Autonomic and Adrenocortical Reactivity and Buccal Cell Telomere Length in Kindergarten Children

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Objective: To examine associations between autonomic nervous system and adrenocortical reactivity to laboratory stressors and buccal cell telomere length (BTL) in children. Methods: The study sample comprised 78 children, aged 5 to 6 years, from a longitudinal cohort study of kindergarten social hierarchies, biologic responses to adversity, and child health. Buccal cell samples and reactivity measures were collected in the spring of the kindergarten year. BTL was measured by real-time polymerase chain reaction, as the telomere-to-single-copy gene ratio. Parents provided demographic information; parents and teachers reported children’s internalizing and externalizing behavior problems. Components of children’s autonomic (heart rate, respiratory sinus arrhythmia [RSA], and pre-ejection period [PEP]) and adrenocortical (salivary cortisol) responses were monitored during standardized laboratory challenges. We examined relationships between reactivity, internalizing and externalizing behaviors, and BTL, adjusted for age, race, and sex. Results: Heart rate and cortisol reactivity were inversely related to BTL, PEP was positively related to BTL, and RSA was unrelated to BTL. Internalizing behaviors were also inversely related to BTL (standardized β = −0.33, p = .004). Split at the median of reactivity parameters, children with high sympathetic activation (decreasing PEP), and parasympathetic withdrawal (decreasing RSA) did not differ with regard to BTL. However, children with both this profile and high cortisol reactivity (n = 12) had significantly shorter BTL (0.80 versus 1.00; χ² = 7.6, p = .006), compared with other children. Conclusions: The combination of autonomic and adrenocortical reactivity was associated with shorter BTL in children. These data suggest that psychophysiological processes may influence, and that BTL may be a useful marker of, early biologic aging. Key words: autonomic reactivity, adrenocortical reactivity, buccal cell telomeres, internalizing, stress, children.

INTRODUCTION

Telomeres are the protective ends of chromosomal deoxyribonucleic acid (DNA); short length leads to cell senescence. Blood cell telomere length (TL) has frequently been used to measure biologic aging and may underlie cardiovascular aging. In adulthood, chronic stress has been associated with shorter TL (1), and shorter telomeres have been associated with heart disease (2,3) and more rapid cardiovascular disease mortality (4,5). TL reflects genetic, cellular, and environmental factors and is thought to decline throughout life (6); longer telomeres may confer a survival advantage (7,8).

As a generalized measure of biologic aging, telomeres could be helpful in establishing the early impact of risk factors for health outcomes, including heart disease. In fact, differences in TL among individuals may materialize early in life. Previous researchers have demonstrated that TL shortens rapidly during early childhood, with significant shortening by the age of 4 years, concurrent with children's rapid growth (9,10). There are, however, obstacles in measuring telomeres in children. Collection of blood samples, which is generally acceptable in adults, is relatively invasive for children. However, although leukocyte TL is an increasingly well-established measure of cellular aging, telomeres can be measured using DNA from any cell type, and buccal epithelial (oral mucosa) cells may provide an alternative source for assessment of TL. There is evidence that the rate of attrition of telomeric base pairs differs by organ tissue (11) and that the rate of attrition of buccal cell TL (BTL) may differ from that of leukocyte TL (12). However, BTL has been used to predict health outcomes including oral, lung, and bladder cancers (13,14) and Alzheimer's disease (15). TL in other types of epithelial cells has also predicted age-related disease (16).

To establish the potential usefulness of BTL as a marker of biologic aging in children, we wanted to evaluate its possible associations with a biologically well-characterized, trait-like variable (17) with potential ramifications for aging during the life course. We used profiles of autonomic nervous system (ANS) and hypothalamic-pituitary-adrenal (HPA) axis responses to stress to accomplish this goal.

ANS and HPA axis responses to stress and adversity take place in a coordinated and temporally sequenced manner that maximizes the organism’s capacity for survival and recovery. Complementary ANS and HPA axis responses produce a shift to a state of biologic and behavioral preparedness, increasing heart rate (HR) and blood pressure, mobilizing cellular nutrients, preferentially redirecting energy resources and perfusion to the brain, and inducing behavioral states of vigilance and fear (18–22). Autonomic reactivity, occurring over seconds, uses the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS), which counts SNS activation by down-regulating activity within the...
same set of target end organs. The HPA axis, involving the sequential secretion of corticotropin-releasing and adrenocorticotropic hormones, activates the adrenal cortex, triggering secretion of cortisol, the principal human glucocorticoid, regulating blood pressure, glucose metabolism, and immune competence. The HPA axis response, occurring over minutes, also serves to temper and control sympathetic activation.

The coordinated, counterbalancing roles of the SNS, PNS, and HPA axis in the integrated stress response can be indexed using a variety of accessible and peripheral physiologic measures. SNS activation can be assessed using the cardiac cycle pre-ejection period (PEP), a measure reflecting the timed length of the period of isovolumetric contraction, whereas PNS withdrawal can be measured as a diminution in the magnitude of respiratory sinus arrhythmia (RSA), the portion of HR variability driven by the respiratory cycle. HPA axis activation can be ascertained using salivary cortisol, which reliably reflects the concentration of free cortisol in plasma. Furthermore, the concurrent, coordinated, or dysregulated activation of these systems can be described in reactivity profiles reflecting joint patterns of SNS, PNS, and HPA axis responses to a challenge (23–25). A maximally vigorous response profile might invoke the combination of high ANS reactivity (decreasing PEP by SNS activation and decreasing RSA by PNS withdrawal) and high HPA axis activation (increasing salivary cortisol).

Therefore, in this study, we examined associations between such profiles of stress reactivity and BTL. We hypothesized that children who are “most reactive” to stress, that is, those characterized by high sympathetic activation, parasympathetic withdrawal, and/or high cortisol response and especially those characterized by all three, may exhibit more accelerated aging of mitotic cells in the first few years of life and that this will be reflected in shorter BTL. In addition, because highly “stress-reactive” children tend to have more internalizing symptoms (26–30), we hypothesized that children with higher internalizing behaviors would have shorter BTL. We examined associations of externalizing behaviors with BTL to evaluate whether relationships were specific to internalizing versus any behavioral problems.

MATERIALS AND METHODS

Study Population

The study sample included 78 kindergarten children in the San Francisco Bay Area who were recruited from a longitudinal study of social dominance status, biologic responses to adversity, and mental and physical health (Peers and Wellness Study [PAWS]). These children came from a single cohort of 141 children entering kindergarten in 2005, that is, the third of three cohorts of kindergarten children participating in the study (n = 338). Other details of data collection have previously been reported (31).

The final study sample (n = 78) consisted of children who had parental written permission to participate; who provided buccal cell samples; who had complete information on ethnicity, age, sex, and reactivity measures; and who were not taking human growth hormone or exogenous glucocorticoids, medications known to alter salivary cortisol levels (32). Because our hypothesis about temperament and BTL was subsidiary to the primary hypothesis, we did not require complete data on internalizing or externalizing behavior, and these analyses included 73 children.

The sample was ethnically diverse (13% African American, 15% Asian, 6% Hispanic, 45% white, and 21% multiracial or other) but limited to children who spoke English and whose parents spoke English or Spanish. School districts were chosen to ensure students were from a wide socioeconomic range. However, despite variation in income, parents in PAWS were more educated than the general population; more than 50% of mothers were college graduates compared with less than 30% in the general population (33).

Data Collection

Data for the study were collected in the fall (Time 1) and spring (Time 2) during the kindergarten year. Data on age and body mass index (BMI [kg/m2]) were collected in the fall, with information on height and weight collected by nurse practitioners. Data on reactivity, internalizing symptoms, externalizing symptoms, and BTL were collected in the spring. Parents’ informed consents and children’s assents were obtained before the start of data collection, and all study procedures were approved by the Committee for the Protection of Human Subjects at the University of California, Berkeley, and the Committee on Human Research at the University of California, San Francisco.

Telomere Length

We measured TL in buccal (cheek) cells that were collected specifically for this study. Buccal cell samples were collected by a nurse practitioner at the end of the children’s kindergarten year by brushing the oral mucosa, using 10 strokes with a sterile nylon bristle cytology brush. A separate brush was used for each cheek to maximize the amount of DNA obtained. Samples were processed and DNA purified using the Puregene DNA Isolation Kit (Gentra Systems, Inc, now known as Qiagen, Gaithersburg, MD) for analyzing DNA from buccal cells (34).

TL values were measured from DNA by a quantitative polymerase chain reaction assay that determines the ratio of telomere repeat copy number to single-copy gene copy number (T/S) in experimental samples as compared with a reference DNA sample. Higher T/S ratio signifies longer telomeres (35). We repeated the assays for five samples and found a coefficient of variation of 9.1%. BTL assays were conducted blinded to other participant information.

Internalizing and Externalizing Symptoms

Parents and teachers completed the internalizing and externalizing scales from the MacArthur Health and Behavior Questionnaire (27,36). The internalizing behavior problems scale assesses children’s symptoms of depression and anxiety. The externalizing behavior problems scale reflects children’s aggressive or impulsive behaviors. Respondents were asked to report on the child’s behavior within the past 6 months, based on a three-point Likert scale ranging from “never or not true” to “often or very true.” Higher scores on both forms indicate higher symptoms. The Berkeley Puppet Interview is a semistructured interview measure of young children’s perceptions of their family environment, school context, relationships with teachers, social skills and behaviors, and internalizing and externalizing symptoms (J. C. Ablow, unpublished instrument, 1993) (37). The scores from the three reporters were combined using principal components analysis (38), yielding a “core” score that reflected concurrent impressions on internalizing and externalizing symptoms provided by the teachers, parents, and children.

Reactivity Protocol

A 20-minute reactivity protocol was completed by the children in a separate, quiet room at their elementary school. A child completed four sets of paired tasks (one control condition and one challenge condition each). The challenge tasks were designed to elicit autonomic responses to challenges across social (39), cognitive (40), sensory (41), and emotional (42) domains, geared for 4- to 6-year-old children (43,44). The protocol included four tasks: a) a structured child interview taken from the Gesell School Readiness Test (39) and administered by an unknown examiner, b) a digit span recitation task from the Kaufman Assessment Battery for Children (40), c) a drop of lemon juice placed on the child’s tongue, and d) an emotion-evoking videotape, chosen to elicit fear (45). Roughly half of the entire cohort showed a decrease in PEP or RSA score for each task. Because the challenges involved some degree of potentially confounding psychomotor activity, each challenge task was preceded by a nonchallenging “control task” that paralleled the psychomotor demands of that challenge task (31). Before and after the set of four challenges, children were...
read a 2-minute calming story to procure resting physiologic measures. Other details have previously been reported (31).

**ANS Reactivity**

Children's ANS reactivity was assessed using changes in RSA, PEP, and HR reactivity in response to the series of challenges. Four spot electrodes (two current and two impedance electrodes) were placed in the standard tetrapolar configuration on the child's neck and chest, and electrocardiograph (ECG) electrodes were placed on the right clavicle and lower left rib. ANS measures (HR, RSA, and PEP) were monitored continuously during the protocol. Data were acquired using the Biopac MP150 (Biopac Systems, Santa Barbara, CA) interfaced to a PC-based computer. RSA refers to fluctuations in HR related to the respiratory cycle and gated by efferent fibers of the vagus nerve. RSA is an index of the PNS's capacity to regulate responses to positive and negative environmental demands (46,47). RSA was estimated as the natural logarithm of the variance of the heart period within the frequency bandpass associated with respiration at this age (i.e., 0,15–0,80 Hz) (48,49). PEP time intervals were calculated based on the time in milliseconds from the ECG Q wave (corresponding to the onset of ventricular depolarization) to the B point of the dZ/dt waveform (corresponding to the onset of left ventricular ejection (50)). HR is an integrative, multiply determined physiologic parameter under the influence of autonomic and other biologic factors. HR was ascertained from interbeat interval data acquired using an ECG digitized at 500 Hz, analyzed with the detection algorithm of Berntson and colleagues (51). Other details have previously been reported (31,43,44,52).

ANS reactivity responses during each of the control tasks were used as baseline reference values. Mean RSA, PEP, and HR magnitudes for each of four tasks were calculated for 1-minute intervals and averaged within each task (52). The four difference scores were then averaged within each measure of ANS reactivity. Reactivity scores were computed as ANS activity during the challenge task minus that during the paired control task. Negative RSA and PEP difference scores reflect autonomic reactivity via PNS withdrawal and SNS activation, respectively. HR difference scores measure HR acceleration and provide an integrated measure of sympathetic and parasympathetic reactivity. Henceforth, the abbreviations RSA, PEP, and HR signify reactivity or change.

**Adrenocortical (HPA Axis) Reactivity**

At the beginning (baseline) and end of the reactivity protocol, saliva was collected by instructing the child to chew on a cotton roll for 20 to 30 seconds. Wet cotton rolls were deposited in salivette tubes and stored at −7°C until assayed. Salivary cortisol samples were assayed using a commercial immunoassay with chemiluminescence detection (Cortisol Luminescence Immunoassay; IBL, Hamburg, Germany). Other details have previously been reported (53). Given that cortisol levels in saliva reach their peak approximately 15 to 20 minutes after stressor onset, cortisol values collected at the beginning of the session were considered baseline reference values. The average session lasted 27 minutes; cortisol values collected at the end of the session were considered a measure of reactivity to a novel, mildly stressful context. Slightly more than one third of the cohort showed an increase in cortisol. We created standardized difference scores by subtracting the preprotocol baseline values from postprotocol cortisol values. Positive cortisol reactivity values indicated a stress response.

**Data Preparation**

Reactivity scores were standardized to a mean of 0 and a standard deviation of 1. We subsequently created autonomic reactivity profiles (54,55) from the cross-classification of positive (>0) and negative (≤0) difference scores for PEP and RSA. Children in the four cross-tabular cells were classified as showing coactivation, coinhibition, reciprocal parasympathetic activation, or reciprocal sympathetic activation (24,56). Theoretically, the most reactive children, with regard to ANS response, should be those characterized by reciprocal sympathetic activation (sympathetic activation accompanied by parasympathetic withdrawal).

However, because a maximally reactive response to stress should provoke both ANS and adrenocortical responses, we additionally computed a reactivity profile based on the cross-classification of reciprocal sympathetic activation (PEP ≤0 and RSA ≤0, yes or no) and high versus low (standardized cortisol reactivity score, >0 versus ≤0) cortisol response (Fig. 1).

**Statistical Analyses**

We used ttests to compare the children in the telomeres subsample with the entire cohort. We evaluated the distribution of BTL with univariate analyses (SAS PROC UNIVARIATE, SAS version 9.2, Cary, NC, SAS Institute Inc., 2008). We computed the Pearson correlation between age and BTL. Using analysis of covariance, we regressed potentially confounding variables against categories of reactivity adjusted for continuous age (Table 1).

**Analyses of Subcomponents of ANS and HPA Axis Stress Reactivity**

We evaluated possible outliers. The cortisol reactivity measure was left-skewed because of two influential outliers for linear cortisol reactivity and BTL. We evaluated linear associations with and without these points. Through histograms and univariate analyses, we verified that other telomere and reactivity measures were approximately normally distributed.

We regressed standardized BTL scores separately against continuous HR, PEP, RSA, and cortisol difference scores, adjusted for age (continuous), race, and sex. To overcome issues with influential outliers for cortisol, we regressed BTL against a dichotomous cortisol variable, defined as higher versus lower than median cortisol difference scores.

**Analyses of High Stress-Reactive Profiles**

Using generalized linear models (SAS PROC GENMOD), we regressed standardized BTL values against linear reactivity variables and then categories of autonomic reactivity profiles based on information on RSA and PEP, with children characterized by reciprocal parasympathetic activation or as the “least reactive” (i.e., low sympathetic response and high, unchanging parasympathetic tone) as the reference. Because of the nonnormal nature of categorical reactivity variables, we used contrast statements to compare children who were defined to be the most reactive to other groups of children and evaluated significance with Wald χ² tests.

We additionally evaluated the combination of ANS and adrenocortical reactivity by regressing BTL against the cross-classification of high and low cortisol reactivity and high and low ANS reactivity (high reactivity defined here as the presence of reciprocal sympathetic activation; Fig. 1). The reference group was composed of children with low cortisol responses who were not characterized by reciprocal sympathetic activation.

**Analyses of Internalizing and Externalizing Symptoms**

We regressed standardized BTL score against continuous internalizing and externalizing symptoms scores, adjusted for age (continuous), race, and sex.

**Approach to Covariate Adjustment**

We sought to ensure parsimonious models and to avoid inappropriate adjustment. All models were adjusted for demographic characteristics including...
age, race (White [reference], African American, Hispanic, Asian, and other), and sex. Additional adjustment for maternal education had little notable influence on associations, so reported findings did not include it.

We considered models adjusted for BMI. However, adjustment for BMI would be inappropriate if genes for reactivity and body mass are autosomal linked or alternatively, if they are pleiotropic traits. Kagan and Snidman (17) reported that reactive children tended to have characteristics including blue eyes, light hair, and thinner faces and bodies. In our data, reactivity was inversely associated with BMI, consistent with the work of Kagan and Snidman (17), suggesting that adjustment may inappropriately constrain variability in reactivity variables of interest. Although we report BMI-adjusted findings, primary models were not adjusted for BMI.

All analyses were conducted using SAS 9.2.

## RESULTS

Children in the telomere substudy (78/141) were similar to the rest of the same-grade cohort from PAWS with regard to age, sex, race, and sex. Additional adjustment for maternal education had little notable influence on associations, so reported findings did not include it.

We considered models adjusted for BMI. However, adjustment for BMI would be inappropriate if genes for reactivity and body mass are autosomally linked or alternatively, if they are pleiotropic traits. Kagan and Snidman (17) reported that reactive children tended to have characteristics including blue eyes, light hair, and thinner faces and bodies. In our data, reactivity was inversely associated with BMI, consistent with the work of Kagan and Snidman (17), suggesting that adjustment may inappropriately constrain variability in reactivity variables of interest. Although we report BMI-adjusted findings, primary models were not adjusted for BMI.

All analyses were conducted using SAS 9.2.

### TABLE 1. Selected Characteristics by Category of Reactivity Among Participants in the Peers and Wellness Study, n = 78

<table>
<thead>
<tr>
<th>ANS and Adrenocortical Reactivity Profile</th>
<th>Low Reactivity Profile</th>
<th>ANS Reactivity Without HPA Axis Reactivity</th>
<th>HPA Axis Reactivity Without ANS Reactivity</th>
<th>ANS and HPA Axis Reactivity or Most Reactive</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>38</td>
<td>14</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>Female sex, a,b, %</td>
<td>60.5</td>
<td>58.3</td>
<td>48.8</td>
<td>66.8</td>
</tr>
<tr>
<td>Ethnicity, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>44.9</td>
<td>37.9</td>
<td>40.9</td>
<td>57.6</td>
</tr>
<tr>
<td>African American</td>
<td>15.8</td>
<td>7.8</td>
<td>13.6</td>
<td>8.4</td>
</tr>
<tr>
<td>Asian</td>
<td>18.3</td>
<td>18.1</td>
<td>10.3</td>
<td>8.9</td>
</tr>
<tr>
<td>Hispanic/Latino</td>
<td>5.3</td>
<td>7.2</td>
<td>7.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Multiethnic/other</td>
<td>15.8</td>
<td>28.9</td>
<td>28.2</td>
<td>25.0</td>
</tr>
<tr>
<td>Height, cm</td>
<td>115.9</td>
<td>115.7</td>
<td>114.6</td>
<td>118.2</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>16.7</td>
<td>15.5</td>
<td>16.4</td>
<td>15.5</td>
</tr>
<tr>
<td>Internalizing score b,c</td>
<td>0.01a</td>
<td>−0.64b</td>
<td>−0.04</td>
<td>−0.00</td>
</tr>
<tr>
<td>Externalizing score b</td>
<td>−0.23</td>
<td>−0.58</td>
<td>−0.13</td>
<td>−0.13</td>
</tr>
<tr>
<td>College graduate mothers, b, %</td>
<td>71.2a</td>
<td>81.6</td>
<td>82.8</td>
<td>99.4b</td>
</tr>
<tr>
<td>Child reactivity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR score b,c</td>
<td>−0.3a</td>
<td>0.6b</td>
<td>0.4b</td>
<td>1.1b</td>
</tr>
<tr>
<td>PEP score b,c</td>
<td>0.3a</td>
<td>−0.8b</td>
<td>0.0a</td>
<td>−1.4b</td>
</tr>
<tr>
<td>RSA score b,c</td>
<td>0.2a</td>
<td>−0.3b</td>
<td>0.3a</td>
<td>−0.3b</td>
</tr>
<tr>
<td>Cortisol difference score b,c</td>
<td>−2.5a</td>
<td>−1.8a</td>
<td>1.8b</td>
<td>1.6b</td>
</tr>
</tbody>
</table>

a All variables are age adjusted.
b Different letters indicate significant (p < .05) differences between groups.
c Standardized scores.

ANS = autonomic nervous system; HPA = hypothalamic-pituitary-adrenal; HR = heart rate; PEP = preejection period; RSA = respiratory sinus arrhythmia.

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**Figure 2.** Standardized reactivity difference scores and buccal cell telomere length (BTL) in the Peers and Wellness Study, n = 78. * Heart rate reactivity was inversely related to BTL. Externalizing symptoms were not related to BTL. PEP = preejection period; T/S = telomere repeat copy number to single-copy gene copy number; ANS = autonomic nervous system; RSA = respiratory sinus arrhythmia.
and continuous reactivity scores including PEP, RSA, and cortisol difference scores. However, those in the substudy were more likely to be white (46% versus 26%, \( p < .001 \)), to have mothers who were college graduates (55% versus 32%, \( p = .008 \)), and to have higher HR reactivity scores (score, 2.6 versus 1.7; \( p = .03 \)), compared with those from the full cohort.

We collected sufficient DNA to obtain TL for each child (10 ng to 1 μg). BTL as measured by T/S ratio ranged from 0.33 to 1.59, with a mean of 0.97 (standard deviation = 0.26) and a median of 0.96. The distribution of BTL seemed to be approximately normal. Within the sample, age was weakly inversely associated with BTL (\( r = -.20, p = .08 \)).

**Analyses of Subcomponents of ANS and HPA Axis Stress Reactivity**

Using Spearman correlations, PEP reactivity was positively correlated with RSA (\( r = .29, p = .01 \)), BMI (\( r = .35, p = .004 \)), and BTL (\( r = .23, p = .04 \)) and inversely correlated with HR reactivity (\( r = -.40, p = .0003 \)). RSA reactivity was also inversely related to HR reactivity (\( r = -.48, p < .0001 \)). HR reactivity was positively related to cortisol (\( r = .27, p = .02 \)) and inversely related to BMI (\( r = -.29, p = .01 \)). Besides HR reactivity, cortisol was not significantly related to other variables.

In linear regression models, adjusted for age, sex, and race, HR reactivity was inversely related (standardized \( \beta = -0.22, p = .03 \)), PEP change was positively related (standardized \( \beta = 0.24, p = .02 \)), and RSA change was not associated (standardized \( \beta = 0.11, p = .29 \)) with BTL (Fig. 2). An inverse association of the linear cortisol reactivity score with BTL became nonsignificant when two outliers were removed. However, although children with cortisol reactivity to the stressor had significantly shorter BTL than those who were nonreactive (0.90 versus 1.01; \( \chi^2 = 4.2, p = .04 \)), exclusion of the same two children with two outlier values had no material influence on the results from analysis of the dichotomous variable.

Adjustment for BMI diminished linear associations of HR and PEP with BTL, which became nonsignificant, but did not diminish associations between dichotomous cortisol and BTL.

**Analyses of Stress Reactivity Profiles**

**Descriptive Differences Between Reactivity Profile Groups**

Of the four groups, the mothers of the least reactive children, those not characterized by either reciprocal sympathetic activation or with HPA axis reactivity, were less likely to have a college degree than the mothers of those children characterized as most reactive. Children with ANS reactivity but without HPA axis reactivity had lower internalizing scores than children characterized as having a low reactivity profile. Other than the expected differences in measures of reactivity, there were no other significant differences between groups. Differences were significant (\( p < .05 \); Table 1).

**BTL Differences Between Reactivity Profile Groups**

Reactivity categories based on the combination of PEP and RSA were not significantly associated with BTL (likelihood \( \chi^2 = 2.5, df = 3, p = .48 \)). However, children who showed simultaneous activation of systems—who were characterized by reciprocal sympathetic activation and demonstrated cortisol reactivity to the stressor—had shorter BTL than other groups (T/S ratio, 0.80 versus 1.00; \( \chi^2 = 7.5, p = .006 \); likelihood \( \chi^2 = 9.2, df = 3, p = .03 \) for group difference; Fig. 3). Adjustment for maternal education and BMI did not substantially alter these associations.

**Analyses of Internalizing and Externalizing Symptoms**

Internalizing symptoms were inversely associated (standardized \( \beta = -0.33, p = .004 \)) with BTL, but externalizing symptoms were not associated with BTL (standardized \( \beta = -0.11, p = .38 \)). Adjustment for BMI did not substantially attenuate associations between internalizing symptoms and BTL. We considered a final model adjusted both for the cross-classified measure of ANS and adrenocortical reactivity and internalizing symptoms. Simultaneous adjustment did not substantively influence either the association of reactivity or internalizing with BTL; each was independently associated with the outcome.

**DISCUSSION**

Consistent with our hypotheses, autonomic and adrenocortical reactivity in response to a series of standardized laboratory challenges was inversely associated with BTL in this sample of 78 kindergarten children. In particular, children with the combination of higher sympathetic reactivity, greater parasympathetic withdrawal, and higher cortisol response to an acute stressor had the shortest BTLs. As predicted, internalizing but not externalizing symptoms were also inversely related to BTL. These findings suggest the utility of buccal cells to assess TL in children and demonstrate that factors early in childhood may already be influencing the aging process. These findings are novel and need replication.

The findings are also consistent with a growing literature linking stress arousal in adults to shortened telomeres. Leukocyte

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**Figure 3.** Reactivity profiles and buccal cell telomere length. * Children with reciprocal sympathetic activation and high cortisol response differed from other reactivity profiles (T/S ratio, 0.80 versus 1.00; \( \chi^2 = 7.5, p = .0006 \). T/S = telomere repeat copy number to single-copy gene copy number; ANS = autonomic nervous system; HPA = hypothalamic-pituitary-adrenal.
TL has been linked to greater urinary stress hormones (cortisol and catecholamine) (57,58). Parasympathetic withdrawal to an acute stressor has been associated with low telomerase, an enzyme shown to help maintain TL (57). There are few biomarkers of health in children because the appearance of chronic disease generally occurs many decades later. Although researchers have suggested that differences in cardiovascular trajectories materialize early in the life span, with the exception of one study of cardiovascular risk factors in 6- to 7-year-old children (59), most studies of youth have examined outcomes in later childhood and adolescence. These, moreover, have primarily evaluated risk factors for disease including BMI and blood pressure, which have uncertain trajectories given the long potential horizon to the development of a chronic disease. Thus, because of methodological and technological limitations, it has been difficult to demonstrate the impact of risk factors on biologic aging early in life. If BTL measures early biologic aging or disease risk, it has great potential utility in research on human development and aging. To our knowledge, this is the first study to examine associations of childhood stress reactivity and TL.

The mechanism through which reactivity to stress might contribute to telomere attrition in children is unknown. Early in life, there is rapid shortening of leukocyte telomeres, with greater declines than in any other period of life (10). The fact that adverse events early in life are predictive of health outcomes throughout life (60) suggests that this could be a critical point in the life course during which childhood exposure to adverse stress could augment early biologic aging. However, no studies have examined whether stress or psychosocial risk factors might accelerate this early decline. In adults, chronic stress and greater perceptions of stress seem to contribute to telomere shortening (1). The mechanisms of stress-induced shortening are unknown but may involve increased exposure to stress hormones, inflammation, and oxidative stress (57,61,62). Mechanisms linking stress reactivity and TL (63) in children are likely to be similar to those in adults, although particular cardiovascular risk factors unique to early childhood such as low birth weight, “catch-up growth,” prenatal exposures, or epigenetic influences may promote telomere attrition through unique pathways not yet delineated (6).

Strengths of this study included a normative sample of 5- and 6-year-old children, reactivity measures with controls for psychomotor activity, and blinded examinations of TL in children, using noninvasive means. In addition, our method using cheek swabs rather than a mouthwash rinse, which may produce a large proportion of leukocytes (64), could have generated a larger proportion of buccal epithelial cells.

Study limitations included a relatively small sample size and a measure of TL from only one cell type (buccal cells), at only one time point. Future research will ideally enable exploration of reactivity with longitudinal change in telomeres. The children who participated in the substudy were of higher socioeconomic status (SES) than study participants generally. Furthermore, the entire PAWS sample was more educated than the general population, in keeping with the demography of the East San Francisco Bay Area. Results may thus not generalize to children of extremely low SES. Nevertheless, given the stress experienced by children from low socioeconomic environments (65), it is likely that a sample with a greater representation of children of low SES might have even larger variability in TL. Previous work suggests that children of both low SES and high SES may be more reactive than children of middle SES (20,66). Thus, it is possible that the findings in this study underestimate the association between reactivity and TL.

Methodologically, it would be preferable to be able to compare associations with both leukocyte and BTL because leukocyte DNA has been more often used in research. Indeed, Thomas and colleagues (15) found no correlation between absolute leukocyte TL and BTL, calling into question the utility of BTL. However, a more recent article by Gadalla and colleagues (67) found a high correlation between relative measures of BTL and leukocyte TL, suggesting a minimum that BTL may be a useful means of providing relative rankings for TL, if not absolute length, and as such may be useful for differentiating risk in children. Buccal cell DNA has also been successfully used to measure DNA methylation, another aging-related epigenetic modification in DNA, like BTL (M.T. Essex, W.T. Boyce and colleagues, unpublished data, 2011). Further research is needed to clarify the importance of TL in different cell types for disease prediction.

Other limitations include a lack of information on birth weight, paternal age at birth, parental health, or sibship characteristics. Most notably, paternal age at birth has been positively related to leukocyte TL in offspring (68). However, a recent study reported paternal age to be positively related to leukocyte TL in offspring (68). However, a recent study reported paternal age to be positively related to externalizing but not internalizing behaviors (69), suggesting paternal age is unlikely to explain our findings.

We noted a relatively large coefficient of variation in reliability testing of the telomere assay—up to one third of the total variation. Nonetheless, associations for internalizing symptoms, HR, PEP, and the dichotomous reactivity measure defined by ANS and cortisol response with BTL approached significance even when effect sizes were reduced by one third. The general consistency in the pattern of associations between reactivity and BTL (M.T. Essex, W.T. Boyce and colleagues, unpublished data, 2011). Further research is needed to clarify the importance of TL in different cell types for disease prediction.

To summarize, high reactivity was associated with shorter BTL in children. This study is a first step toward examining BTL in a psychosocial context and in children. Although in need of replication, the association of reactivity with BTL suggests that children with greater sensitivity to social stressors and adversities may prove more susceptible to aging-related biologic changes (60). BTL may also serve as a marker of health in studies in children, although more data are needed to establish the clinical significance of BTL in childhood and subsequently to adult health.
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