starting school age, parental educational attainment, socioeconomic status, medication intake, routine changes, behavioral changes, prior stressful events, previous napping, prior physical activity, crying, strategy changes, number of mistakes committed in the matching task, 4 or 6 p.m. shift, season (Winter / Autumn; Spring / Summer), number of trials, task duration and emotion scores.

GLMs were used to examine inter-subject confounder effects on biomarker trajectories, while Welch's F tests and Spearman correlations assessed confounder influences on baseline and AUC scores. We also tested correlations between individual saliva samples and AUCs. Sex differences were evaluated in emotion scores and saliva samples with Welch's F tests, and sex-specific Spearman correlations were conducted between AUC and emotion scores. Finally, Welch's F tests were applied to analyze differences in the number of trials completed according to responder status.

As expected, Kolmogorov-Smirnov test indicated non-normal distribution of the salivary cortisol and alpha-amylase data, which were log-transformed for statistical analysis. Untransformed values are, however, reported for descriptive purposes. Outliers exceeding three SDs were winsorized (Field, 2024). Statistical analyses were conducted using SPSS 23.0 (IBM Corp., Armonk, N.Y., USA).

Sensitivity power analyses were conducted using G*Power (α =.05, 1–β =.80). For repeated-measures GLMs, the design was sensitive to effects of $f \geq .217$ ($\eta^2p \geq .045$) for within-subject cortisol (N = 57) and $f \geq .234$ ($\eta^2p \geq .052$) for alpha-amylase (N = 49), and to group-by-time interactions of $f \geq .138$ ($\eta^2p \geq .019$) and $f \geq .149$ ($\eta^2p \geq .022$), respectively. For *t*-tests, the minimum detectable effects were $dz \geq 0.378$ (cortisol) and $dz \geq 0.409$ (alpha-amylase) for paired comparisons, and $d \geq 0.756$ (cortisol), $d \geq 0.822$ (alpha-amylase), and $d \geq 0.750$ (emotions) for independent comparisons (including Welch's F). For correlations, sensitivity was $r \geq .361$ (cortisol) and $r \geq .388$ (alpha-amylase); when split by sex, $r \geq .508$ (cortisol) and $r \geq .556$ (alpha-amylase) in boys, and $r \geq .485$ (cortisol) and $r \geq .500$ (alpha-amylase) in girls.

3. Results

3.1. Sample characteristics

Fifty-eight children and their primary caregivers participated in the study. Eleven saliva samples were identified as outliers and winsorized (five alpha-amylase, six cortisol). Due to insufficient sample volume, alpha-amylase data from nine children were unavailable (analyzable N = 49; 27 girls, 22 boys). In addition, one mother reported corticosteroid use after the visit; her child's cortisol data were excluded (analyzable N = 57; 30 girls, 27 boys). Of the other 5 children who reported medication intake, three had taken paracetamol, and two multivitamin supplements. Participants were homogeneous in terms of medium-to-high socioeconomic status and high parental educational attainment, with a narrow age range (see Table 1).

3.2. Effectiveness of the SRTP

Results of the stress reactivity task are presented in Table 2, whereas descriptive statistics, effect sizes, and *t*-test outcomes for SRTP variables are reported in Table 3. The mean alpha-amylase AUCg was 17,465.80 (SD = 2682.93), with a mean AUCi of 282.59 (SD = 1574.21). For cortisol, the mean AUCg was 13,992.25 (SD = 2148.60), and the mean AUCi was 175.47 (SD = 1412.18).

Regarding the SNS, 77.55 % of children were classified as AARs. GLM revealed a significant main effect of time course, $F(3.468, 166.469) = 3.539$, $p = .012$, $\eta^2p = .069$, and a significant quadratic trend, $F(1.000, 48.000) = 7.791$, $p = .008$, $\eta^2p = .140$ (Fig. 2). Exploratory paired *t*-tests showed an uncorrected significant increase in alpha-amylase from S1 to S2 ($t(48) = -2.911$, $p = .005$, dz = -.416), followed by several uncorrected decreases across later comparisons, including S2→S6 ($t(48) = 2.996$, $p = .004$, dz = .428), S3→S6 ($t(48) = 2.431$, $p = .019$, dz = .347), S4→S6 ($t(48) = 2.139$, $p = .038$, dz = .306), and S5→S6 ($t(48) = 2.308$, $p = .025$, dz = .330). None of these differences remained significant after applying Bonferroni correction (all p-values ≥.06). The increase from S1 to S2 ($p = .075$) and the decrease from S2 to S6 ($p = .60$) showed marginal significance. All uncorrected and corrected comparisons are presented in the Supplementary Material. Most AAR peaked between 0 and 10 min post-task (68.4 %), and the average percent increase in alpha-amylase from baseline to peak was 83.3 %.

As for the HPA axis, 64.92 % of children were classified as CRs. GLM did not reveal a significant main effect of time course, $F(3.468, 194.191) = 1.740$, $p = .152$, $\eta^2p = .030$. However, polynomial contrasts indicated a significant quadratic trend, $F(1.000, 56.000) = 4.817$, $p = .032$, $\eta^2p = .079$ (Fig. 3). Exploratory paired *t*-tests identified one uncorrected significant increase in cortisol from S2 to S3 ($t(56) = -2.382$, $p = .021$, dz = -.316), and one uncorrected decrease at S5→S6 ($t(56) = 2.012$, $p = .049$, dz = .266). However, none of these differences remained significant after applying Bonferroni correction (all p-values ≥.315). All uncorrected and corrected comparisons are provided in the Supplementary Material. Most CR peaked at either 10–20 min (45.9 %) or 40 min (29.7 %) post-task, and the average percent increase in cortisol from baseline to peak was 62.68 %.

Noteworthy, no significant correlations were observed between

Table 1
Sociodemographic and relevant for stress reactivity variables.

	Total sample (n = 58)	Alpha-amylase sample (n = 49)	Cortisol sample (n = 57)
Variable	**Total number and % / M (SD)**		
Age (in months)	53.71 (4.95) Minimum age = 48 Maximum age = 64	53.61 (5.12) Minimum age = 48 Maximum age = 64	53.75 (4.98) Minimum age = 48 Maximum age = 64
Sex			
Male	27 (46.6 %)	22 (44.9 %)	27 (47.4 %)
Female	31 (53.4 %)	27 (55.1 %)	30 (52.6 %)
Starting school age (in months)	19.18 (11.21)	19.37 (11.31)	19.20 (11.31)
Mother's educational attainment			
Primary education	2 (3.4 %)	1 (2 %)	2 (3.5 %)
Secondary education	14 (24.2 %)	13 (26.5 %)	14 (24.6 %)
University education	42 (72.4 %)	35 (71.4 %)	41 (72 %)
Father's educational	3 (5.2 %)	1 (2 %)	
attainment	28 (48.3 %)	26 (53.1 %)	3 (5.2 %)
Primary education	27 (46.6 %)	22 (44.9 %)	28 (49.2 %)
Secondary education			26 (45.6 %)
University education			
Socioeconomic status			
< 1000€	0 (0 %)	0 (0 %)	0 (0 %)
1000€ - 2000€	12 (20.7 %)	9 (18.3 %)	12 (21.1 %)
2000€ - 3000€	24 (41.4 %)	21 (42.9 %)	25 (43.8 %)
3000€ - 4000€	15 (26 %)	13 (26.5 %)	15 (26.3 %)
> 4000€	7 (12.1 %)	6 (12.3 %)	5 (8.7 %)
Medication intake			
Yes	6 (10.3 %)	5 (10.2 %)	5 (8.8 %)
No	52 (89.7 %)	44 (89.8 %)	52 (91.2 %)
Routine changes			
Yes	3 (5.2 %)	2 (4.1 %)	3 (5.3 %)
No	55 (94.8 %)	47 (95.9 %)	54 (94.7 %)
Substantial behavioral changes			
Yes	3 (5.2 %)	1 (2 %)	3 (5.3 %)
No	55 (94.8 %)	48 (98 %)	54 (94.7 %)
Stressful events that day			
Yes	6 (10.3 %)	6 (12.2 %)	6 (10.5 %)
No	52 (89.7 %)	43 (87.8 %)	51 (89.5 %)
Previous napping			
Yes	16 (27.6 %)	15 (30.6 %)	15 (26.3 %)
No	42 (72.4 %)	34 (69.4 %)	42 (73.7 %)
Shift			
4 pm	30 (51.7 %)	24 (49 %)	29 (50.9 %)
6 pm	28 (48.3 %)	25 (51 %)	28 (49.1 %)
Season			
Winter / Autumn	33 (56.9 %)	25 (51 %)	33 (57.9 %)
Spring / Summer	25 (43.1 %)	24 (49 %)	24 (42.1 %)

alpha-amylase and cortisol values (r ranging from –.156–.222, p = .125–.996), nor between their respective AUC scores. Specifically, cortisol AUCg was not associated with alpha-amylase AUCg (r = .037, p = .798) or AUCi (r = .052, p = .721), and cortisol AUCi showed no correlation with alpha-amylase AUCg (r = –.022, p = .879) or AUCi (r = .086, p = .558).

Experimenter observational ratings showed a significant time effect for both activity, F(1.985, 37.716) = 3.714, p = .034, η^2p = .164, and subjective stress, F(1.683, 31.976) = 20.960, p < .001, η^2p = .525. While caregiver ratings revealed no time effect for activity, F(1.511, 30.223) = 3.333, p = .061, η^2p = .143, but a significant effect for subjective stress, F(1.596, 31.922) = 6.040, p = .009, η^2p = .232.

GLM analyses revealed a significant effect of time for all emotion scores: anger, F(1.875, 76.874) = 8.130, p = .001, η^2p = .165; sadness, F(2.704, 110.870) = 57.469, p < .001, η^2p = .584; nervousness, F (2.439, 100.001) = 29.324, p < .001, η^2p = .417; neutral emotions, F (1.577, 64.653) = 3.886, p = .035, η^2p = .087; and happiness, F(1.127, 46.215) = 4.203, p = .042, η^2p = .093

Table 2
Stress reactivity task results.

Variable	Total sample (n = 58)		Alpha-amylase sample (n = 49)		Cortisol sample (n = 57)	
	% / M (SD)					
	4 p.m. shift	6 p.m. shift	4 p.m. shift	6 p.m. shift	4 p.m. shift	6 p.m. shift
Time of S1 (hour: min)	16:47 (0:28)	18:38 (0:16)	16:38 (0:18)	18:37 (0:11)	16:47 (0:28)	18:38 (0:16)
Time of S2 (hour: min)	17:08 (0:28)	18:59 (0:16)	17:00 (0:18)	18:58 (0:10)	17:09 (0:28)	18:59 (0:16)
Time of S3 (hour: min)	17:21 (0:28)	19:11 (0:17)	17:13 (0:18)	19:10 (0:10)	17:21 (0:28)	19:11 (0:17)
Time of S4 (hour: min)	17:33 (0:28)	19:24 (0:17)	17:25 (0:18)	19:23 (0:10)	17:33 (0:29)	19:24 (0:17)
Time of S5 (hour: min)	17:45 (0:28)	19:36 (0:17)	17:37 (0:18)	19:35 (0:10)	17:45 (0:28)	19:36 (0:17)
Time of S6 (hour: min)	17:57 (0:28)	19:48 (0:17)	17:49 (0:18)	19:47 (0:10)	17:57 (0:28)	19:48 (0:17)
Number of trials completed						
2	4 (6.9 %)		4 (8.2 %)		4 (7 %)	
3	12 (20.7 %)		10 (20.4 %)		11 (19.3 %)	
4	42 (72.4 %)		35 (71.4 %)		42 (73.7 %)	
Crying during the task						
Yes	13 (22.4 %)		10 (20.4 %)		12 (21.1 %)	
No	45 (77.6 %)		39 (79.6 %)		45 (78.9 %)	
Change in strategy						
Yes	28 (48.3 %)		27 (55.1 %)		27 (47.4 %)	
No	30 (51.7 %)		22 (44.9 %)		30 (52.6 %)	
Number of sorting mistakes	6.57 (4.66)		6.30 (4.56)		6.58 (4.71)	
Duration of stress reactivity task (min)	14.08 (2.11)		14.24 (1.85)		14.05 (2.11)	

T-test for all observational measures can be consulted in Table 3.

3.3. Sex differences

A GLM revealed a significant interaction between sex and cortisol secretion, F(3.345, 183.998) = 2.555, p = .045, η^2p = .044, with a more pronounced quadratic trend in females, F(1, 55) = 10.517, p = .002, η^2p = .161 (Fig. 4). No significant sex interactions were found for alpha-amylase secretion, F(3.454, 162.317) = 0.776, p = .525, η^2p = .016. Furthermore, no sex differences emerged in individual saliva samples or AUC scores for either biomarker (Table 4). Complementing these findings, descriptive inspection indicated that response rates did not differ substantially between sexes (CR: boys = 66.67 %, girls = 63.34 %; AAR: boys = 77.27 %, girls = 80.76 %). However, among responders, peak cortisol timing differed: most male CRs peaked between 30–40 min post-stressor (50 %), whereas most female CRs peaked between 10–20 min (57.9 %). Taken together, the significant quadratic trend and the descriptive pattern suggest that females exhibited a faster and more pronounced cortisol response, followed by earlier recovery.

Pertaining to sex differences in emotions, females displayed significantly more sadness throughout the stress task compared to males, Welch's F(1, 55.979) = 6.860, p = .011, d = 0.682, η^2 = .107 (Table 4), and cried more frequently than boys (25.8 % vs. 18.5 %). Spearman correlations also revealed sex-specific associations between emotions and biomarker reactivity. Among boys, greater anger was linked to lower alpha-amylase AUCi (*r* = −.652; *p* = .001), and greater happiness was associated with lower AUCg (*r* = −.470; *p* = .027) and AUCi (*r* = −.491; *p* = .020). Among girls, higher sadness predicted greater cortisol AUCi (*r* = .516; *p* = .004), while greater neutral affect was

Table 3
Descriptive, effect sizes and *t*-test results from SRTP variables.

Biomarkers' descriptives and *t*-test from consecutive samples (n = 49 for alpha-amylase; n = 57 for cortisol)

	S1	S2	*t*; *p*	S3	*t*; *p*	S4	*t*; *p*	S5	*t*; *p*	S6	*t*; *p*	Effect Size S1-S2	Effect Size S2-S3	Effect Size S3-S4	Effect Size S4-S5	Effect Size S5-S6
Alpha-amylase	74.49 ± 55.61	93.05 ± 65.37	–2.911;.005	86.07 ± 64.47	1.457;.152	81.59 ± 61.39	0.764;.448	79.53 ± 55.09	0.087;.931	70.88 ± 50.14	2.333;.024	–0.416	0.206	0.108	0.012	0.330
Cortisol	30.01 ± 16.03	30.10 ± 16.62	0.366;.716	35.40 ± 21.64	–2.382;.021	34.70 ± 22.38	0.713;.479	34.86 ± 22.85	–1.149;.252	32.98 ± 21.22	2.012;.049	0.049	–0.316	0.094	–0.152	0.267

Observational subjective stress and activity descriptives and *t*-test (n = 58)

	M1 (Pre-task)	**M2 (Task)**	***t* ; *p***	**M3 (Post-task)**	***t* ; *p***	**Effect Size M1-M2**	**Effect Size M2-M3**
Activity exp.	1.48 ± 0.77	1.96 ± 0.86	–3.570;.001	2.05 ± 0.75	0.000; 1.000	–0.473	0.000
Activity careg.	1.33 ± 0.543	1.63 ± 0.69	–3.478;.001	1.71 ± 0.64	–0.237;.815	–0.461	–0.052
Stress exp.	1.28 ± 0.55	2.98 ± 0.90	–12.668;.000	1.65 ± 0.67	3.979;.001	–1.663	0.890
Stress careg.	1.29 ± 0.49	2.32 ± 0.85	–8.629;.000	1.67 ± 0.79	1.000;.329	–1.143	0.218

Emotions during the task descriptives and *t*-test (n = 58)

	T1	**T2**	***t* ; *p***	**T3**	***t* ; *p***	**T4**	***t* ; *p***	**Effect Size T1-T2**	**Effect Size T2-T3**	**Effect Size T3-T4**
Anger	0.61 ± 1.16	1.85 ± 2.09	–5.585;.000	1.94 ± 1.89	–3.330;.002	2.06 ± 3.20	1.326;.192	–0.746	–0.514	0.205
Sadness	2.12 ± 1.83	4.24 ± 2.50	–7.013;.000	4.86 ± 2.42	–6.339;.000	2.62 ± 3.87	11.154;.000	–0.937	–0.978	1.721
Nervous-ness	3.73 ± 2.53	6.75 ± 3.02	–8.205;.000	7.78 ± 3.24	–3.553;.001	7.28 ± 3.33	0.761;.451	–1.096	–0.548	0.117
Neutral emotions	13.89 ± 2.74	11.46 ± 2.88	6.998;.000	12.98 ± 2.71	–2.615;.012	12.25 ± 4.83	–0.916;.365	0.935	–0.404	–0.141
Happiness	1.35 ± 2.49	0.70 ± 1.52	3.275;.002	1.44 ± 5.57	–0.888;.380	2.78 ± 6.98	–4.340;.000	0.438	–0.137	–0.670

Note: Effect Size = dz; exp. = experimenter; careg. = caregiver. Untransformed alpha-amylase (U/ml) and cortisol (ng/ml) values are shown for descriptive purposes. Emotions during the task mean scores between raters are shown. Means and standard deviations (Mean ± SD) are reported for each sAA and cortisol sample (S1-S6), as well as for observational stress and activity scales (M1-M3), and emotions ratings (T1-T4). Table also shows paired-samples *t*-tests conducted between consecutive time points for biomarkers (before Bonferroni correction), activity and stress scales, and emotions.

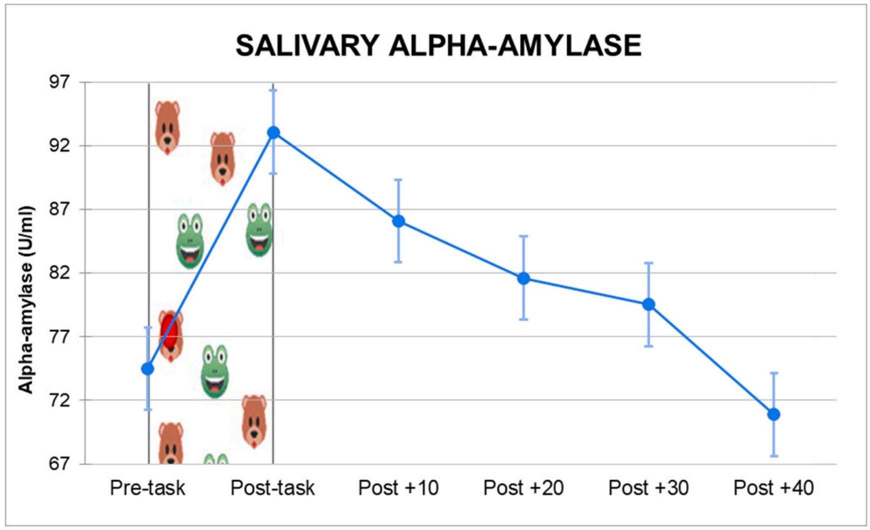

Fig. 2. *SNS reactivity pattern. Note*: untransformed alpha-amylase values with standard error are shown (U/ml); n = 49.

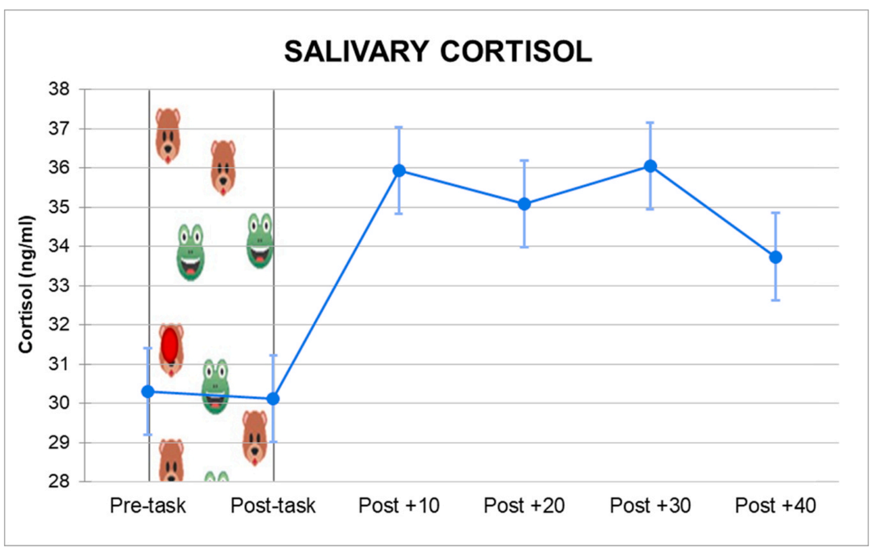

Fig. 3. *HPA reactivity pattern. Note*: untransformed salivary cortisol values with standard error are shown (ng/ml); n = 57.

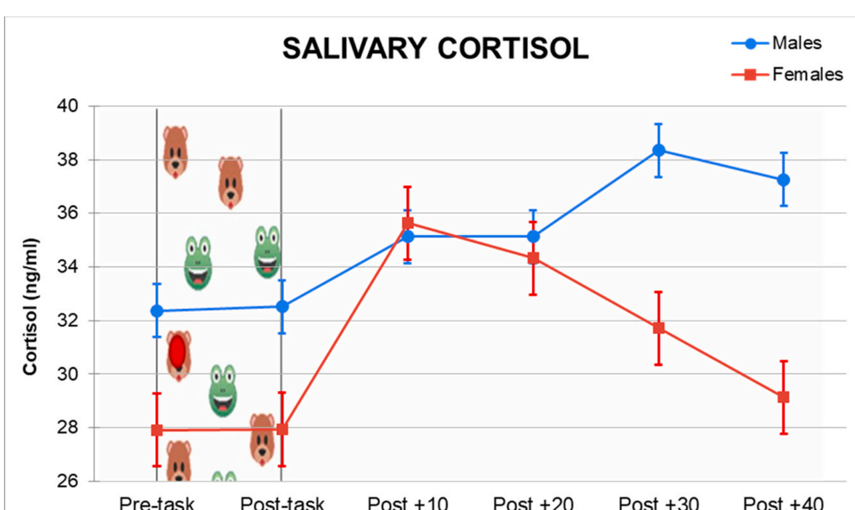

Fig. 4. *Sex differences in cortisol reactivity patterns. Note*: untransformed salivary cortisol values with standard error are shown (ng/ml); n = 57.

Table 4
Sex differences.

Alpha-amylase data	Boys (n = 22) M (SD)	Girls (n = 28) M (SD)	Welch's F (gl1, gl2)	p	d
Alpha-Amylase S1	73.226 (50.984)	75.530 (60.072)	F(1, 43.368) = 0.051	.822	–0.07
Alpha-Amylase S2	85.547 (58.650)	98.957 (70.703)	F(1, 43.000) = 0.623	.434	–0.23
Alpha-Amylase S3	79.245 (66.752)	91.443 (63.328)	F(1, 37.340) = 1.698	.201	–0.39
Alpha-Amylase S4	79.597 (65.472)	83.171 (59.170)	F(1, 42.034) = 0.244	.624	–0.14
Alpha-Amylase S5	75.223 (58.590)	82.918 (53.020)	F(1, 44.825) = 0.356	.554	–0.17
Alpha-Amylase S6	68.218 (49.720)	72.978 (51.290)	F(1, 47.875) = 0.001	.979	0.01
Alpha-Amylase AUCg	17,339.209 (2896.440)	17,565.268 (2552.427)	F(1, 42.220) = 0.083	.774	–0.01
Alpha-Amylase AUCi	122.864 (1774.526)	408.096 (1418.127)	F(1, 39.607) = 0.378	.542	–0.18
Cortisol data	**Boys (n = 27) M (SD)**	**Girls (n = 30) M (SD)**	**Welch's F (gl1, gl2)**	**p**	**d**
Cortisol S1	32.361 (18.889)	27.902 (12.914)	F(1, 52.252) = 0.765	.386	0.23
Cortisol S2	32.522 (19.129)	27.937 (13.971)	F(1, 52.658) = 0.693	.409	0.22
Cortisol S3	35.137 (25.933)	35.639 (17.376)	F(1, 51.978) = 0.342	.561	0.16
Cortisol S4	35.137 (25.380)	34.320 (19.749)	F(1, 53.794) = 0.009	.926	0.02
Cortisol S5	38.355 (27.608)	31.715 (17.436)	F(1, 51.094) = 0.503	.481	0.19
Cortisol S6	37.264 (22.929)	29.138 (19.134)	F(1, 54.926) = 2.426	.125	0.34
Cortisol AUCg	14,154.433 (2254.505)	13,846.290 (2076.331)	F(1, 53.103) = 0.286	.595	0.01
Cortisol AUCi	83.500 (1319.095)	258.250 (1508.651)	F(1, 54.960) = 0.218	.643	–0.12
Emotions data	**Boys (n = 27) M (SD)**	**Girls (n = 31) M (SD)**	**Welch's F (gl1, gl2)**	**p**	**d**
Anger	5.167 (3.932)	5.919 (5.901)	F(1, 56.610) = 0.334	.566	–0.15
Sadness	9.685 (5.621)	13.887 (6.596)	F(1, 55.979) = 6.860	**.011***	–0.68
Nervousness	24.574 (9.995)	20.710 (7.986)	F(1, 49.650) = 2.594	.114	0.43
Neutral Emotions	48.574 (12.591)	44.000 (15.395)	F(1, 55.799) = 1.548	.219	0.32
Happiness	4.926 (5.390)	3.064 (3.180)	F(1, 40.897) = 2.471	.124	0.43

Note: * significance for $p < .05$

associated with lower cortisol AUCi ($r = -.496$; $p = .005$).

3.4. *Other confounders' influence on stress reactivity*

GLMs with inter-subject factors revealed that both crying and overall happiness during the task significantly influenced biomarker responses. Crying was significantly associated with the cortisol trajectory, $F(3.582, 196.999) = 3.404$, $p = .013$, $\eta^2p = .058$, showing both linear, $F(1, 56) = 4.947$, $p = .030$, $\eta^2p = .081$, and quadratic trends, $F(1, 56) = 6.847$, $p = .011$, $\eta^2p = .111$ (Fig. 5). This pattern indicates that children who cried exhibited a more reactive cortisol profile, with a steeper post-stressor increase peaking at + 20 min and a more pronounced curvature (sharper rise followed by steeper recovery) compared to non-crying children. In contrast, overall happiness exhibited a significant interaction with alpha-amylase secretion, $F(3.689, 173.366) = 2.914$, $p = .026$, $\eta^2p = .058$, following a linear trend, $F(1, 56) = 6.477$, $p = .014$, $\eta^2p = .121$, such that higher levels of happiness were associated with lower alpha-amylase responses.

Children who cried during the task displayed higher cortisol AUCi, Welch's $F(1, 17.104) = 8.181$, $p = .011$, $\eta^2 = .132$. The number of trials completed was not associated with responder status, as indicated by non-significant results for both CRs, Welch's $F(1, 44.369) = 0.603$, $p = .432$, $d = 0.021$, and AARs, Welch's $F(1, 20.502) = 0.346$, $p = .563$, $d = 0.018$.

Season of assessment (autumn/winter versus spring/summer) did not significantly affect cortisol or alpha-amylase indices. For cortisol, Welch's test showed no significant differences in AUCg, $F(1, 54.840) = 3.767$, $p = .057$, although this effect was marginal and reflected a moderate effect size ($d = 0.50$). No differences emerged for AUCi, $F(1, 49.058) = 0.014$, $p = .908$, $d = 0.35$. The same pattern was observed for alpha-amylase: AUCg showed no seasonal variation, $F(1, 46.386) = 0.872$, $p = .355$, $d = –0.20$, and neither did AUCi, $F(1, 45.422) = 0.025$, $p = .876$, $d = –0.01$.

Previous napping showed no significant effect on cortisol secretion, $F(3.451, 189.825) = 0.837$, $p = .489$, $\eta^2p = .015$, or alpha-amylase secretion, $F(3.449, 162.090) = 0.493$, $p = .714$, $\eta^2p = .010$. Similarly, no differences emerged in cortisol AUCg, Welch's $F(1, 36.466) = 1.075$, $p = .307$, or cortisol AUCi, Welch's $F(1, 22.000) = 0.979$, $p = .333$. The same was true for alpha-amylase AUCg, Welch's $F(1, 25.455) = 0.003$, $p = .956$, and alpha-amylase AUCi, Welch's $F(1, 27.363) = 1.443$, $p = .240$.

Medication intake showed no significant effect on cortisol secretion, $F(3.438, 189.095) = 1.035$, $p = .384$, $\eta^2p = .018$, or alpha-amylase secretion, $F(3.463, 162.742) = 0.370$, $p = .802$, $\eta^2p = .008$. Similarly, no differences emerged in cortisol AUCg, Welch's $F(1, 4.669) = 0.001$, $p = .974$, or cortisol AUCi, Welch's $F(1, 5.734) = 2.413$, $p = .174$. The same was true for alpha-amylase AUCg, Welch's $F(1, 4.489) = 0.480$, $p = 523$, and alpha-amylase AUCi, Welch's $F(1, 4.411) = 0.000$, $p = .999$.

Routine changes showed no significant effect on cortisol secretion, $F(3.460, 190.317) = 0.317$, $p = .840$, $\eta^2p = .006$, or alpha-amylase secretion, $F(3.461, 162.690) = 1.028$, $p = .388$, $\eta^2p = .021$. Similarly, no differences emerged in cortisol AUCg, Welch's $F(1, 2.892) = 0.000$, $p = .995$, or cortisol AUCi, Welch's $F(1, 2.238) = 0.912$, $p = .431$. The same was true for alpha-amylase AUCg, Welch's $F(1, 2.135) = 0.005$, $p = .948$, and alpha-amylase AUCi, Welch's $F(1, 2.270) = 2.358$, $p = .250$.

Behavioral changes showed no significant effect on cortisol secretion, $F(3.452, 189.850) = 0.323$, $p = .836$, $\eta^2p = .006$, or on alpha-amylase secretion, $F(3.456, 162.418) = 0.458$, $p = .739$, $\eta^2p = .010$. In contrast, a significant effect emerged for cortisol AUCg, Welch's $F(1, 2.773) = 50.139$, $p = .007$, $d = .305$, whereas no significant effect was found for cortisol AUCi, Welch's $F(1, 4.654) = 2.484$, $p = .180$. For alpha-amylase AUCg and AUCi, there were not enough data to conduct the analyses.

Prior stressful events showed no significant effect on cortisol

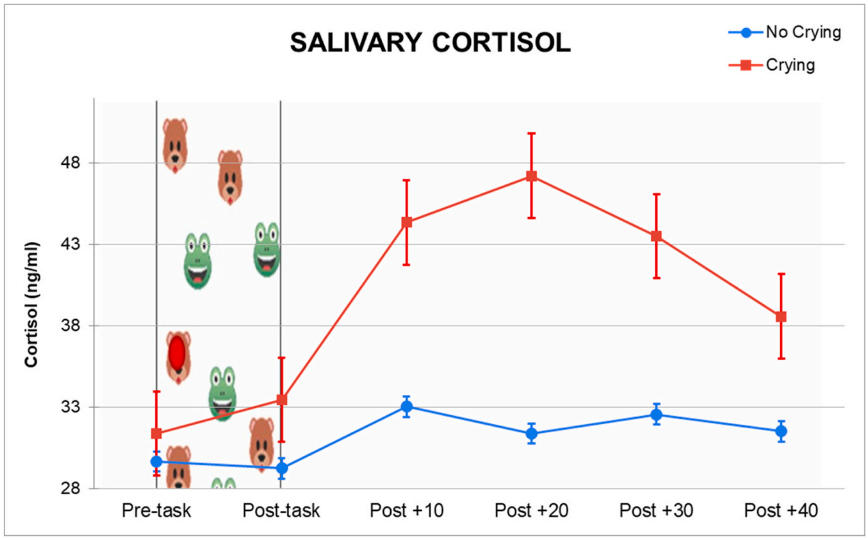

Fig. 5. *Stress reactivity and crying condition. Note*: untransformed salivary cortisol values with standard error are shown (ng/ml); n = 57.

secretion, F(3.470, 190.874) = 0.912, p = .447, η^2p = .016, or alpha-amylase secretion, F(3.436, 161.485) = 0.609, p = .632, η^2p = .013. Similarly, no differences emerged in cortisol AUCg, Welch's F(1, 6.068) = 0.972, p = .362, or cortisol AUCi, Welch's F(1, 5.899) = 1.802, p = .229. The same was true for alpha-amylase AUCg, Welch's F(1, 6.426) = 0.311, p = .596, and alpha-amylase AUCi, Welch's F(1, 6.226) = 0.945, p = .367.

Prior physical activity showed no significant effect on cortisol secretion, F(3.574, 196.570) = 2.062, p = .095, η^2p = .036, or alpha-amylase secretion, F(3.369, 158.333) = 1.470, p = .221, η^2p = .030. Similarly, no differences emerged in cortisol AUCg, Welch's F(1, 5.507) = 0.262, p = .629, or cortisol AUCi, Welch's F(1, 4.543) = 1.822, p = .240. The same was true for alpha-amylase AUCg, Welch's F(1, 5.601) = 3.484, p = .115, and alpha-amylase AUCi, Welch's F(1, 4.772) = 3.380, p = .128.

Strategy changes showed no significant effect on cortisol secretion, F (3.387, 176.125) = 0.544, p = .674, η^2p = .010, or alpha-amylase secretion, F(3.505, 161.233) = 0.966, p = .420, η^2p = .021. Similarly, no differences emerged in cortisol AUCg, Welch's F(1, 51.577) = 0.012, p = .911, or cortisol AUCi, Welch's F(1, 51.992) = 0.040, p = .842. The same was true for alpha-amylase AUCg, Welch's F(1, 46.772) = 1.600, p = .212, and alpha-amylase AUCi, Welch's F(1, 46.751) = 0.303, p = .585.

Finally, the shift (4 p.m. vs. 6 p.m.) showed no significant effect on cortisol secretion, F(3.436, 188.979) = 0.634, p = .615, η^2p = .011, or alpha-amylase secretion, F(3.476, 163.369) = 1.443, p = .227, η^2p = .030. Similarly, no differences emerged in cortisol AUCg, Welch's F(1, 53.474) = 0.455, p = .503, or cortisol AUCi, Welch's F(1, 51.844) = 0.865, p = .357. The same was true for alpha-amylase AUCg, Welch's F(1, 47.751) = 1.292, p = .261, and alpha-amylase AUCi, Welch's F(1, 47.209) = 0.602, p = .442.

Finally, Spearman correlations showed influence in the AUC scores for several confounders: age, starting school age, socioeconomic status, task duration, total anger, total neutral emotions. Correlations are presented in Table 5.

Table 5
Confounders' correlations.

	n = 49		n = 57	
	Alpha-amylase AUCg	Alpha-amylase AUCi	Cortisol AUCg	Cortisol AUCi
Age	-.089	.107	**.348****	**.264****
Starting school age	.015	.062	**.286***	.027
Mother's educational attainment	-.093	.114	.003	-.056
Father's educational attainment	-.013	-.044	-.113	.175
Socioeconomic status	.335	**.441***	.270	.272
Number of mistakes committed in the matching task	-.044	.256	-.117	.053
Number of trials	-.013	.266	-.003	-.178
Task duration	.023	.020	-.097	**-.264***
Anger during the task	.128	**-.285***	.234	.048
Sadness during the task	.171	.073	.132	.192
Nervousness during the task	.163	.103	.109	.025
Neutral emotions during the task	-.056	.144	**-.266***	**-.286***
Happiness during the task	**-.309***	-.140	.113	-.022

Note: Spearman's *r* values are presented in the table.
* = $p < .05$;
** = $p < .001$

4. Discussion

The main aim of the present study was to confirm that the SRTP elicits a stress response in Spanish preschoolers. We hypothesized activation of both the SNS and HPA axis, with earlier and stronger SNS activity, and associations between stress reactivity and confounders including napping, crying, season, sex, parental education, and socioeconomic status. As expected, the SRTP triggered stress responses in both systems, as reflected by significant quadratic secretion trends and high responder rates, with alpha-amylase peaking earlier and more strongly than cortisol. Several hypothesized influences were also supported: girls showed a faster cortisol rise and recovery than boys, and crying during the task was strongly associated with cortisol reactivity. In contrast to our predictions, socioeconomic status was positively—rather than negatively—associated with alpha-amylase reactivity. Likewise, napping, season and parental education were not related to biomarker reactivity. However, findings related to napping and season should be interpreted with caution due to low statistical power.

To further elaborate on the SNS response, sAA showed the expected quadratic pattern, rising significantly from pre- to post-task and returning to baseline by 40 min. This study is the first to confirm this pattern in preschoolers using a stress-inducing task.

Regarding the HPA axis response, a significant quadratic trend showed that cortisol rose between 0 and 10 min post-task, remained stable until 30 min, and then started to decrease. This pattern aligns with Kryski et al. (2011) and Roos et al. (2017), but contrasts with Send et al. (2019), who reported an atypical response with a delayed peak (30–40 min) and no subsequent decline.

Responder criteria for both biomarkers were based on previous pediatric cortisol studies, defining responders as participants whose peak values exceeded baseline by more than twice the mean assay variability (Send et al., 2019; Tolep and Dougherty, 2014). In our sample, the AAR criterion aligned with the thresholds typically used in adult research (Becker and Rohleder, 2020; O'Donohue et al., 2024) and resulted in a slightly higher proportion of responders than previously reported (Becker and Rohleder, 2020). In contrast, both the cortisol increase criteria and the proportion of cortisol responders were consistent with those observed in earlier preschool studies (de Weerth et al., 2013; Send et al., 2019; Tolep and Dougherty, 2014).

Furthermore, alpha-amylase peaked earlier and more sharply than cortisol, consistent with findings in older children and adolescents (Allwood et al., 2011; Gordis et al., 2006) and with the faster SNS activation relative to the HPA axis. Although no relation was found between the salivary alpha-amylase and cortisol samples, nor between their AUC scores—suggesting SNS–HPA asymmetry (Gordis et al., 2008)—, this absence should be interpreted cautiously given the limited power to detect small-to-medium associations.

Beyond physiological indices, the protocol effectively elicited observable signs of activation, subjective stress and negative emotions in preschoolers, complementing the biological findings. Consistent with Spinrad et al. (2009), negative emotions (anger, sadness, nervousness) increased during the unfair phase, while neutral and positive affect decreased, confirming its frustrating nature.

A key contribution of this study is the analysis of sex differences in stress reactivity. Although AUC and individual sample analyses showed no significant effects, limited power may have masked small group differences. Still, the cortisol secretion curve revealed a faster rise and decline in girls than in boys (peaking around 20 min earlier), which suggests potential sex-related variations in stress regulation dynamics even in the absence of significant mean-level effects. This secretion pattern contrasts with Kryski et al. (2011), who reported no sex interactions, but aligns with Send et al. (2019), who observed a marginally stronger response in girls. Likewise, studies focusing on specific biomarkers or summary indices found no significant sex differences (Allwood et al., 2011; de Weerth et al., 2013; Kryski et al., 2011), while meta-analytic evidence indicated that samples with more girls show greater cortisol reactivity, although this effect did not emerge when gender composition was tested categorically (i.e., male-only, mixed, female-only) (Seel et al., 2025). Together, these findings highlight the need to examine sex-specific secretion patterns beyond isolated samples or aggregate indices such as AUC.

Psychological factors may partly explain these sex differences. Cortisol reactivity in preschoolers is especially sensitive to motivated performance under social-evaluative threat and linked to self-conscious emotions such as shame, embarrassment, and heightened negative affect (Dickerson and Kemeny, 2004; Lewis and Ramsay, 2002; Tolep and Dougherty, 2014). Accordingly, girls in our study showed more sadness and cried more often, suggesting that their emotional and cognitive appraisal may have accelerated the physiological response, leading to a faster rise and recovery. This pattern is noteworthy, as elevated cortisol reactivity in girls has been associated with heightened risk for internalizing problems—difficulties that tend to be reported more frequently in preschool-aged girls than in boys (Spinrad et al., 2009).

Sex differences also emerged in emotional responses and their links to biomarker reactivity. Girls showed more sadness, and both greater sadness and lower neutral emotions were associated with higher cortisol reactivity. In boys, anger and happiness were associated with lower alpha-amylase reactivity. This pattern contrasts with studies reporting no sex differences or greater negative emotions in boys (Kryski et al., 2011; Spinrad et al., 2009; Tolep and Dougherty, 2014), which may help explain why Kryski et al. (2011) found no sex effects in cortisol patterns. These findings suggest that emotional expression is differentially linked to physiological responses in boys and girls.

The protocol's ability to detect sex differences has important implications. Because physiological sensitivity to stress emerges early, identifying sex-specific reactivity patterns is key to understanding emotional and behavioral dysregulation (Allwood et al., 2011). Such patterns may help explain the higher prevalence of anxiety and depression among females (Koszycki et al., 2019; McLean et al., 2011), in whom sex-related differences in cortisol reactivity have been observed (Zorn et al., 2017). These results highlight the need for sex-specific risk models, as boys and girls may respond to stress through distinct physiological and behavioral mechanisms (Spinrad et al., 2009).

Regarding SNS confounders, anger and happiness were unexpectedly associated with lower alpha-amylase reactivity, contrasting with Spinrad et al. (2009). This pattern may reflect reduced task engagement—angry children being absorbed by negative affect and happy children less focused on performance. Additionally, higher socioeconomic status was associated with higher alpha-amylase reactivity, possibly reflecting stronger parental expectations and performance-related pressure.

Regarding HPA confounders, the GLM showed that children who cried during the task had greater cortisol reactivity. On one hand, crying may reflect low frustration tolerance, often observed in children with less developed emotion regulation skills or limited experience with similar task demands (Knaus, 2006). On the other hand, it could represent an early coping strategy to elicit caregiver support or reduce task demands. In both scenarios, such responses carry a physiological cost. Other associations between cortisol reactivity and less-neutral emotions, shorter task duration, or older age lacked sufficient power and should be interpreted cautiously. Still, they suggest that positive emotions may buffer cortisol responses (Kryski et al., 2011; Tolep and Dougherty, 2014), longer duration may reflect reduced motivation or engagement (Kryski et al., 2011), and older children may be more aware of the task's social-evaluative nature.

The main limitation of this study concerns statistical power, especially for some non-significant confounders. Power did not reach the conventional 80 % threshold for detecting small-to-medium effects or medium-to-large between-group differences, indicating that some nonsignificant results—and specific significant findings mentioned above—should be interpreted with caution. In particular, several confounding variables showed very low frequencies, further reducing the ability to detect small-to-moderate effects: routine changes, behavioral changes, prior stressful events, medication intake, napping, sorting mistakes, and number of trials completed. These variables should not be ruled out as potential confounders for sAA and cortisol; rather, their analyses should be considered exploratory, indicating only that medium-to-large effects were not detected in the present study, while smaller effects may remain undetected due to limited power.

Beyond statistical power, additional limitations should be acknowledged. First, extending saliva sampling beyond 40 min post-stressor would better capture cortisol recovery, particularly in boys. The 40-minute limit reflected a balance between scientific aims and feasibility, as frequent early sampling was needed to track rapid alpha-amylase and initial cortisol responses while ensuring children's tolerance, limited laboratory time, and financial constraints. Future studies should extend sampling to 60 min to more accurately characterize recovery. Second, the 30-minute acclimatization period may have been shorter than ideal; however, prior participation in the cohort likely reduced anticipatory stress. Third, the absence of a control condition limits the ability to separate task-induced from context-related stress. Additionally, fear was not coded among observed emotions, which could have provided additional insight into children's stress reactions. Moreover, children's wake times were not assessed, which limits the interpretation of diurnal variation in salivary biomarkers. Finally, we note that the presence of the caregiver may have attenuated children's stress responses, and that future research should explicitly assess relationship quality and consider including conditions with and without caregiver presence to better understand its impact on HPA and SNS activity

This study also presents several strengths. Frequent sampling of both SNS and HPA activity enabled precise mapping of secretion patterns, and the analysis of confounders highlighted several meaningful influences—namely sex, crying, and socioeconomic status.

5. Conclusions

Our findings confirm that the SRTP protocol is an effective laboratory paradigm for assessing stress reactivity in Spanish preschoolers at both the SNS and HPA axis levels, while also providing valuable insight into confounding variables that may influence these responses in early childhood.

Using this protocol may help identify children at greater risk for psychopathology and inform personalized preventive interventions. Although cognitive factors were not directly assessed in this study, we sought to minimize their potential influence through the sample's developmental homogeneity, narrow age range, and socioeconomic context. Nevertheless, cognitive factors may still affect stress reactivity, and future research should explore how preschoolers' SNS and HPA responses relate to cognitive development, behavioral problems, and perinatal stress, and further investigate cross-system asymmetry—an index that has been associated with behavioral difficulties and exposure to chronic stress or maltreatment (Allwood et al., 2011; Gordis et al., 2008).

Ethical information

The study was approved by the Human Studies Ethics Committee of the University of Granada (Spain; 968/CEIH/2019) and conducted in accordance with the Ethical Principles of Psychologists and Code of Conduct of the American Psychological Association (APA), as well as the European Union's Good Clinical Practice Directive (Directive 2005/28/EC). Sample collection followed the 1975 Helsinki Declaration and its subsequent revisions. After reading the information sheet, each mother signed a consent form authorizing their child's participation.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work the author(s) used ChatGPT in order to enhance the clarity and quality of the English language. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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CRediT authorship contribution statement

María Isabel Peralta-Ramírez: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Raquel González-Pérez:** Supervision, Resources, Formal analysis. **Javier De Echarri-Lorente:** Investigation, Formal analysis, Data curation. **Ahmed F. Fasfous:** Writing – review & editing, Supervision, Investigation. **Miguel Ángel Baos-González:** Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Carolina Mariño-Narváez:** Visualization, Supervision, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2025.107720.

Data availability

Anonymous data is available upon request to the first author: mbaos@ugr.es

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