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Stress and telomere shortening among central Indian conservation refugees

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Research links psychosocial stress to premature telomere shortening and accelerated human aging; however, this association has only been demonstrated in so-called "WEIRD" societies (Western, educated, industrialized, rich, and democratic), where stress is typically lower and life expectancies longer. By contrast, we examine stress and telomere shortening in a non-Western setting among a highly stressed population with overall lower life expectancies: poor indigenous people—the Sahariya—who were displaced (between 1998 and 2002) from their ancestral homes in a central Indian wildlife sanctuary. In this setting, we examined adult populations in two representative villages, one relocated to accommodate the introduction of Asiatic lions into the sanctuary (n = 24 individuals), and the other newly isolated in the sanctuary buffer zone after their previous neighbors were moved (n = 22). Our research strategy combined physical stress measures via the salivary analytes cortisol and α-amylase with self-assessments of psychosomatic stress, ethnographic observations, and telomere length assessment [telomere–fluorescence in situ hybridization (TEL-FISH) coupled with 3D imaging of buccal cell nuclei], providing high-resolution data amenable to multilevel statistical analysis. Consistent with expectations, we found significant associations between each of our stress measures—the two salivary analytes and the psychosomatic symptom survey—and telomere length, after adjusting for relevant behavioral, health, and demographic traits. As the first study (to our knowledge) to link stress to telomere length in a non-WEIRD population, our research strengthens the case for stress-induced telomere shortening as a pancultural biomarker of compromised health and aging.

telomeres | stress | India | indigenous peoples | human displacement

Psychosocial stress is associated with elevated risk for a range of human diseases and curtailment of human life expectancy (1–16). Telomeres—repetitive and stabilizing features of chromosomal termini that cap and protect them—have also been shown to be associated with aging and disease (17–19). Telomere length erodes normally with cell division and generally with aging, triggering cellular senescence once telomere length eclipses a threshold, contributing to tissue degeneration and organ decline with longevity (20–25). Given evidence that stress can elevate the risk of human mortality, and that premature telomere shortening can serve as a proxy for increased risk of disease and mortality, it is reasonable to posit that stress is also associated with telomere shortening (23, 26, 27). Indeed, research provides evidence of telomere shortening in a range of stress-inducing life situations, including among primary caregivers of chronically ill children and Alzheimer's patients (28, 29), children spending more time in orphanages or experiencing other forms of neglect and adversity (30–32), women suffering from intimate partner violence (33), patients suffering from stress-related mood disorders (34, 35), and individuals of low socioeconomic status (SES) (36). [However, not all research demonstrates the expected associations between stress and telomere length, as in studies failing to identify links between low SES and telomere shortening (27, 37).]

Of note, studies relating stress and telomere maintenance typically unfold in what have been termed "WEIRD" societies (38, 39)—i.e., within Western, educated, industrialized, rich, and democratic populations, a small slice of total humanity—potentially limiting our understanding and generalizability of the association between biopsychosocial stressors and telomere maintenance. Populations in WEIRD societies generally enjoy higher life expectancies (40–42) and are relatively insulated from the traumas, stressors, and political coercions common throughout the developing world. Insofar as telomere length proxies for life expectancy, and that psychosocial well-being increases with economic development (43), studies involving WEIRD populations can be construed as potentially sampling high on the telomere length dimension and low on the stress dimension.

By contrast, we assessed associations between stress and telomere length in the context of tumultuous life changes experienced by an indigenous population—the Sahariya—displaced from their ancestral homes in a central Indian wildlife sanctuary. There, we examined adult heads of household in two representative villages, one relocated from their forest homes in the ecological core of the sanctuary to accommodate the future introduction of Asiatic lions into the sanctuary, and the other newly isolated in the sanctuary's buffer zone after their previous neighbors were relocated, with this second group of villagers also facing more restricted forest access in this buffer zone (44). Consistent with prior research on the lasting psychosomatic costs of

Significance

Recent research links life stress to premature telomere shortening and human aging. However, this association has only been demonstrated in Western contexts, where stress is typically lower and life expectancies longer. Using innovative approaches, we show significant associations between stress and telomere shortening in a non-Western setting among a highly stressed population with lower life expectancies: poor indigenous people—the Sahariya—who were displaced between 1998 and 2002 from their central Indian wildlife sanctuary homes. Our research strengthens the case for stress-induced telomere shortening as a pancultural biomarker of compromised health and aging.

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displacement, dispossession, and loss of homeland, a phenomenon found mainly in the developing world, our sample of villagers presented with levels of psychosomatic suffering that approximate populations receiving psychiatric care (45, 46). Developing and underdeveloped societies are estimated to host 80% of the estimated count of refugees and displaced peoples worldwide (46). Indeed, adult Sahariya villagers expressed deep uncertainty about the future, particularly with respect to the welfare of their children, and wished to return to their predisplacement lives.

Although displacement of human populations is an intrinsically compelling phenomenon, with an estimated 51.2 million internally displaced peoples worldwide (46, 47), our investigation of the relationship between stress and telomere length in a non-WEIRD setting is motivated in this context by other scientific reasons. Specifically, our population of indigenous Sahariya potentially demonstrates compromised telomere maintenance (given measurably lower life expectancies in rural parts of central India), while being higher on the stress dimension (given the high levels of reported psychosomatic suffering and disease burden), providing valuable information on a unique and understudied population, as well as advancing efforts to more fully characterize statistical associations between stress and telomere maintenance.[†]

It is also important to recognize that such real-life studies are often characterized by methodological limitations. For example, research linking life stress and telomere shortening typically rely on psychosocial distress scales or comparisons between presumed or perceived stressed and nonstressed control groups, rather than physical measures such as salivary stress analytes (like cortisol): e.g., orphans vs. nonorphans, abused vs. nonabused women, low- vs. high-SES individuals, and the mentally disordered vs. mentally healthy (19, 28, 30–35, 55). In vitro research demonstrates that the stress hormone cortisol can interfere with maintenance of telomere length by reducing telomerase activity (56). It has also been shown that oxidative stress preferentially damages telomeric DNA compared with other genomic regions, and that antioxidants can delay "replicative senescence" (57–59). However, in vivo research linking self-reported levels of experienced psychosocial stress, stress biomarkers (i.e., catecholamines and glucocorticoids), and telomere maintenance are few (60, 61), severely limiting our understanding of potential connections and hypothesized mechanisms, such as inflammation and oxidative stress (60, 62), in naturally occurring contexts. Furthermore, current population-based stress and telomere studies typically rely on quantitative real-time PCR methodology, which evaluates all of a cell's DNA to assess an average telomere length across many different cell types (19, 28, 63). Although certainly representing progress in the field, such an approach is still limited by lack of specificity, not only in regard to cell type, but also in evaluation of particular populations of telomeres (e.g., distributions of shortest and/or longest).

In contrast to previous studies, here we combined physical measures of the psychobiology of the stress response (salivary cortisol and α-amylase), collected in these Indian village contexts using minimally invasive field-appropriate techniques (64–72), with Sahariya self-assessments of psychosomatic stress, ethnographic observations, and high-resolution telomere length measurement. The value of microscopy-based techniques for evaluation of telomere length on a cell-by-cell basis has been effectively demonstrated (73–77). We developed an innovative approach for assessment of telomere length that combined telomere–fluorescence in situ hybridization (TEL-FISH) (73) using a modified protocol to collect and process the obtained samples (78), with 3D reconstruction of individual cell nuclei to facilitate analysis of all visible telomere signals in the entire extension of sampled cells, rather than just a fraction (presumably the longest) of them (75). Our approach allowed us to evaluate the length of hundreds of thousands of individual telomeres in a single class of putative buccal basal stem or progenitor cells, greatly improving the specificity and quantification of telomere length, including the important ability to define distributions of the shortest telomeres (76, 77). Such a strategy afforded us particularly high-resolution data amenable to multilevel statistical analysis.

To summarize, with innovative methodologies and strategies that mapped community and individual variation in life stress in a unique non-Western setting, our research explores the case for stress-related telomere shortening as a pancultural biomarker of compromised health and aging.

Study Setting

The Kuno-Palpur Wildlife Sanctuary (referred to here as Kuno) is a dry tropical deciduous forest reserve of 345 km^2—1,245 km^2 when Kuno's buffer zone forests are included—lying in the Sheopur District of northwestern Madhya Pradesh, India (Fig. 1). In 1981, the area was declared a state wildlife sanctuary in accordance with India's 1972 Wildlife Protection Act, but serious nature conservation efforts did not begin in Kuno until the mid-1990s, when it was designated as the proposed home for Asiatic lions to be brought from Gujarat. To relocate these lions, the Indian state decreed that human–lion cohabitation was not possible in the area comprising the core of the sanctuary. From 1998 to 2002, all 24 villages within the sanctuary's core area, totaling about 8,000 individuals, were moved to "denotified" Forest Department land (whose protected status was revoked), much of it degraded and heavily deforested, about 10 km east of Kuno's core. Every male above 18 y of age was considered the head of a separate family household and given goods and services worth 1 *lakh*, or 100,000 rupees (about US $2,100) (44). This included the following: two hectares of agricultural land, a separate plot of land for home construction, paid transport for household belongings, fodder for animals, a small cash incentive, and community facilities like wells, schools, health clinics, and paved roads. As Kuno lies in the northwest corner of India's central "tribal belt," most inhabitants are *Janjatis*, a common Hindi term for these groups, although they are also referred to as *Adivasis*—literally "first inhabitants" or "aboriginals." More specifically, most of Kuno's villages are dominated by Sahariyas, classified along with 75 other groups across 15 states as a "primitive tribe" because of their protoagricultural practices, reliance on foraging, poverty, and high illiteracy rates, as well as their geographical remoteness and vulnerability.

Kuno's relocated inhabitants' loss of homeland was accompanied by acute stress during initial relocation, followed by sustained and more chronic stress of various kinds, as Kuno's denizens adjusted to life as settled farmers in government-constructed residences in unfamiliar and more urbanized surroundings. Nonrelocated villagers remaining behind in Kuno's buffer zone did not experience the trauma of loss of homeland. However, they did experience a radical curtailment of access to core and also buffer zone natural and cultural resources, impacting their livelihoods and existence. Furthermore, they were effectively deserted by all their relocated neighbors, leaving them relatively isolated in a few scattered forest villages and vulnerable to the predations of bandits and politically powerful herding communities who visit the area, wreaking intermittent havoc on homes and crops. They also suffer other unexpected social sleights, for example, in the way

[†]Within a WEIRD context, recent studies on US ethnic and racial minorities are beginning to illuminate precisely these posited population-level relationships, demonstrating, for example, that blacks and Hispanics, as high-stress and lower-life expectancy groups, do in fact possess shorter telomeres relative to whites (48–50). However, this literature is divided, with other primarily cross-sectional research demonstrating either no racial differences or that blacks have longer telomeres compared with whites (51, 52). As a possible explanation for these contradictory findings, research suggests longitudinal differences in black compared with white telomere maintenance, with blacks' telomeres degrading at a faster rate (from a sometimes longer initial starting point), especially at older ages (48–51, 53, 54).

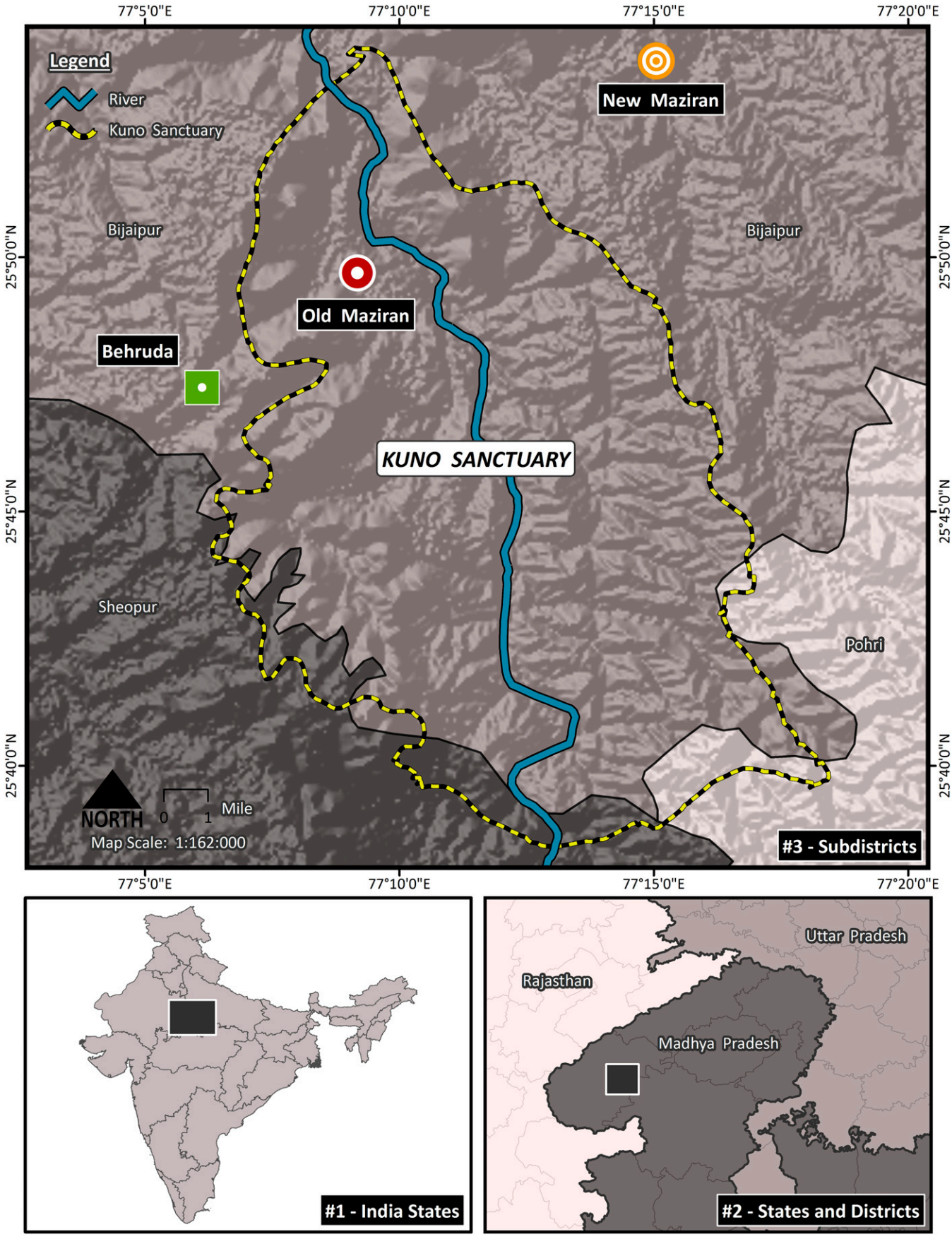

Fig. 1. Kuno-Palpur Sanctuary and village locations of Maziran (before and after relocation) and Behruda (isolated).

their kin no longer wish to intermarry with such isolated and thus "wild" people. Villagers remaining in the forest also suffer from a kind of benign neglect from the Indian state. These Sahariya were not economically compensated for their "restriction of access" to traditional resources—like lucrative grass-cutting contracts and the hunting and gathering of wild foods in Kuno's core—that technically also qualifies them as "displaced peoples" by new World Bank standards established in 2002 (79–81). Instead, their needs for water, schools, hospitals, and other state services go largely unheard, as the state focused more fully on relocated Sahariya populations. Both of these Sahariya populations—the recently relocated and also the newly isolated—then, have experienced a form of policy-generated "disaster," in the sense of collectively experienced traumatic events, which are acute and time-delimited in their initial impact, and followed by sustained and more chronic problems and stressors that compromise health and well-being (82–86).

Individual- and Village-Level Sampling

In Spring 2012, Snodgrass, Chakrapani, and their field research team collected saliva and buccal cells (cheek swabs) from 46 individuals, 24 male or female heads of household from the physically relocated village of Maziran and 22 similar individuals from the now-isolated village of Behruda. (Village names are pseudonyms.) Buccal cells were collected on a single occasion from these 46 individuals, saliva over separate 9-d periods in each village. These individuals were a randomly chosen subsample of an original Summer 2011 baseline health, well-being, and social change survey distributed to 159 Sahariya heads of households, 78 in the relocated village context and 81 in the buffer zone village. Typically, separate households were defined locally and also in our study as nuclear families composed of a married couple and their children, functioning as independent economic and social units. In Maziran, there were ∼150 such households, and 120 in Behruda. (Additional families had migrated to Maziran postdisplacement, but the predisplacement populations of these villages were nearly identical.)

Village selection was informed by ethnography conducted by Snodgrass and his field research team during the summers of 2006–2008 and 2010 in all 24 physically displaced villages, and in the three villages remaining in the buffer zone and closest to Kuno's core. The villages of Maziran and Behruda were judged to exemplify the different destinies of Kuno's "relocated" vs. "isolated" inhabitants. Also, these two villages are very similar in size, age and sex structure, economy, and ethno-religious practices. Maziran, typifying the stresses associated with physical displacement (those who were moved out of this sanctuary's core), is a government-built village of displaced Sahariyas. Behruda, a buffer zone Sahariya settlement located just outside the core of the sanctuary's core ecological zone (Fig. 1), typifies sudden isolation and restriction of traditional lifestyle. Before intervention, the two villages had particularly close (even daily) social and economic interaction, including frequent intermarriage. If not for the capricious draw of the sanctuary boundary, resulting in the removal of villagers in Maziran and the profound isolation of villagers in Behruda, the coevolution of these villages would have continued undisturbed.

Ethnographic observations and interviews were ongoing throughout all phases of the study, continuing until Spring 2013, allowing us not only to intelligently sample this non-WEIRD population but also to develop mutual trust and understanding with the indigenous Sahariya, increasing our ability to meaningfully interpret our findings. More details on the collection and measurement of our biomarkers, survey measures, and statistical procedures are provided in *Materials and Methods*.

Research Hypotheses and Analytical Plan

We assessed associations between telomere length and psychobiological stress, adjusting for known correlates of premature telomere shortening. Our stress markers included the two salivary analytes cortisol and α-amylase. Cortisol is a stress hormone of particular interest to our analysis, as it is commonly tracked in saliva as a marker of hypothalamic–pituitary–adrenal (HPA) axis activation (64, 65, 67, 69). Salivary α-amylase (sAA) is a surrogate marker for stress-related changes in the body that reflects activity and arousal of the autonomic nervous system (66, 71, 87). Analytically, these measures represent interrelated but distinct aspects of the psychobiology of stress and the stress response: i.e., the HPA axis and the sympathetic nervous system (64–66, 88). Salivary analytes such as these are increasingly used to assess psychosomatic stress in non-WEIRD contexts, thus providing both methods and interpretive frameworks for our own research (9, 16, 69, 89–95). Alongside the two physiological analytes, we also elicited responses from Sahariya to the Bradford Somatic Index (BSI) (96), a psychiatric scale validated to capture mental health disorders among South Asian populations. As mentioned, we used TEL-FISH and 3D imaging of buccal stem/progenitor cell nuclei to quantify telomere length, providing us with the ability to track the lengths of individual telomeres (73), giving us a total of 126,410 telomeres across our 46 sampled individuals.

With respect to cortisol, a healthier arousal profile is indicated by higher morning vs. evening levels of the hormone, because research has shown that a healthy condition implies a decrease in cortisol concentration over the course of the day (64, 65). For α-amylase, a healthier diurnal profile, the inverse of cortisol, is indicated by lower morning relative to evening levels of the hormone, given that research demonstrates amylase to increase over the course of the day in healthy individuals (88). Finally, a lower score on the BSI indicates less somaticized distress. With this in mind, and insofar as stress impacts telomere length, we anticipated the following: hypothesis 1: telomere length will increase with a healthier cortisol profile, that is, with a higher morning compared with evening cortisol level; hypothesis 2: telomere length will increase with a healthier sAA profile, that is, with lower morning compared with evening sAA levels; hypothesis 3: telomere length will increase with lower levels of BSI self-reported psychosomatic distress. [These hypotheses are stated with respect to the least-squares model. Insofar as stress operates uniformly across the conditional distribution of telomere length, hypotheses can be restated as the odds of a telomere exceeding a specified threshold will (*i*) increase in a healthier cortisol profile, (*ii*) increase in a healthier sAA profile, and (*iii*) decrease with higher psychosomatic distress.]

We use random-effects generalized least-squares and logistic regression procedures to test our hypotheses. Random-effects models are used when an outcome variable, in our case telomere length, is clustered in groups. If telomeres drawn from the same cluster resemble each other quantitatively (i.e., high intraclass correlation), the statistical regression assumption of independence of observations is violated. In our case, more than 120,000 telomeres are drawn from over 1,900 buccal cells taken from 46 persons that reside in one of two villages. Diagnostic analyses revealed that the likeness of telomeres drawn from the same cell is substantially greater than telomeres drawn from the same person and telomeres drawn from the same village. [For instance, in random-effects logistic regression null models—that is, models emptied of explanatory variables—estimating the likelihood of an individual telomere exceeding the 50th percentile in length, we find that the intraclass correlation within cell ($\rho = 0.217$) is more than twice the size of the intraclass correlation within person, and ∼80 times greater within village.] By incorporating a cell-specific random intercept in regression models, we statistically account for the combined effects of omitted cell characteristics that appear to influence the fluorescence of in-

dividually measured telomeres. To account for intraclass correlations within village and person, statistical models incorporate a village-level fixed effect (with 1 = villager in Maziran; 0 = villager in Behruda), and a person-level random effect captured by taking the average telomere length of a sampled adult minus the expected mean over all persons sampled. Practically, this means that our regression analyses are conducted at the level of individual telomeres—preserving length information on more than 120,000 telomeres—while statistically accounting for the nonindependence of telomeres retrieved from villages, persons, and buccal cells. [Theoretically, analyses of small endogamous populations on a substantially heritable trait like telomere length may, absent a substantial shock of stress that operates unevenly across persons and village settings, violate the nonindependence assumption in statistical regression by design.]

In addition to the above corrections for clustering, to isolate the unique relationships between stress measures and telomere shortening, our analyses control for a variety of person-level correlates likely associated with shortened telomeres, including smoking (97), diet and nutritional deficiencies (98), disease burden (malaria in this case) (99), SES (36), and demographic characteristics of respondents (17–19, 33). Our models also controlled for the count of telomeres retrieved per cell to overcome a selection bias common to cytological imaging procedures of this kind,[‡] as well as the count of cells sampled per person. More detail on measurement and statistical procedures are described in *Materials and Methods*. The results presented below describe relationships between stress measures and telomere length in our non-WEIRD population.

Results

Within- and Between-Village Descriptive Statistics on Telomere Length. Fig. S1 *A–C* display histograms of telomere fluorescence intensity (TFI), which we use as a surrogate measure of telomere length in our village settings. In Fig. S1*A*, the distribution of TFI is displayed for all persons, in Fig. S1*B*, for isolated villagers in Behruda, and in Fig. S1*C*, for relocated villagers in Maziran. TFI is mildly positively skewed across (S = 1.26) and within ($S_{Behruda}$ = 1.30; $S_{Maziran}$ = 1.23) village contexts. Villages are near identical with respect to age and sex structure. The mean age of our Behruda telomere subsample was 35.5 y (min = 19; max = 60), and the mean age in Maziran was 36.25 y (min = 20; max = 60). With respect to sex, in both villages exactly one-half of measured respondents are male. The age and sex composition of our subsample approximates the population structure of both villages.

Random-Effects Regression Models. In Table 1, column 2, parameter estimates from the random-effects least-squares model are reported, and columns 3–5 report odds ratios corresponding to threshold exceedance models at the 25th, 50th, and 75th percentiles in telomere fluorescence.[§] As described more fully in *Materials and Methods*, all models control for sex, age, animal protein consumption, tobacco use, household earnings, whether the respondent suffered a malaria incident, the SD in the estimation of telomere fluorescence, and the count of telomeres retrieved per cell.[¶]

Village differences. Starting with Table 1, column 2, we find that villagers in the relocated Maziran have statistically significantly shorter telomeres compared with those in the isolated Behruda. Other things held equal, telomere fluorescence is 0.65 pixels per μm lower in the Maziran population (95% CI : −1.20, −0.11). Expressed in percentage terms, villagers in Behruda present with telomeres that are 1.76% longer than telomeres among villagers in Maziran. In columns 3–5, log odds are presented, corresponding to random-effects logistic regression models on the likelihood that an individual telomere exceeds the 25th, 50th, and 75th percentile in length, respectively. We find that adults sampled in the relocated village of Maziran are 20.6% (95% CI: 40.9, 0.02) and 28.0% (95% CI: 46.3, 9.7) less likely to possess telomeres exceeding the 25th and 50th percentiles in length, respectively.

Salivary cortisol. The salivary analyte of cortisol—where a higher morning compared with evening level is considered a healthier arousal profile (64, 65)—significantly predicts telomere length and in the direction hypothesized. Other things held equal, an increase in the difference between morning and evening cortisol levels (in micrograms per deciliter) increases expected telomere length by 0.48 (95% 0.23, 0.73) pixels per μm. Likewise, the odds of eclipsing the 25th, 50th, and 75th percentiles in telomere length increase by 21.4 (95% CI: 12.1, 30.6), 20.5 (95% CI: 12.1, 28.9), and 14.0% (95% CI: 5.5, 22.5), respectively, with an increase in the morning vs. evening difference in cortisol levels. Logistic regression results imply that the telomere maintenance benefit of a healthier cortisol profile functions similarly across the distribution of telomere length.

sAA. Also consistent with theoretical expectations, sAA—where a healthy arousal profile is seen in lower morning compared with evening levels (88)—behaves oppositely of the analyte cortisol with respect to the association with telomere length. Again, other things held equal, we find that the morning minus evening level of sAA significantly reduces the expected length of telomeres by –0.47 (95% CI: –0.71, –0.23) μm per pixel, a magnitude approximately offsetting the gains to telomere length from a healthier

[‡]Although our 3D TEL-FISH approach for assessment of telomere length increased visualization of shorter telomeres, cell samples still varied in the number of telomeres observed. The median number of telomeres measured per cell was 69, and the interquartile range was 18 (25th, 59; 75th, 77). We account for this detection bias by adjusting predictions of telomere fluorescence by the count of telomeres observed per cell. Results from our multilevel least-squares model indicate that telomere fluorescence declines by 0.06 μm per pixel (95% CI: –0.08, –0.05) for an additional telomere observed per cell. A correction factor of 0.06 μm per pixel is not trivial. For instance, other things held equal, predicted telomere fluorescence decreases ∼3% in going from the 25th to the 75th percentile in the count of telomeres measured per cell. Because telomere detection bias operates on smaller telomeres, taking the average length of observed telomeres—a more common practice in telomere science—will result in upward biased estimates. In addition, insofar as the number of observable telomeres per cell is determined by the health of the organism, and assuming healthy organisms have longer telomeres, differences derived in comparisons of healthy and unhealthy populations will be biased downward. It should also be noted that some cells had implausibly high telomere count, >92. We restricted analyses to cells with <92 telomeres observed. Cells with excess telomeres were probably cycling (i.e., in S or G_2). In these cases, the number of telomeres present could be more than 92 (as expected in G_0/G_1 cells). Of note, these measurement biases would also occur within more standard PCR telomere length assessment techniques, but given that only an average telomere length is calculated, rather than that of each individual telomere, it cannot be corrected.

[§]For instance, although base buccal cells are categorically different in phase position and morphology, the process of cell division is continuous. Therefore, limiting samples to base phase cells cannot guarantee that all buccal cells fall exactly on the continuum of cell division. By accounting for the cell identity from which a telomere is retrieved, one can crudely account for phase position cell characteristics that may influence observed telomere lengths. Model statistics reported in Table 1 show $\rho = 0.35$ in the random-intercept least-squares model, and ρ = 0.45–0.50 in random-effects logistic regression models. ρ is the intraclass correlation. With respect to random-effects logit models 1–4, ρ pertains to a latent variable reflecting the propensity of a telomere to eclipse a specified threshold—either the 25th, 50th, or 75th percentile. A ρ value of 0.50 (corresponding to our random-effects logit model in column 4) indicates that ∼50% of the variance in the underlying propensity of a telomere to exceed the 50th percentile in fluorescence (or length) is attributable to cell id.

[¶]Although our analytic emphasis is on associations of telomere length and measures of stress, it is worth noting the intuitive behavior of control variables, lending validity to our telomere fluorescence measurement procedure. With respect to the random intercept logistic model (in column 4), tobacco/bidi consumption ($b = -0.10$; $P < 0.01$), age ($b = -0.01$; $P < 0.01$), male sex ($b = -0.53$; $P < 0.01$), and malaria incident ($b = -0.25$; $P < 0.05$) are significantly negatively associated with telomere fluorescence, whereas household income ($b = 0.22$, $p < 0.01$) and animal protein consumption ($b = 0.14$, $p < 0.01$) are significantly positively correlated with telomere fluorescence. Consistent with the technical criticism that telomere measurement efforts are plagued by selection bias (i.e., longer telomeres are more likely to be observed than shorter telomeres), we find that the probability that an observed telomere exceeds the 50th percentile in length declines significantly with the number of telomeres/per cell observed ($b = -0.01$; $P < 0.01$). We also find that telomere fluorescence decreases significantly in the count of cells retrieved per person ($b = -0.05$; $P < 0.01$). Finally, we find that the odds of an individual telomere exceeding the 50th percentile in length increase significantly in the standard deviation of the estimated length of a telomere. The inclusion of this term is meant to adjust for the error in length estimation by 3D imaging that captures both the mean and the distribution around the estimated mean in microns per pixel.

Table 1. Results from random-effects generalized least-squares and logistic regressions of telomere fluorescence

Variables	Least-squares coefficient (Model 1)	Logistic ≥ $P_{0.25}$ coefficient (Model 2)	Logistic ≥ $P_{0.50}$ coefficient (Model 3)	Logistic ≥ $P_{0.75}$ coefficient (Model 4)
Village	−0.653*	−0.206*	−0.280**	−0.147
	(0.279)	(0.104)	(0.933)	(0.093)
Diurnal cortisol	0.479**	0.214**	0.205**	0.140**
	(0.130)	(0.047)	(0.043)	(0.043)
Diurnal sAA	−0.470**	−0.110*	−0.089*	−0.149**
	(0.122)	(0.046)	(0.040)	(0.041)
BSI	−0.508†	−0.694**	−0.347**	0.045
	(0.188)	(0.109)	(0.099)	(0.101)
Log-likelihood		−20,548.97	−25,985.48	−19,844.89
ρ	0.347	0.500	0.455	0.446
N	126,410	126,410	126,410	126,410
Overall R^2	0.874			

Models control for sex, age, animal protein consumption, tobacco use, household earnings, whether the respondent suffered a malaria incident, the SD in the estimation of telomere fluorescence, the count of cells per person, the count of telomeres retrieved per cell, and TFI random effect for person. SEs are in parentheses. $^{**}P < 0.01$, $^{*}P < 0.05$, $^{\dagger}P < 0.10$.

cortisol profile. Similarly, probabilities of exceeding the 25th, 50th, and 75th percentiles in telomere length decrease by 11.0% (95% CI: 20.0, 1.9), 8.9% (95% CI: 16.8, 1.0), and 14.9% (95% CI: 22.9, 7.0), respectively, with unit changes in morning vs. evening difference in sAA levels. Given overlapping intervals of confidence, logistic regression results indicate that an unhealthy sAA profile operates uniformly negatively on telomeres of varying length.

BSI. Finally, with respect to our nonanalyte measure of stress, as anticipated, a count of psychosomatic complaints on the BSI > 9 (reflecting higher distress) is associated with a −0.51 unit decrease in telomere fluorescence, where $P < 0.10$. Holding other things equal, telomeres taken from adults presenting with high somatic suffering are 69.4% (95% CI: 90.8, 48.0) and 34.7% (95% CI: 54.0, 15.3) less likely to eclipse the 25th and 50th percentiles in telomere length, respectively.

Discussion

Consistent with the premise that life stress contributes to loss of telomere length, each of our study's three key indicators of stress, the two salivary analytes (cortisol, α-amylase) and the BSI (somatic suffering), were associated with telomere length. More specifically, we found significant associations between less healthy cortisol and sAA profiles and telomere shortening. These results are strengthened by the fact that we collected saliva over separate 9-d periods in each village, allowing us to identify stable salivary analyte profile traits as opposed to more transitory states (64, 100). Furthermore, we found that persons scoring high on the BSI—reflecting greater self-reported psychosomatic distress in South Asian contexts (96)—presented with shorter telomeres. All results held after controlling for other known correlates associated with telomere shortening—such as age, health indicators like malarial incidents, nicotine use, SES, and dietary indicators like meat consumption—with these controls also behaving in ways consistent with theoretical predictions.

We used a particularly sensitive and high-resolution telomere length assessment strategy that facilitated a deeper understanding of telomere biology in this unique context. TEL-FISH coupled with 3D imaging of buccal cell nuclei allowed us to select specific target cell populations (in this case, putative basal stem or progenitor cells) for telomere length determination on a cell-by-cell basis and to scan the entire cell volume so that telomeres were not occluded in ways that would bias our estimates (73). This approach provided greater precision than other techniques that give only a single average telomere length across cells of all types within a given individual. Of note, our 3D TEL-FISH telomere measurement strategy permitted use of multilevel statistical analyses, allowing us to identify and estimate sampling biases pertaining to the number of telomeres drawn from each cell and the number of cells drawn from each person. The presence of these biases necessitates exercising care when interpreting results from studies relying on a single average telomere length estimate for each person sampled.

Although we found consistent differences in regard to telomere length between villagers in the isolated Behruda (longer) and the relocated Maziran (shorter), the absence of pretest measures warrants using caution with respect to interpreting the differential stressors accompanying isolation vs. physical displacement. Although the two villages were similar in size, age, and sex structure, were separated by only a short distance, and regularly exchanged social and economic goods before displacement, differences in telomere length observed between the villages may be attributable to unobserved predisplacement differences. It is thus difficult to say with certainty that the stress of isolation and restriction of access to the sanctuary experienced by the people of Behruda is less distressing than the experience of physical relocation visited on the villagers of Maziran.

Whether or not differences in telomere length between villagers are attributable to the capricious draw of the sanctuary boundary, Sahariya in both contexts presented with high levels of somatic suffering comparable to populations receiving psychiatric care (45). A now substantial development studies literature speaks of the traumatic toll of human displacement, be it actual physical relocation or a restriction of access to traditional lands and resources (44, 79–86). Indeed, our own ethnographic interviews and observations revealed that suicidal ideation was common in our Sahariya respondents, and our field team witnessed an actual 2012 suicide attempt (using rat poison) by a young woman suffering domestic strife exacerbated by her family's poverty and husband's alcoholism. These details illuminate the particularly high levels of psychosomatic duress characteristic of both of these Sahariya villages, which are not adequately sampled and captured in stress and telomere studies carried out in wealthier and more securely developed WEIRD societies.

In these terms, and to conclude, by meticulously tracing and predictively modeling links between stress and telomere maintenance in a highly distressed non-WEIRD population, our research strengthens the analytic value of stress-related telomere shortening as a broadly human marker of compromised health and aging.

Materials and Methods

Telomere Length: Sampling, Storage, and Assays. The research described in this paper, including the use of appropriate informed consent procedures, has been reviewed and approved by the Colorado State University Institutional Review Board (IRB) for the protection of human subjects. Buccal basal cells

were collected from 46 individuals in the field, including 22 from Behruda and 24 from Maziran.[||] Upon arrival at the laboratory in the United States (~7 d after collection), samples were vortexed for 10 min, then centrifuged (581 × *g*) for 10 min at room temperature. Supernatant was removed to ~1 mL of cell suspension and ~15 mL of buccal cell (BC) buffer (1.6 g of Tris·HCl, 38.0 g of EDTA, and 1.2 g of sodium chloride) added. Cells were pelleted and washed twice in this manner. The pellet was then resuspended in 5 mL of BC buffer, with cells drawn by a syringe fitted with an 18-gauge needle 20 times (without producing bubbles). The suspension was passed through a 100-μm nylon filter into another 50-mL tube to remove large cell aggregates. The filtered suspension was transferred to a 15-mL centrifuge tube and brought to a final volume of 8 mL with BC buffer. Cells were fixed by slowly dropping 500 μL of freshly prepared Carnoy fixative (3:1 methanol to glacial acetic acid) down tube walls and into the cell suspension. After centrifugation and resuspension of the pellet in 5 mL of fixative, cells were treated with 5 μL/mL Cytoclear (Genial Genetic Solutions) for 10 min at room temperature. Cells were centrifuged, and the pellet washed by resuspending in fixative twice. Cell suspensions were dropped onto slides, air dried, and aged overnight. Remaining cell suspensions were preserved in pure methanol at 4 °C.

Slides were incubated in Pepsin (200 μL of 40 units/mL in 0.01 M HCl; Sigma-Aldrich) for 15 min at 37 °C, then washed in PBS for 2 min, dehydrated through a graded ethanol series, and air dried. A Cy3-labeled peptide nucleic acid telomere probe (TTAGGG3; Biosynthesis) was prepared by diluting 5 μL of probe in 36 μL of formamide (Sigma-Aldrich), 12 μL of 0.05 M Tris buffer, 2.5 μL of 0.1 M KCl (Sigma-Aldrich), and 0.6 μL of 0.1 M MgCl (Sigma-Aldrich) for a final concentration of 300 ng/mL. Fifty microliters of probe mixture was applied to each slide, and then coverslipped and denatured at 73 °C for 3 min. Slides were incubated at 37 °C for 2 h, and then washed in a series of 43.5 °C washes for 2.5 min each; washes 1/2: 50% formamide in 2× sodium citrate (SSC); washes 3/4: 2× SSC; and washes 5/6: 2× SSC plus 0.1% Nonidet P-40. Slides were counterstained with 50 μL of DAPI (in Prolong Gold Antifade; Invitrogen), coverslipped, and stored at −20 °C. Slides were processed in sets of two, one sample from each village, to minimize variability between sample runs.

Image *Z* stacks were taken using a Zeiss Axio Imager.Z2 microscope, capturing fluorescent images with a Coolsnap ES2 camera running Metamorph software (Molecular Devices). A total of 50 images was obtained, each consisting of 22 stacks (0.2 μm per plane) in two different wavelengths (Dapi and Cy3). Three-dimensional deconvolution and (*Z* conversion 3D to 2D) was performed with ImageJ software rsb.info.nih.gov/ij/ under 3D blind parameters. Point spread function was calculated for each picture to obtain a maximum projection of the 22 stacks, allowing analysis of all telomere signals visible in the whole extension of each cell nuclei.

Analysis of TFI was performed using TELOMETER (available as a download from demarzolab.pathology.jhmi.edu/telometer/download.html). Telomere signals from 45 nuclei per sample were analyzed using custom program settings (minimum object size: 1; maximum object size: 350; despeckle ratio: 0.3; rolling ball size: 1). TFI, our response variable, is measured as the average number of pixels per micrometer. In total, 124,610 telomeres retrieved from 1,989 cells from 46 villagers were analyzed. The procedures described above meaningfully improve quantification of telomeres for three reasons. First, by targeting basal progenitor cells, we limit confounding arising from nonrandom sampling of cell type mix. Second, by measuring individual telomeres on a cell-by-cell basis, our methodology facilitates per-cell, per-individual, and per-population telomere length profiles and distributions, statistical properties of potential relevance to telomere biology. Third, by scanning the entire cell volume (*X*, *Y*, and *Z* dimensions), we approach the capture of all telomeres within a cell, minimizing a detection bias plaguing other procedures.

Of further note, we implemented safeguards to reduce telomere measurement error. The most standard way to control for reproducibility is to perform measurements of objects that will not change in intensity over time. We used 0.2-μm fluorescent microbeads to calibrate the optical system of the microscope. This provided an idea of the 3D space reconstructed, and of the fluorescence variation due to the light source and its deformation. Once the system was ready for a given experiment, we measured microbeads several times during the period of image acquisition. Specifically, we measured the fluorescence of the beads at the beginning, and then again at 1/4, 1/2, 3/4, and ending time intervals. One can think of this process as a form of test–retest reliability. Our measurement strategy produced small variation (less than 5%) across tests, indicating very high test–retest reliability. The fluorescence measurement procedure used in the current study was also externally validated in our previous research of chronic kidney disease in felines (75), where parallel measurements of telomere length were taken from a control cell line (or cell type) using both microbeads and cell type controls, and thereby arriving at satisfactorily reproducible telomere measurements in all experiments.

Cortisol and sAA: Sampling, Storage, and Assays. Following recent innovations in minimally invasive collection of salivary stress analytes in anthropological field settings (69, 70, 72), saliva was sampled twice daily (morning and evening and sometimes in the afternoon) from a subpopulation of 46 individuals over 9-d periods in each village. Saliva was collected as passive drool in small plastic vials, refrigerated, and shipped with ice packs to Salimetrics Laboratories, where they were stored at –80 C until assay. On the day of assay, samples were centrifuged at 3,000 rpm (1,500 × *g*) for 15 min to remove mucins. Samples were assayed for cortisol and sAA following Granger et al. (66). Briefly, a high-sensitivity salivary expanded-range, high-sensitivity salivary cortisol enzyme immunoassay (Salimetrics) was used, which has a range of sensitivity from 0.003 to 3.0 μg/dL. Cortisol assays were run in duplicate, and the average of the duplicates for each sample was used in the analyses. Salivary cortisol is measured in micrograms per deciliter.

sAA was measured using a kinetic reaction assay that employs a chromagenic substrate, 2-chloro-*p*-nitrophenol, linked to maltotriose. The enzymatic action of sAA on this substrate yields 2-chloro-*p*-nitrophenol, which can be measured spectrophotometrically at 405 nm using a standard laboratory plate reader. The amount of sAA activity present in the sample is directly proportional to the increase (over a 2-min period) in absorbance at 405 nm. Results were computed in units per milliliter of sAA by the following: [absorbance difference per minute × total assay volume (328 mL) × dilution factor (200)]/[millimolar absorptivity of 2-chloro-*p*-nitrophenol (12.9) × sample volume (0.008 mL) × light path (0.97)]. For both cortisol and sAA, average intraassay and interassay coefficients of variation were less than 10% and 15%, respectively. To derive diurnal slopes,** the average morning readings of salivary cortisol and sAA were subtracted from average evening readings corresponding to each analyte. In regression analyses, diurnal salivary cortisol and sAA measures are standardized with $\mu = 0$, $\sigma = 1$.

Survey Measures. Our key indicator of psychosomatic distress is the 21-item, 2-point scale version of the BSI, which has been validated to capture psychiatric disorders among South Asian populations, unfolding as they do more typically in a bodily rather than psychic language of distress (96). A villager received a score of 1 if she/he had a BSI score >75th percentile, and 0 otherwise. Because other factors contribute to telomere shortening, including smoking (97), diet, and nutritional deficiencies (98), and certain diseases (99), the survey also included measures of tobacco/bidi use (₹100 units), animal protein consumption (₹100 units), household income (₹1,000 units), demographic characteristics (age and sex of respondent), and whether or not a respondent suffered a malaria incident in the last 30 d.

Statistical Approaches and Procedures. We rendered a series of random-effects generalized least-squares and logistic regression models, accounting for the nonindependence of telomeres sampled from the same cell, telomeres sampled from the same person, and telomeres drawn from persons sharing the same village. Diagnostic tests revealed that telomeres drawn from the same cell are significantly more alike than telomeres drawn from the same person or telomeres drawn from the same village. To account for within-village and within-person correlations, models include a dummy variable for village residence, and a random effect for sampled persons corresponding to the average telomere length of person *k* minus the expected mean in telomere length over all persons sampled. To account for unmeasured cell

[||]A modified protocol described by Thomas et al. (78) was used. Briefly, each participant rinsed their mouth vigorously with water for approximately 15 s to eliminate contaminants. A small-headed toothbrush (2 cm) was rotated 10 times against the inside cheek wall in a circular motion starting from the middle, increasing gradually in circumference as an outward spiral. The head of the brush was then placed into a 50-mL tube containing 15 mL of Saccomanno's fixative of 50% (vol/vol) ethanol and 2% (vol/vol) polyethylene glycol diluted in water, and shaken to dislodge and suspend cells. This process was repeated with a new toothbrush on the opposite cheek wall to obtain two samples for each individual. The tubes were sealed and shipped to the laboratory for processing and analysis. Based on individual cellular morphology, the buccal cells scored were basal cells (progenitors), deriving from basal stem cells. Basal cells give rise to the other cells of the upper layers through proliferation and differentiation. In Thomas et al. (78), figure 2, the morphology of the different cell types is described, as are the layers they form in the buccal mucosa.

**Because villagers did not typically wear watches and frequently left their homes immediately after waking, it was impossible to reliably collect two saliva samples on awakening separated by 30-min intervals, which would have allowed us to additionally assess a cortisol awakening response, motivating the decision to rely on diurnal slopes for this stress measure. The signal-to-noise ratio, calculated as the mean diurnal slope divided by the SD of the slope across days, is 11.03 for diurnal cortisol and 1.43 for sAA.

characteristics that implicate variation in telomere fluorescence, we allow each cell to have its own intercept. Letting L_{ijk} denote the fluorescence (length) of an observed telomere i in cell j from person k, our random intercept regression estimator is modeled as follows:

$$L_{ijk} = \beta_0 + \beta_1 V_k + \beta_2 C_k + \beta_3 H_k + \beta_4 B_k + \Gamma_1 M_k + \Gamma_2 P_k + \Gamma_3 T_k + \Gamma_4 I_k + \Gamma_5 G_k + \Gamma_6 A_k + \lambda_1 N_j + \lambda_2 C_k + \lambda_3 \sigma_i + \lambda_4 \mu_k + u_j + e_{ij}, \quad [1]$$

where V_k assumes a value of 1 if the respondent resides in the village of Maziran, C_k is the average diurnal cortisol slope of a sampled person, H_k is the average diurnal sAA slope of a sampled person, B_k is the sampled person's score on the BSI (with 1 = nine or more complaints), M_k is equal to 1 if the respondent suffered a malaria incident in the most recent month, P_k is the amount of money (₹100 units) a sampled villager spends on animal protein per month, T_k is the amount of money (₹100 units) a sampled villager spends on bidi and tobacco per month, I_k is the household income per capita of the sampled respondent (in ₹1,000 units), G_i assumes a value of 1 if the sex of the sampled person is male, A_i is the respondent's age in years, N_j is the number of telomeres retrieved per cell, C_k is the number of cells retrieved per person,[††] σ_i is the SD of the estimated fluorescence of telomere i, and μ_k is a person-level random effect corresponding to the average telomere length of person k minus the expected mean over all persons sampled. Unlike the ordinary least-squares model, the random-effects model residual term is divided in two parts: (*i*) a cell-specific error component, given by u_j; and (*ii*) a telomere-specific error component, which varies between telomere and cell, given by e_{ij}. The cell level residual u_j is meant to capture the combined effects of omitted cell characteristics (101). Both residual terms are assumed to be Gaussian with zero means: $u_j \sim N(0, \sigma_u^2)$ and $e_{ij} \sim N(0, \sigma_u^2)$.

[††]Including terms for the number of telomeres measured per cell and the number of cells per person are crucial statistical adjustments, as the average fluorescence of telomeres declines measurably in the number of telomeres measured per cell and in the number of cells retrieved per person.

Next, we perform a series of random-effects logistic regression models. As with the random-effects least-square model, the random-effects logistic regression model relaxes the assumption of conditional independence in the standard logistic regression by adding a cell-specific random intercept (ζ_j) to the binary prediction equation. Unobserved characteristics or conditions at the cell level are integrated in ζ_j. With the definition of terms carrying from Eq. **1**, our model is defined as follows:

$$\text{logit}\left\{\Pr\left(y_{ijk}=1 \mid x_{ijk}, \zeta_j\right)\right\} = \beta_0 + \beta_1 V_k + \beta_2 C_k + \beta_3 H_k + \beta_4 B_k + \Gamma_1 M_k + \Gamma_2 P_k + \Gamma_3 T_k + \Gamma_4 I_k + \Gamma_5 G_k + \Gamma_6 A_k + \lambda_1 N_j + \lambda_2 C_k + \lambda_3 \sigma_i + \lambda_4 \mu_k + \zeta_i. \quad [2]$$

With respect to random-intercept logistic regression models, we estimate likelihoods of the fluorescence of telomere i exceeding the 25th, 50th, and 75th percentile thresholds in telomere fluorescence. Such logistic regression analyses allow one to investigate whether predictors influence telomere fluorescence differently or uniformly across the distribution of telomere fluorescence.

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