Received: 4 April 2020 | Revised: 23 September 2020 | Accepted: 24 September 2020

DOI: 10.1002/ajhb.23515

ORIGINAL RESEARCH ARTICLE

American Journal of Human Biology WILEY

Lifetime stress and war exposure timing may predict methylation changes at NR3C1 based on a pilot study in a warrior cohort in a small-scale society in Kenya

Bilinda Straight[1] | **Georgiana Fisher**[2] | **Belinda L. Needham**[3] | **Amy Naugle**[4] | **Charles Olungah**[5] | **Puntipa Wanitjirattikal**[2] | **Cecilia Root**[6] | **Jen Farman**[6] | **Todd Barkman**[7] | **Claudia Lalancette**[8]

[1]Department of Anthropology, Western Michigan University, Kalamazoo, Michigan

[2]Department of Statistics, Western Michigan University, Kalamazoo, Michigan

[3]Department of Epidemiology, University of Michigan, Ann Arbor, Michigan

[4]Department of Psychology, Western Michigan University, Kalamazoo, Michigan

[5]University of Nairobi Institute of Anthropology, Gender & African Studies, Nairobi, Nairobi, Kenya

[6]Unaffiliated (Western Michigan University Department of Anthropology Alum), Kalamazoo, Michigan

[7]Department of Biological Sciences, Western Michigan University, Kalamazoo, Michigan

[8]University of Michigan Epigenomics Core, Ann Arbor, Michigan

Correspondence
Bilinda Straight, Department of Anthropology, Western Michigan University, Kalamazoo, MI.
Email: bilinda.straight@wmich.edu

Funding information
National Science Foundation, Grant/Award Number: 0413431; Western Michigan University, Grant/Award Number: College of Arts & Sciences (CAS) & Office of Resea

Abstract

Objectives: Candidate gene methylation studies of NR3C1 have identified associations with psychosocial adversity, including war trauma. This pilot study (sample sizes from 22 to 45 for primary analyses) examined NR3C1 methylation in a group of Kenyan pastoralist young men in relation to culturally relevant traumatic experiences, including participation in coalitional lethal gun violence.

Methods: Adolescent and young adult Samburu men ("warriors") were recruited for participation. DNA was obtained from whole saliva and methylation analyses performed using mass spectrometry. We performed a data reduction of variables from a standardized instrument of lifetime stress using a factor analysis and we assessed the association between the extracted factors with culturally relevant and cross-culturally comparative experiences.

Results: Cumulative lifetime trauma exposure and forms of violence to which warriors are particularly susceptible were associated with DNA methylation changes in the NR3C1 1_F promoter region but not in the NR3C1 1_D promoter region. However, sensitivity analyses revealed significant associations between individual CpG sites in both regions and cumulative stress exposures, war exposure timing, and war fatalities.

Conclusions: This study supports the importance of NR3C1 methylation changes in response to challenging life circumstances, including in a global south cultural context that contrasts in notable ways from global north contexts and from the starkly tragic examples of the Rwandan genocide and war-associated rape explored in recent studies. Timing of traumatic exposure and culturally salient means to measure enduring symptoms of trauma remain important considerations for DNA methylation studies.

1 | INTRODUCTION

Growing evidence shows that environmental exposures at critical developmental periods, including gestation, early life, and adolescence, epigenetically modulate gene expression. Studies have focused especially on Cytosine-phosphate-Guanine (CpG) DNA methylation (DNAm) because of its responsiveness to environmental conditions (including stress), its role in gene expression, and because it is relatively accessible for both field and laboratory conditions. Yet DNA methylation research is largely lacking in low- and middle-income countries, and studies of ethnic marginals within these countries are almost nonexistent (Buffa et al., 2018; Herba, Glover, Ramchandani, & Rondon, 2016; McDade et al., 2017). Additionally, more work is needed on potential epigenetic modifications during the developmentally dynamic adolescent to young adult period, particularly in relation to violence and other stressors (van der Knapp et al., 2014; Vukojevic et al., 2014; Yehuda et al., 2013). Anthropologists, including cultural anthropologists, are well positioned to address these lacunae, while maintaining caution in interpreting results given that the field is still developing (Thayer & Non, 2015).

Both animal and human studies have examined DNAm in the glucocorticoid receptor (GR) because of the role of GR in stress response. In humans GR is encoded by the NR3C1 gene (Nuclear Receptor Subfamily 3, Group C, Member 1), a protein coding gene located on chromosome 5, which can act as a transcription factor and as a regulator of other transcription factors. Stress activates the hypothalamic-pituitary-adrenal (HPA) axis, a major neuroendocrine system. In humans, the paraventricular nucleus of the hypothalamus secretes corticotropin-releasing hormone (CRH), which in turn regulates the adrenal cortex, producing glucocorticoids (mainly cortisol), which bind to the glucocorticoid and mineralocorticoid receptors.

Human candidate gene DNAm studies of NR3C1 in association with psychosocial stressors as well as psychological outcomes have focused especially on the Promoter 1_F region (Palma-Gudiel, Córdova-Palomera, Leza, & Fañanás, 2015), although an increasing number of studies have examined other regions (Watkeys, Kremerskothen, Quidé, Fullerton, & Green, 2018). Promoter 1_F region has been of early interest because it contains the NGF1-A binding region that Weaver et al. (2004) reported in their path-breaking rat study that showed that increased licking and grooming associated to increased GR expression in adult offspring, which was mediated by differential methylation of NGF1-A in the exon 1_7 GR promoter (1_F in humans). Moreover, these DNAm changes persisted into adulthood but were reversed through cross-fostering. Numerous human studies have found DNAm changes in the 1_F region associating to stressors, especially at sensitive time periods (gestation, early life, and adolescence). However, the results have been mixed in whether the direction of change is hyper- or hypomethylation, and there have been more null than positive results in individual CpG sites (Watkeys et al., 2018). We selected the 1_F region for the present study because of its importance in the literature. We additionally selected the 1_D region because it has been associated with changes in response to stress, with the direction of effect not well understood (Palma-Gudiel et al., 2015, 2018; Turecki & Meany, 2016).

Studies of NR3C1 exon 1_F DNAm in relation to early childhood, adolescent, and lifetime trauma have typically reported hypermethylation if there were significant results, while few studies reported significant results related to trauma in other regions (Palma-Gudiel et al., 2015; Turecki & Meany, 2016; Watkeys et al., 2018). Romens, McDonald, Svaren, and Pollak (2015) examined the 1_F region (using whole blood) in U.S. children ages 11 to 14 years and found greater methylation associated with childhood maltreatment. Efstathopoulous et al. (2018) examined the 1_F region (using whole saliva) in Swedish adolescents ages 13 to 14 years and reported hypermethylation in association with adolescent self-reports of being bullied. Tyrka, Price, Marsit, Walters, and Carpenter (2012) examined the 1_F region (using whole blood) in healthy U.S. adults and found increased methylation in association with retrospective reporting of early childhood adversity such as parental death, desertion, and maltreatment.

With respect to the less-studied 1_D region, Tyrka et al. (2015) examined the 1_D, 1_F, and 1_H regions (using whole saliva) in U.S. children ages 3 to 5 and found higher levels of methylation in the promoter regions of exon 1_F and exon 1_D in association with child maltreatment but no significant associations with exon 1_H. Parent et al. (2017) conducted a longitudinal study of the exon 1_D and exon 1_F promoter regions (using whole saliva) in U.S. preschool children, half of whom had experienced maltreatment in the past 6 months (baseline). They found child maltreatment to associate with higher levels of NR3C1 exon 1_D methylation but not exon 1_F promoter methylation at baseline, and contextual stress associated with poverty and trauma to associate with higher levels of NR3C1 exon 1_F promoter methylation at baseline. Additionally, they found a higher rate of change in the direction of reduced methylation in most of the 1_D CpG sites and in two 1_F CpG sites (27 and 29) at a repeat measure 6 months later.

Gene expression studies are also important to refining our understanding of the biological relevance of DNA

methylation changes identified in studies examining potential associations between DNA methylation changes and psychosocial (and other) stressors. Vukojevic et al. (2014) included a key component in their study of Rwandan genocide survivors by linking NR3C1 methylation with gene expression and memory function in healthy Swiss study participants. (Saliva DNA was used for both Rwandan and Swiss participants.) In Swiss participants, researchers found increased NR3C1 methylation associated with reduced NR3C1 expression in both males and females, but reduced picture recognition in men only, in a task in which participants were shown and asked to identify new pictures and pictures they had been shown in an earlier task. They additionally found an association in men only between NR3C1 methylation and neuroimaging during success in performing a memory task. Vukojevic and colleagues' study points to the importance of DNA methylation and gene expression in enhancing or suppressing potentially traumatic memories—an essential component in long term coping with experiences culturally encoded as emotionally painful. The study's findings also point to potential post-transcriptional gender differences in the effects of glucocorticoids on memory. Such studies of gene expression are particularly important to support the potential biological relevance of findings of human field studies in the global south (including the present study), where gene expression is typically difficult to examine.

The present pilot study is based on work with 82 young Samburu pastoralist men in northern Kenya of the warrior (*lmurran*) age grade, all of whom are eligible to be tasked with care and defense of the community and its livestock herds with AK-47 and G3 assault rifles if necessary. In a previous paper (Straight et al., 2019), we examined Samburu warfare and prosocial emotion in evolutionary perspective, focusing particularly on motivated behaviors in contrast to defensive warfare and the moral conflict of killing. To do so, we compared DNAm in NR3C1 and MAOA genes to individual combat behaviors, including the choice to kill or spare the lives of enemies and the choice of engaging in offensive livestock raids against enemies in contrast to experiencing attacks on one's own herds. Additionally, we examined the motivating consequences of Samburu warfare in the form of acquired livestock in contrast to experiencing livestock losses. Finally, we compared provisional diagnoses of posttraumatic stress disorder (PTSD) to DNAm and combat variables, finding significant association comparing PTSD to livestock losses but null findings in the comparison between PTSD and DNAm. In this article, we report for the first time on these young men's cumulative lifetime stressors of all kinds. Our primary hypothesis predicted DNAm changes in NR3C1 in response to cumulative lifetime stress exposure. Our sensitivity analyses examined significant findings in relation to individual CpG sites and included comparisons to war stressor timing and exposure to fatalities as relevant to significant findings.

The present study is potentially important with respect to statistically significant associations between lifetime stressors and DNA methylation differences. It is also potentially important in focusing on young men in a small-scale society (Samburu) in a low-income country, who are chronically exposed to coalitional gun violence, interpersonal violence, food and water insecurity, and other stressors. This high stress load offers a unique opportunity to closely examine and compare the biological impact of several types of stressors in the same study while also providing information relevant for humanitarian relief efforts. Additionally, the majority of DNAm research to date has been on global north populations and thus does not adequately capture the range of human biocultural variation (Buffa et al., 2018; Herba et al., 2016; McDade et al., 2017). Finally, the relatively greater socioeconomic homogeneity of Samburu communities compared to most populations targeted for DNAm reduces the impact of confounders.

2 | METHODS

2.1 | Ethics statement

Approval was obtained from Western Michigan University's Human Subjects Institutional Research Board, conforming to the U.S. Federal Policy for the Protection of Human Subjects. An explanation of the study was provided in a meeting with Samburu elders prior to beginning data collection and informed consent was obtained from all participants. Additional meetings were held with participants and elders in December 2015 and July 2016, including a question and answer session discussing DNA and DNA methylation in lay terms with Samburu metaphors.

2.2 | Participants and study community

Samburu are pastoralists inhabiting northern Kenya's semi-arid lands, a region subject to cyclical drought and also periodic extreme droughts that appear to be increasing in frequency based on climate change (Cook & Vizy, 2012, 2013; Funk, 2011; Opiyo, Wasonga, Nyangito, Schilling, & Munang, 2015; Tierney, Ummenhofer, & deMenocal, 2015). Food and water insecurity are chronic and disease burden is high (particularly malaria, typhoid,

upper respiratory infections, and gastrointestinal infections). Additionally, the ruggedness of the semi-arid terrain in combination with tasks necessary to livestock husbandry lead to frequent injuries, and low access to adequate medical care translate to high infection rates even from simple cuts. These factors, together with chronic intercommunity violence and domestic violence add up to a high physiological and psychosocial stress load.

Initiating adolescent and young adult men into the *lmurran* ("warrior") age grade is a central feature of Samburu society that has persisted through colonialism, Kenyan independence, and ongoing social change that has accelerated with globalization. Historically, young Samburu lmurran were tasked with protecting the herds from predators such as leopards, hyenas, and lions; and with defending the community and the herds from attack by neighboring communities. These young men likewise were celebrated within their communities for attacking neighboring communities to steal livestock (Holtzman, 2016; Kasfir, 2007; Straight, 2005).

The need for defense from predators and neighboring groups has not subsided but the style and reasons for intergroup attacks has changed. In the 21st century, violence between northern Kenyan pastoralist communities is characterized in the literature as chronic, low-intensity warfare, fought with military assault rifles for reasons that vary widely between territorial expansion, livestock recovery or theft for the sake of individual and community interests, and large scale livestock theft as mercenaries for political elites and a clandestine economy (Bollig & Oesterle, 2008; Greiner, 2013; Holtzman, 2016; Straight, 2009).

At the same time, increased opportunities for education and for occupations such as teachers, civil servants, and medical workers has created a Samburu warrior age grade that for some, means caring for the herds and exposure to coalitional gun violence, while for others, it may mean some degree of livestock husbandry on weekends or school breaks with little or no exposure to coalitional gun violence.

The study's participants were all active adolescent and young adult Samburu warriors of the then-current generational cohort (named *Lkishami*). Participants were recruited through word of mouth and announcements at a livestock market in the study's lowland catchment area. Fifty-one participants in their late teens to early adulthood who were still active in the lmurran role were enrolled in the first phase (July 2015). Of those 51, 3 were excluded due to lack of corroboration of combat reports, reducing the number to 48. In December 2015, 34 more participants were enrolled, bringing the total sample to 82. Of these, 57 were available in December 2015 for DNA saliva assays, with one refusal for the assay only, for a total of 56 participating in the DNAm component.

Data collection was carried out in July and December 2015, with a July 2016 follow-up, and included culturally relevant questions about combat experiences and culturally-modified trauma exposure (Life Exposure Checklist for DSM-5). The study also employed PTSD instrumentation (PTSD Checklist for DSM-5), a vulnerability questionnaire designed for this specific population, data on parental death (loss of a parent, timing), self-identified most significant lifetime stressful event (description of event, timing), and ethnographic interviews.

Coalitional gun violence is illegal in northern Kenya. Therefore, identifiers were excluded and demographic data kept to a minimum at first visit, with partial demographic data collected at the second visit in December 2015 and additional demographic and additional stressor data collected at the July 2016 follow up. Given this population's obligations to engage in long distance herding, some participants were unavailable at subsequent visits, which resulted in some missing demographic and stressor values. This was an unavoidable but reasonable cost of collecting reliable combat data. Of the 82 participants, 37 reported never engaging in coalitional gun violence (raiding) and 45 reported at least one experience.

The demographic data consisted of age (estimated), wealth, education, and employment data, collected for descriptive purposes and as potential confounders, using culturally meaningful criteria and peer-corroborated self-assignment into high wealth, typical wealth, and low wealth in relation to their neighbors (Table 1). All participants live in one of Samburu county's most arid and remote regions and engage fully in the livestock economy even as some members of every family participate in wage labor and the cash economy for the benefit of the family as a whole. Participants' own employment (yes/no) and highest grade, and also their parents' highest grade and employment were collected.

Since Samburu do not keep track of their ages, ages were estimated based on known date of initiation, the lead authors' own knowledge in a number of cases (based on knowing them since 1992), and resorting to known events and participants' appearance. It is possible that some participants were younger than estimated but they would be unlikely to be older because of events timing and cultural practices surrounding initiation. All but the youngest had been initiated between 2006 and 2008 when they were ages ~11 to mid-twenties and had therefore been active as warriors for 9 years by the time of the study. The youngest had been initiated 3 years before the study (in 2013) and was certain of his age (17).

TABLE 1 Descriptive statistics

Variable	N	%				
Wealth self-rating	55	40% poor; 47% medium/average; 13% wealthy				
Participant ever employed?	39	35% yes				
Either parent ever employed?	39	31% yes				
Mother deceased?	54	9% yes				
Father deceased?	54	39% yes				
Variable	**N**	**Range**	**Min**	**Max**	**Mean**	**SD**
Estimated age in years	65	19	17	36	27.67	3.970
Highest grade—participant	39	12	0	12	1.67	3.311
Highest grade either parent	39	7	0	7	0.26	1.141
LEC score	82	16	0	16	6.97	4.157
#Years since mother deceased	54	22	4	26	12.00	9.924
#Years since father deceased	54	29.75	0.25	30	7.39	6.547
#Years since most recent war exposure	82	12.58	0.42	13	2.25	5.070
#Years since first war exposure	82	19	1	20	4.10	11.600
#Years since worst stressor	39	25.17	0.83	26	6.48	37.563
#Samburu fatalities	82	13	0	13	1.28	7.389
#Enemy fatalities	82	23	0	23	4.18	17.456

2.3 | Psychological instrumentation

This study employed the *Life Experience Checklist for Diagnostic and Statistical Manual of Mental Disorders* (DSM), fifth Edition (LEC-5) (Weathers et al., 2013). Our methods of translation and tests for reliability of this implementation for our study population are described in Straight et al., 2019. The lead author is trained as a cultural anthropologist and spent the first half of her research career with Samburu (1992-2008) engaged exclusively in ethnographic participant-observation and other cultural anthropological methods, with an emphasis on gender, material culture, illness and death, warfare, embodiment, and the nature of human experience. Previous attempts to use standardized instruments in Samburu beginning early in 2009 demonstrated that questions based on global north symptomology are challenging at best. This pilot study used the LEC-5 in a Samburu community for the first time in order to focus on experience (quantifiable as "exposures"), which is at the heart of cultural anthropology's toolkit. We used the brief LEC-5 checklist rather than the extended interview, combining this with culturally specific combat experience interviews. In a subsequent study of Samburu women, we have used the full interview version of the LEC-5, alongside questions tailored to the Samburu (papers in preparation). In both cases, ethnographic participant-observation preceded and informed the interviews.

For the warrior pilot, a group of Samburu community members conferred on the questions and made suggestions on modifications, which included eliminating the two questions on sexual assault for the pilot, contextualizing natural disaster and other questions to make sense for Samburu, and including in the captivity question, the Samburu-specific experiences of forced marriage and other forms of restricted freedom. Not only girls, but also young men of the warrior age-grade can be forced to marry against their will and can have their freedom curtailed in other ways, with humiliating forms of capture and corporal punishment used for transgressions. For each potentially traumatic event, participants were asked whether they had ever experienced the event themselves, whether they had witnessed it, heard of a specific case in which it had occurred to someone else, had experienced it during employment, or had never heard of it happening at all.

To offer nuance particularly in applying the LEC-5 to a remote global south community, and for data reduction purposes, a factor analysis was performed for the results reported on here, assuming that there are latent variables linearly related to the original variables. Since warriors experience extraordinarily high rates of multiple potentially traumatic experiences, we only scored events reported as directly experienced for purposes of the factor analysis. However, we have included both witnessed and directly experienced events for calculating a composite, cumulative score as a separate variable from the factor

scores. For comparison and due diligence, we also performed a secondary factor analysis that included both witnessed and directly experienced events. We scored as 0 any events that were only heard about because the level of potentially traumatic events is so high that everyone in the study is vicariously exposed to the most difficult stressors of war, death, potentially fatal illness, etc.

Unweighted least squares was used as the method of factor extraction. Since the original variables were binary, a polychoric correlation was used for estimation. The number of factors retained was based on the eigenvalue criteria, thus we retained only four factors for the primary factor analysis with an eigenvalue greater than 1. Factor scores were computed as estimates of the underlying latent constructs. The proportion of variance explained by the rotated factors were 11.05%, 11.47%, 20.40%, and 10.71%, respectively (Tables 2 and 3).

2.4 | DNA methylation

For DNA sampling, we used Oragene-500 kits (whole saliva) following the instructions for the kits. Saliva sampling was well tolerated. Saliva consists of epithelial cells and leukocytes in proportions that vary between individuals. Studies comparing DNAm obtained from saliva compared to whole blood have nevertheless pointed to the applicability of saliva for DNAm studies (Langie et al., 2016; Smith et al., 2015). Smith and colleagues compared DNA methylation obtained from saliva, blood, and brain tissues and found that DNAm patterns in saliva were more similar to the brain regions examined in the study than DNAm obtained from blood samples. This is extremely relevant to the present study, which focuses on the psychosocial impacts of human lived experiences. At the same time, they affirmed the need for adjustments for cellular heterogeneity in saliva, which was not possible for the current study. While computationally reference-free methods are available (eg, Houseman, Molitor, & Marsit, 2014), these are computationally intensive methods designed for epigenome-wide association studies. Our study is a candidate gene study.

DNA extraction was performed at Western Michigan University with the Gentra Puregene Blood Kit (Qiagen Inc.) using the DNA Purification from Body Fluid protocol. Genomic DNA (gDNA) samples were sent to the University of Michigan Epigenomics Core for EpiTYPER MassARRAY. All samples passed the core's QC. Samples were processed in batches for bisulfite conversion and PCR amplification. 500 ng of gDNA was bisulfite converted using Zymo's EZ DNA Methylation kit. 20 ng of converted DNA was used in a 10 μl PCR amplification volume. 5ul of the PCR reaction was used to verify that an amplicon of the proper size was obtained for each reaction (done electrophoresis on a 1.5% agarose-EtBr gel). For each sample, 4 PCR reactions were performed; one for each primer pair. Both 1_F and 1_D are located in a CpG island spanning 3 kb along the proximal promoter region (see Table 4 for primer pairs). We selected the promoter regions of 1_F comprising 47 CpG sites and 1_D comprising 28 CpG sites. All the PCR showed amplicons at the expected size. The amplicons were sent to the DNA sequencing core for the remainder of the assay. To minimize batch effects, all the amplicons generated were deposited on the same spectroChIP II array for analysis on the Sequenom MassARRAY instrument. Eleven samples lacked data: DNA and PCR products looked good, but they did not give any fragments at the MassARRAY

TABLE 2 Rotated loading matrix for LEC-experienced-events factors[a] (loadings lower than absolute 0.300 omitted; higher loadings in absolute value indicate higher association of the corresponding variables with the factors)

Variable	F1	F2	F3	F4
1 Disaster	0.963			
3 Transportation accident				0.986
4 Serious accident	0.356			
5 Toxic substance				−0.566
6 Physical assault			0.569	
7 Weapon assault			0.953	
10 Combat or exposure to war			0.906	
12 Life threatening illness/injury	0.317			
13 Severe human suffering	0.485	0.512		
14 Violent death		0.830		
15 Accidental death		0.623		
16 Harm you caused			0.714	

[a]Questions 8 and 9 were excluded based on Samburu expert panel and need for a qualitative study devoted to those questions (Straight et al., 2019).

TABLE 3 Explained variance and reliability of LEC rotated factors (Mislevy & Bock, 1990)

Factor	Variance	Proportion of variance explained	Reliability estimate
1	1.436	11.05%	0.933
2	1.491	11.47%	0.787
3	2.652	20.40%	0.954
4	1.392	10.71%	0.999

TABLE 4 NR3C1 primers (lowercase part of the primer indicates the adapter; uppercase indicates the part of the primer specific to the NR3C1 gene)

Sequence description	Sequence
NR3C1_P2_10F (1_D region)	5′-aggaagagagTTTTTAGTTTAAGGGGAAGGGAA-3′
NR3C1_P2_T7R (1_D region)	5′-cagtaatacgactcactatagggagaaggctCTTCAAAATATCAAAACAAAAAAAACC-3′
NR3C1_P1_10F (1_F region)	5′-aggaagagagTTTAATTTTTTAGGAAAAAGGGTGG-3′
NR3C1_P1_T7R (1_F region	5′-cagtaatacgactcactatagggagaaggctCCCTAAAACCTCCCCAAAAA-3′

TABLE 5 Rotated loading matrix for DNA methylation principal components

Gene region	Principal component (PC label)	CpG sites[a]	% variance explained
NR3C1-Primer 1 (1_F region)	PC1	38_39, 47	23
NR3C1-Primer 1 (1_F region)	PC2	12_13, 20_21, 35	21
NR3C1-Primer 1 (1_F region)	PC3	17_18, 36	19
NR3C1-Primer 2 (1_D region)	PC1	7, 8_9_10_11, 13, 18_19	23
NR3C1-Primer 2 (1_D region)	PC2	3_4, 5_6, 21_22_23	19
NR3C1-Primer 2 (1_D region)	PC3	1_2, 13, 15, 16, 17	18
NR3C1-Primer 2 (1_D region)	PC4	24_25_26	11

[a]CpG sites on the same fragment analyzed by mass spectrometry are linked, indicated by underline: e.g., 6_7.

assay. This might have happened at the generation of the antisense RNA/cleavage, deposition onto the spectroCHIP, or at the analysis in the mass spectrometer. This reduced the study's DNAm sample size from 56 to 45. All 56 samples are stored for future use that conforms to the informed consent approvals.

2.5 | Statistical tests

For statistical analyses of DNA methylation, we used the data reduction method of principal component analysis (PCA) given that PCA substantially reduces the number of tests performed and thus partly alleviates concerns about multiple testing, particularly for exploratory analyses (Ho, Ashbury, Taylor, Vanner, & King, 2016; Mulligan, D'Errico, Stees, & Hughes, 2012; Shumay, Logan, Volkow, & Fowler, 2012). Our complete assay and NR3C1 DNAm principal component analysis methods are detailed in Straight et al., 2019. The rotated loading matrix for our DNAm principal components by CpG site and proportion of variance explained are in Table 5.

We performed bivariate associations between the study's DNAm outcome variables and the potential confounders of age, wealth, and education using Spearman or Wilcoxon rank sum tests as appropriate to the variable. None of the covariates was significant except for parents' highest grade, which was weakly significant in a DNAm PC that did not significantly associate with any exposure variable. Therefore, covariates were set aside for analyses, and there was no need to adjust for them in multivariate models. Additionally, the sample sizes were small and the data violated the normality assumptions in ways that could not be easily addressed using simple transformations. Age might be expected to be significant in comparison to DNAm. The lack of significant association might be in part due to age estimation of our participants or the

fact that they are close together developmentally. However, other studies with participants of precise, documented ages have likewise found no significant associations when examining DNAm in NR3C1 exons 1F and 1D specifically (Cicchetti & Handley, 2017; Parent et al., 2017).

In order to examine NR3C1 in association with lifetime cumulative stress and our LEC factor variables, we created two sets of bivariate analyses using our NR3C1 DNA methylation principal components for each region (1_F and 1_D): First, we compared principal components derived from DNA methylation of exon 1_F and exon 1_D promoter regions, to LEC cumulative score (all reported stressors). Second, we compared the same DNAm principal components to the four LEC factors. We performed adjustment for multiple comparisons for the primary hypothesis results, setting the alpha at 0.1. This alpha was warranted to avoid Type II error due to the exploratory nature of the analyses and the difficulty of defining the family of tests (Feise, 2002). Additionally, more complex adjustment methods have been developed that are more suitable to DNAm candidate gene approaches and with higher power but these are not yet available in R, SPSS, or SAS.

We also performed sensitivity analyses. For the sensitivity analyses, we examined all eligible CpG sites. Eligibility criteria were that the CpG could not be on the same fragment with other CpG sites and must be sufficiently methylated for meaningful comparison. In CpG sites 14 and 47 in exon 1_F, only one and two participants respectively had methylation above zero. CpG sites 9, 19, 35 and 36 in exon 1_F, and CpG sites 7, 13, 15, 16, and 17 in exon 1_D were used for the sensitivity comparisons. Each of these CpG sites was compared to LEC cumulative exposure score and LEC Factor 3 score (due to its significance in primary hypothesis testing). Additionally, to further explore the experiences associated with LEC-5 Factor 3, we used participant combat experience interview data to create two war exposure timing variables (number of years since first war exposure and number of years since most recent war exposure) and two war fatality variables (Samburu and enemy fatalities in raids in which the participant participated). Samburu warfare is characterized by organized coalitional gun violence, with attacks that can occur on family home compounds, on livestock camps where warriors spend a lot of time caring for animals, or in the bush while herding. While some warriors engage in offensive attacks, others may be forced to defend their animals against attack but never engage in offensive attacks. For analyzing war fatalities, we created a count variable of exposure of 0, 1, 2, and 3 or more fatalities because of a tendency to overestimate enemy fatalities, underestimate friendly fatalities, and general imprecision at larger numbers. Based on cross comparison for the same battle raids, 0, 1, and 2 counts were precise, while a cut point of 3 ensured accuracy above 2. Descriptive statistics (Table 1) reflect the full range of reported fatalities.

Software: Factor version 10.3 software was used for the factor analysis of the LEC variables. Other data analysis was performed using SAS software, Version 9.4 of the SAS system for Windows, Copyright © 2013 SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, North Carolina. (Lorenzo-Seva & Ferrando, 2006); and IBM SPSS software, version 26 of the SPSS system for Mac, Copyright © 1989, 2019 IBM Corporation and its licensors. To compute *P*-values adjusted for multiple comparisons, R version 4.0.1 for Mac (2020-06—06), "See Things Now" was used. Copyright © 2020 The R Foundation for Statistical Computing.

3 | RESULTS

Descriptive statistics characterizing the sample are provided in Table 1 and a correlation matrix of covariates and stressor variables is provided in Table 6. Mean age of participants at measurement was 27.67 years. On the wealth self-rating (in relation to neighbors), 40% self-identified as poor, 47% as average and 13% as wealthy. Over a third (35%) had been employed in some form of wage labor or cash generating activity, while 31% of one of their parents had. With respect to specific stressors, 9% reported that their mothers were deceased and 39% reported that their fathers were deceased. Figure 1 provides a bar chart of percentages reported for what participants identified as the worst lifetime stressor. Family death ranked highest (just over 40%), followed by war (10%), with just over 30% stating that nothing rose to the level of a severe enough stressor to report. LEC cumulative life events score was significantly associated with both war exposure timing and fatality variables (Spearman's $rho = 0.455$ and 0.523, $P \leq .0001$). Timing of mothers' death was associated with either parents' highest grade (Spearman's $rho = 0.389$, $P = .01$). Both timing of mother's death and timing of first war exposure were associated with timing of worst stressor (Spearman's $rho = 0.417$, $P = .008$ for mother's death; Spearman's $rho = 0.323$, $P = .05$ for first war exposure).

Associations for the study's primary hypotheses are shown in Table 7. NR3C1 1_F DNAm Principal Components 3 (accounting for 19% of DNAm PC variance) was significantly associated with cumulative score on the LEC-5 (Spearman's $rho = 0.435$, $P = .005$). NR3C1 1_F

TABLE 6 Correlation matrix of primary variables and covariates[a]

	LEC score	#Years mother deceased	#Years father deceased	#Years most recent war	#Years first war exposure	#Years worst stressor	#Samburu fatalities
LEC score	1.000	0.044 (0.75)	0.163 (0.23)	0.455*** (<0.0001)	0.523*** (<0.0001)	0.268 (1.0)	0.547*** (<0.0001)
N	82	54	54	82	82	39	82
#Years since mother dec	0.044 (0.75)	1.000	−0.019 (0.89)	−0.143 (0.30)	−0.130 (0.35)	0.417** (0.008)	−0.063 (0.65)
N	54	54	54	54	54	39	
#Years since father dec	0.163 (0.23)	−0.019 (0.89)	1.000	0.340* (0.01)	0.338* (0.01)	0.227 (0.17)	0.176 (0.21)
N	54	54	54	54	54	39	53
#Years since most rec war	0.455*** (<0.0001)	−0.143 (0.30)	0.340* (0.01)	1.000	0.922** (<0.0001)	0.188 (0.25)	0.434*** (<0.0001)
N	82	54	54	82	82	39	82
#Years since first war	0.523*** (<0.0001)	−0.130 (0.35)	0.338* (0.01)	0.922*** (<0.0001)	1.000	0.323 (0.45)	0.582*** (<0.0001)
N	82	54	54	82	82	39	82
#Years since worst stressor	0.268 (1.0)	0.417** (0.008)	0.227 (0.17)	0.188* (0.25)	0.323* (0.05)	1000	0.221 (0.18)
N	39	39	39	39	39	38	
Samburu fatalities	0.547*** (<0.0001)	0.063 (0.65)	0.176 (0.21)	0.434*** (<0.0001)	0.582*** (<0.0001)	0.221 0.18	1000
N	82	53	53	82	82	38	82
Enemy fatalities	0.526*** (<0.0001)	0.049 (0.73)	0.264 (0.06)	0.507*** (<0.0001)	0.652*** (<0.0001)	0.323* 0.05	0.862*** (<0.0001)
N	82	53	53	82	82	38	82

Note: Not shown: participant age, education, and wealth not significant with any of the study's variables. Parents' highest grade significant with #years mother deceased (Spearman's rho = 0.389, $P = .01$).

[a]Coefficients and P value obtained from Spearman's correlation test.

*Statistical significance at .05.

**Statistical significance at .01.

***Statistical significance at <.0001.

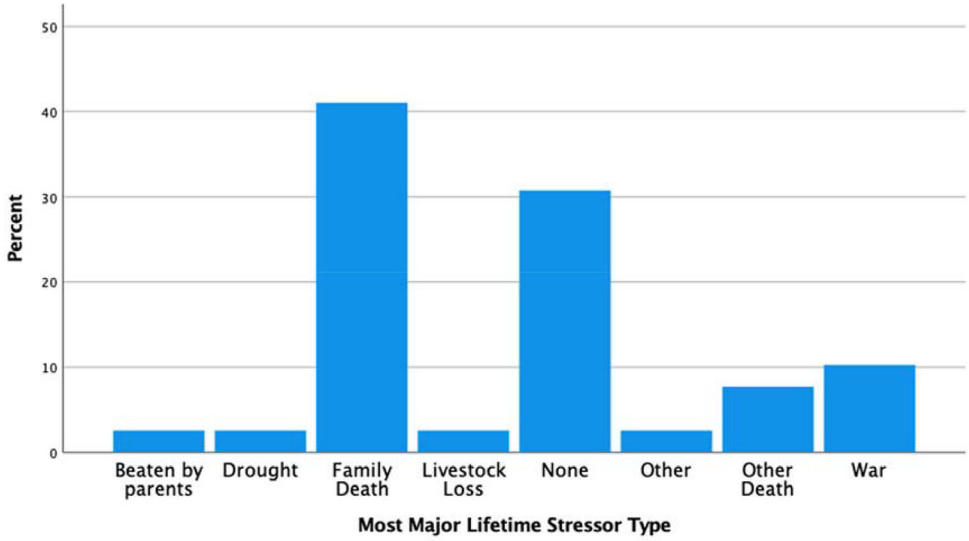

FIGURE 1 Bar chart showing participants' self-identified most major lifetime stressor by % of total (N = 39). Percentages are for each category relative to all categories endorsed, including "no major stressor" (none)

DNAm Principal Component 2 (accounting for 21% of DNAm PC variance) was significantly associated with LEC-5 Factor 3 Score (physical and weapons assault, war exposure, and "harm you caused" (accounting for 20.40% of LEC factor variance) (Spearman's *rho* = −0.427, *P* = .006). The 1_F region was not significantly associated with LEC-5 Factor 1 (disasters, illnesses, and uncontrollable events), Factor 2 (deaths and severe suffering), or Factor 4 (modern exposures for the study population of toxic substance and transportation accidents). The NR3C1 1_D region was not significantly associated with any of our exposure variables of interest.

Table 8 shows results of sensitivity analyses for all eligible CpG sites, comparing to LEC score, war exposure timing (# years since first and most recent war exposures), and two war fatality variables (#Samburu and enemy fatalities). Of the 9 CpG sites examined, 5 were significantly associated with the study's variables. In NR3C1 1_F, CpG 9 was less methylated (negatively correlated) with increasing years since most recent war exposure (Spearman's *rho* = −0.531, *P* = .01, DNAm change = −1.1%). CpG 36 was more methylated with higher LEC score (Spearman's *rho* = 0.368, *P* = .01, DNAm change = +5%), higher LEC Factor 3 score (Spearman's *rho* = 0.306, *P* = .04, DNAm change = +0.5%), increasing years since first war exposure (Spearman's *rho* = 0.425, *P* = .004, DNAm change = +7.5%), and most recent war exposure (Spearman's *rho* = 0.496, *P* = .001, DNAm change = +14%). Figures 2 and 3 provide scatter plots based on medians of most first and most recent war exposures in comparison to CpG 36. In NR3C1 1_D, CpG 7 was less methylated (negatively correlated) with higher LEC score (Spearman's *rho* = −0.352, *P* = .02, DNAm change = −6.3%), higher LEC Factor 3 score (Spearman's *rho* = −0.357, *P* = .02, DNAm change = −7.5%), and higher number of war fatalities (for Samburu fatalities, Spearman's *rho* = −0.318, *P* = .03, DNAm change = −7.1%; for enemy fatalities, Spearman's *rho* = −0.463, *P* = .001, DNAm change = −7.6%). Figures 4 and 5 provide box plots of Samburu and enemy fatalities in comparison to Cpg 7. CpG 15 had more methylation with higher Samburu fatalities (Spearman's *rho* = 0.343, *P* = .02, DNAm change = +5.1%). CpG 17 was more methylated with increasing years since first war exposure (Spearman's *rho* = 0.330, *P* = .03, DNAm change = +4.5%) and most recent war exposure (Spearman's *rho* = 0.389, *P* = .008, DNAm change = +6%).

Our primary interest in this article is in events warriors directly experienced. However, for comparison to our primary LEC-5 factor analysis focusing exclusively on experienced events, we performed a secondary factor analysis that included events warriors experienced vicariously in addition to those directly experienced (results not shown in tables). In NR3C1 1_F, the first factor (accounting for 14.12% of variance) of this experienced + witnessed factor analysis was significantly associated with DNAm Principal Component 3 (Spearman's *rho* = 0.388, *P* = .013) and CpG 36 (Spearman's *rho* = −.359, *P* = .017). In NR3C1 1_D, the first factor was significantly associated with CpG 7 (Spearman's *rho* = 0.309, *P* = .041) and Cpg 13 (Spearman's *rho* = −0.391, *P* = .009), while the second factor was significantly associated with Principal Component 2 (Spearman's *rho* = 0.388, *P* = .019).

4 | DISCUSSION

The population of young men targeted for this study engages in risky and violent behaviors, as well as high energetic expenditure, in contexts of chronic food and water insecurity. Samburu warriors are universally

TABLE 7 Association of DNA methylation principal components with LEC

Variable	Correlation coefficient (Spearman's rho) (*P* value)[a]						
	NR3C1-1_F PC1	NR3C1-1_F PC 2	NR3C1-1_F PC 3	NR3C1-1_D PC1	NR3C1-1_D PC2	NR3C1-1_D PC3	NR3C1-1_D PC4
LEC Score	0.223 (0.16)	−0.082 (0.61)	0.435** (0.005)	−0.198 (0.24)	−0.214 (0.21)	0.111 (0.51)	−0.005 (0.98)
N	41	41	41	37	37	37	37
LEC Factor 1	0.069 (0.67)	−0.274 (0.09)	0.206 (0.20)	−0.259 (0.13)	0.004 (0.98)	−0.089 (0.61)	−0.244 (0.15)
LEC Factor 2	0.176 (0.28)	0.253 (0.12)	0.212 (0.19)	−0.055 (0.75)	−0.118 (0.50)	0.245 (0.15)	0.219 (0.20)
LEC Factor 3	0.226 (0.16)	−0.427** (0.006)	0.275 (0.07)	−0.101 (0.56)	−0.265 (0.12)	−0.139 (0.42)	−0.056 (0.75)
LEC Factor 4	−0.078 (0.63)	−0.172 (0.29)	−0.043 (0.79)	0.087 (0.61)	−0.021 (0.90)	−0.054 (0.76)	−0.049 (0.77)
N for all LEC Factors	41	41	41	37	37	37	37

Note: LEC Score adjusted significant *P* value was significant at alpha of .1 (adjusted *P* value = .1). Adjusted *P* value of LEC Factor 3 = 0.107. Both were computed with false discovery rate (FDR) method using all tests from this table and sensitivity analyses (Table 8). Age, wealth, and education covariates were not significantly associated with any exposure variables nor with any DNAm PCs and thus were set aside for analyses.

[a]Coefficients and *P* value significance obtained from Spearman's correlation test.

**Statistical significance at .01.

expected to defend their families' livestock against predators and neighboring enemy groups and walk long distances with their livestock. Some of them may nevertheless engage in the modern activities of formal education and wage labor, and all of them interact with cash market economies to buy and sell livestock.

Our study employed qualitative methods that were important to reliable data collection and coding and enhanced interpretation of the results. The LEC-5 Factors on their own (as cultural data, independent of DNAm) revealed patterns that make sense for young men in a drought-prone, resource scarce region. The clusters of events in Factors 1, 2, and 3 routinely affect Samburu families; cultural practices and memory forms address them. Moreover, this clustering of events fits warrior experience specifically because—as captured by Factor 3—warriors uniquely experience violent assault, combat, and harm caused to others within the same cultural framework of their duties as *lmurran*. This is in contrast to uncontrollable natural events by which Samburu understand their identity as a people (Factor 1), forms of death for which Samburu have extensive cultural practices related to contagion (Factor 2) (Straight, 2005, 2007, 2009; Straight, Lane, Hilton, & Letua, 2016), and accidental or deliberate acts involving the technologies of modernity (Factor 4) (Straight, Pike, Hilton, & Österle, 2015).

What the LEC failed to differentiate was the enormity of death itself, as its own optic for the Samburu to a degree paralleled only in other societies with equally high rates of premature death from multiple causes. To spend time in Samburu, witnessing death is unavoidable (Straight, 2007, 2019). Death is distributed across all four of the LEC factors, whether violent, natural, or accidental (from "modern" or "traditional" causes). Given the likelihood of experiencing death within one's family before reaching adulthood, it is no wonder that warriors identified family deaths most frequently as their worst life stressor, with other deaths ranking third (behind war at second). As the overall emphasis of the original data collection was on empathy and warfare experiences, we collected ample, nuanced data in the initial phases that permitted us to capture exposure to war-caused violent deaths for our sensitivity analyses. While we attempted to collect data on family deaths in the third/final phase of data collection, the sample sizes were small due to dry-season caused attrition. With the further parsing of the reduced DNAm sample, the sample size for family deaths was prohibitively small. This kind of lacuna motivated us to employ the full interview version of the LEC-5 for our subsequent study and create a Samburu-specific coding scheme for the creation of weighted variables (papers in preparation).

TABLE 8 Association of DNA methylation in eligible CpG sites with LEC cumulative score plus stress exposure timing from sensitivity analyses

Correlation coefficient (Spearman's rho) (*P* value)[a]									
Variable	**NR3C1-1$_F$ CpG 9**	**NR3C1-1$_F$ CpG 19**	**NR3C1-1$_F$ CpG 35**	**NR3C1-1$_F$ CpG 36**	**NR3C1-1$_D$ CpG 7**	**NR3C1-1$_D$ CpG 13**	**NR3C1-1$_D$ CpG 15**	**NR3C1-1$_D$ CpG 16**	**NR3C1-1$_D$ CpG 17**
CpG Min, Max %	0.0% 100%	0.0% 52%	0.0%, 81%	0.0%, 19%	0.0%, 21%	0.0%, 6%	0.0%, 17%	0.0%, 9%	0.0%, 23%
CpG SD	0.1851	0.0900	0.2247	0.0052	0.0469	.0154	.0364	.0212	.0385
N	22	36	45	45	45	45	45	45	45
LEC Score	−0.281 (0.21)	−0.069 (0.69)	−0.147 (0.33)	0.368* (0.01)	−0.352* (0.02)	−0.098 (0.52)	0.165 (0.28)	−0.057 (0.71)	0.190 (0.21)
% change[b]				+5%	−6.3%				
LEC Factor 3 Score	−0.065 (0.78)	−0.038 (0.82)	−0.255 (0.09)	0.306* (0.04)	−0.357* (0.02)	−0.289 (0.05)	0.185 (0.23)	−0.141 (0.36)	0.063 (0.68)
% change[b]				+0.5%	−7.5%				
First war # years ago	−0.356 (0.10)	−0.134 (0.44)	−0.060 (0.70)	0.425 ** (0.004)	−0.172 (0.26)	0.010 (0.95)	0.109 (0.48)	0.09 (0.85)	0.330* (0.03)
% change[b]				+7.5%					+4.5%
Most recent war # years ago	−0.531* (0.01)	−0.085 (0.62)	−0.096 (0.53)	0.496** (0.001)	−0.136 (0.37)	−0.001 (1.00)	0.129 (0.40)	0.058 (0.70)	0.389** (0.008)
% change[b]	−1.1%			+14%					+6%
Samburu fatalities	−0.87 (0.70)	−0.150 (0.38)	−0.057 (0.71)	0.202 (0.18)	−0.318* (0.03)	0.038 (0.81)	0.343* (0.02)	−0.064 (0.67)	0.188 (0.22)
% change[b]					−7.1%		+5.1%		
Enemy fatalities	−0.102 (0.65)	−0.158 (0.36)	−0.133 (0.39)	0.179 (0.39)	−0.463** (0.001)	−0.004 (0.98)	0.211 (0.16)	0.006 (0.97)	0.173 (0.26)
% change[b]					−7.6%				

Note: Significant adjusted *P* value of first war experience timing in 1_F CpG 36 is significant at alpha .1 (adjusted *P* value = .1). Significant adjusted *P* value of most recent war timing in 1_F CpG 36 and enemy fatalities in 1_D CpG 7 were both significant at alpha = .05 (adjusted *P* value = .045), computed with FDR method using all tests from this table and primary analyses (Table 7). Covariates were nonsignificant in preliminary analyses and thus were set aside.

[a]Coefficients and *P* value significance obtained from Spearman's Correlation test.

[b]% DNA methylation change in significant CpG sites indicating maximum change from lowest to highest exposure.

*Statistical significance at .05.

**Statistical significance at .01. Significant adjusted *P* value of first war experience timing in 1_F CpG 36 is significant at alpha 0.1 (adjusted *P* value = .1).

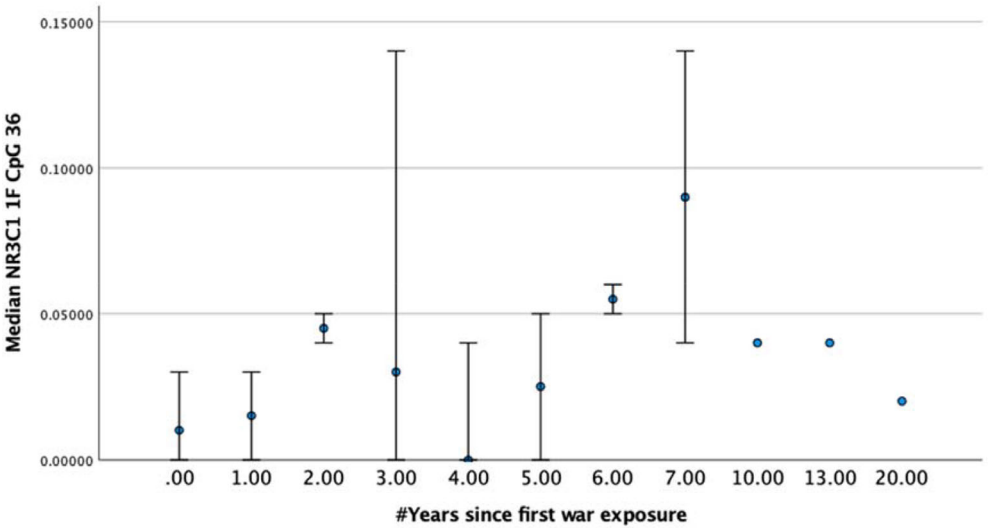

FIGURE 2 Scatter chart with medians for illustration, showing #years since first war exposure in comparison to NR3C1 1_F CpG 36. Error bars show 95% confidence interval. The data are not normally distributed: the study's results (as presented in the tables) are based on nonparametric statistical tests

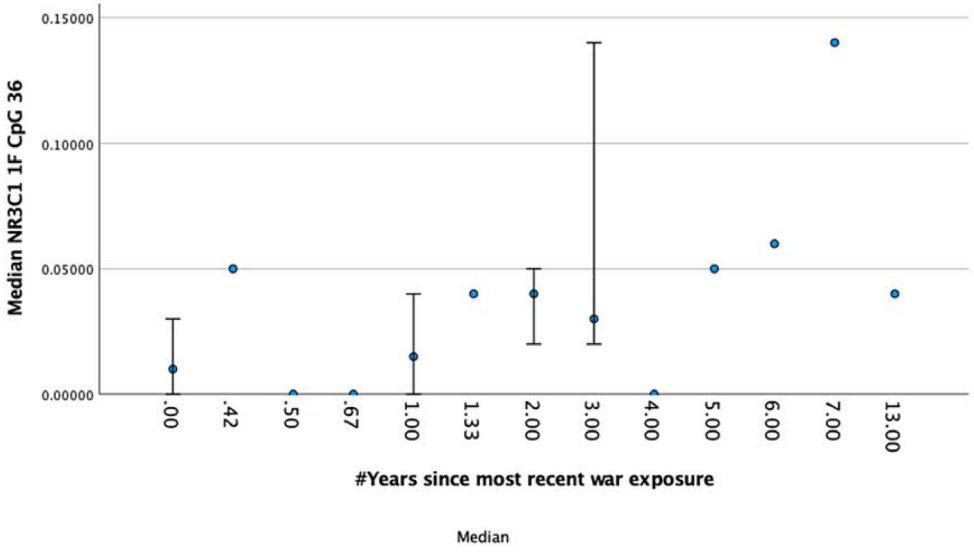

FIGURE 3 Scatter chart with medians for illustration, showing #years since most recent war exposure in comparison to NR3C1 1_F CpG 36. Error bars show 95% confidence interval. The data are not normally distributed: the study's results (as presented in the tables) are based on nonparametric statistical tests

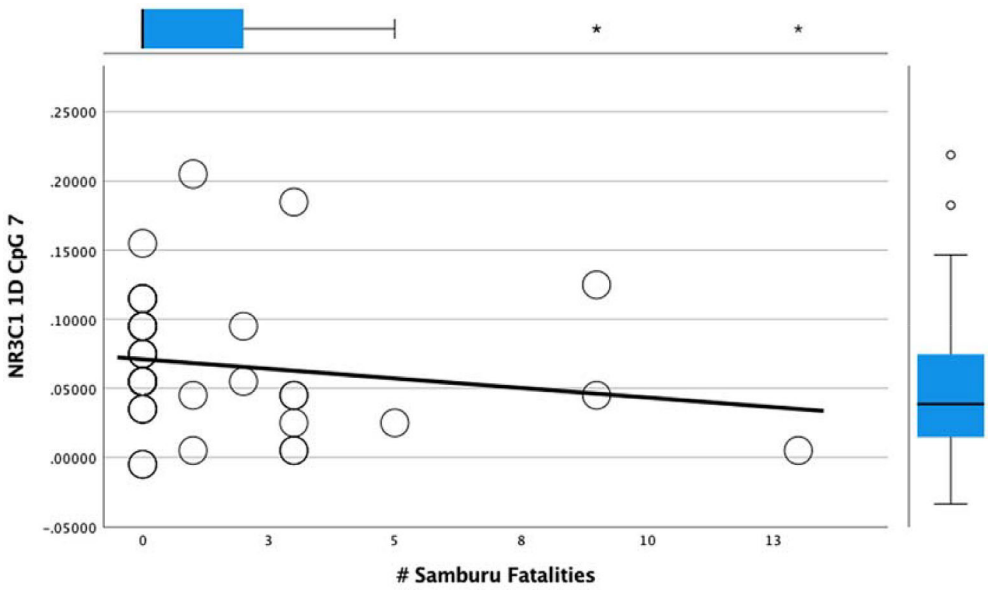

FIGURE 4 Box plot for illustration, showing # of Samburu fatalities in comparison to NR3C1 1_D CpG 7. The data are not normally distributed—the study's results (as presented in the tables) are based on nonparametric statistical tests

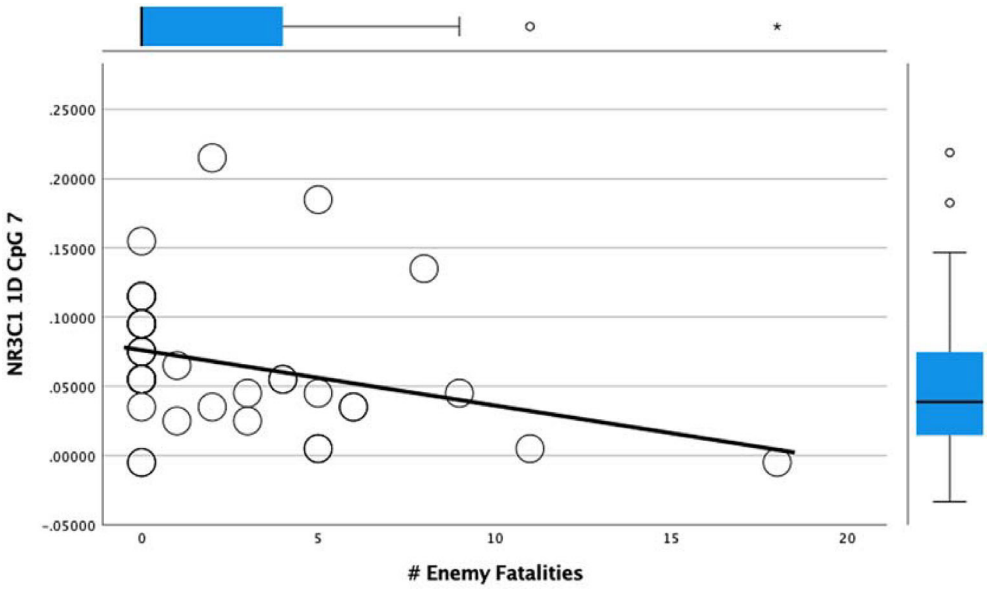

FIGURE 5 Box plot for illustration, showing # of enemy fatalities in comparison to NR3C1 1_D CpG 7. The data are not normally distributed—the study's results (as presented in the tables) are based on nonparametric statistical tests

The distinction in experience between mothers' and fathers' deaths in the full (non-DNAm-reduced) sample is noteworthy. Fathers' deaths are significant in comparison to timing of war exposure, pointing to the possibility that a number of participants' war experiences coincided with their fathers' deaths. This could be an unintended consequence of polygyny, as Samburu men are typically in their mid- to late thirties at marriage and continue to father children into old age. Thus, there is a strong chance that a Samburu child will lose their father in childhood or adolescence, which makes it a commonly shared experience for children. It is possible that young men may feel emboldened to engage in coalitional gun violence after losing their fathers, but this is speculation without systematic study to replicate and explain this finding in our data. Fathers' brothers do play an important role in decisions affecting the lineage and in managing the family after a loss. Children experience mothers' deaths as particularly devastating, with little to ameliorate the impact. In the Samburu polygynous homestead, mothers care for their own children and children they foster. Given chronic food insecurity, women make difficult choices daily, as to how much milk to draw from livestock to feed humans vs how much to leave for livestock young, and as to how much milk and other food to give to each child and adult in their care. Even in adulthood, Samburu speak painfully about the loss of mothers, typically noting that they were denied food, clothing, and educational opportunities because of being fostered with co-wives or other relatives. In fact, the legend of how Samburu acquired tobacco is rooted in a child's loss of a mother and subsequent ill treatment by the co-wife with whom the child is fostered (Straight, 2007). Moreover, in the case of young men, mothers' power is very strong to intercede forcefully and induce their natal kin to help sons acquire the livestock necessary to marry and get other assistance in time of need. In this context, the strong association between mothers' death and worst lifetime stressor makes sense, although a larger study is needed to replicate this finding and compare it to DNAm.

Turning to the DNAm findings, with respect to our primary hypothesis testing using our DNAm Principal Components, our findings in the PCs for exon 1_D were all null. In exon 1_F, the significant association between Principal Component (PC) 3 (CpG sites 17_18 and 36) of the NR3C1 1_F promoter region and cumulative score on the LEC-5 is consistent with other studies of NR3C1 and a variety of life stressors, such as childhood maltreatment (Romens et al., 2015; Tyrka et al., 2012), bullying (Efstathopoulous et al., 2018), and parental death (Melas et al., 2013; Tyrka et al., 2012). The significant association between the second principal component (CpG sites 12_13, 20_21, and 35) of NR3C1 1_F and LEC Factor 3, violent assault, combat, and "harm you caused" is an illustrative finding for the young men targeted for this study, for which violence and combat are culturally mandated and yet extremely stressful as already noted. The findings from the corpus of DNAm studies related to war experience are more complicated, as discussed below in the context of our sensitivity analyses.

The percentage of DNAm changes in our sensitivity analyses varied from as low as 0.5% to as high as 14%. The range of error in detecting DNAm is 5%. Other studies point to the biological relevance of micro DNAm changes (<10%) associating to phenotypic variation and epigenetic programming events (specific to NR3C1: Leenen, Muller, & Turner, 2016; see also Parent et al., 2017).

Of the four exon 1_F CpG sites that met the criteria for sensitivity analyses, two had significant findings: CpG 9 was less methylated with more distant exposure to war, as measured by the "most recent war" timing variable. In gene expression studies of NR3C1 exon 1_F region, DNA methylation and gene expression have typically (though not always) been inversely correlated, with hypermethylation negatively associated with gene expression, and hypomethylation positively associated with gene expression (Watkeys et al., 2018).

CpG 36 (in the NGF1-A binding region) significantly associated to the most variables in the 1_F region: LEC cumulative score, LEC Factor 3 score, LEC experienced + witnessed Factor 1 score, and years since both the first/earliest and most recent warfare exposure, all in the direction of higher methylation. This is consistent with multiple previous studies reporting CpG 36 as hypermethylated in comparison to early stress (Palma-Gudiel et al., 2015, critical review). The fact that the LEC scores capture a mix of early and more recent stresses that are varied in nature is useful for examining the impact of all-cause stress. At the same time, this is at the cost of nuance and may at least partly explain the weakness of the association in comparison to the more precise variables of war stressor timing.

Of the five exon 1_D CpG sites that met the criteria for sensitivity analysis, four had significant associations: CpG 17 was more methylated in comparison to both war exposure timing variables, while CpG 15 was more methylated in comparison to participating in a raid in which there were "friendly" (Samburu) fatalities (more methylation with higher fatalities). CpG 13 was less methylated in comparison to LEC experienced + witnessed Factor 1 score. CpG 7 significantly associated to the most variables in this region: LEC score, LEC Factor 3 score, LEC experienced + witnessed score Factor 1 score, participating in a raid in which there were Samburu fatalities, and participating in a raid in which there were enemy fatalities, all in the direction of lower methylation with increased exposures. The association was particularly strong for enemy fatalities, which is consistent with the "harm you caused" element of LEC Factor 3. It is likewise consistent with other studies of East African warriors examining the moral conflict of warfare in the context of cultural norms (Straight 2017; Zefferman & Mathew, 2020) and empathy (Straight et al., 2020).

Few studies have examined exon 1_D of the NR3C1 promoter region. Tyrka et al. (2015) found exon 1_D hypermethylated in comparison to a variety of early life stressors in preschool children but null findings in the CpG sites that were significant in our study. Our 1_D findings are consistent with those of Parent et al. (2017), although once again, not in the same CpG sites as our study. They were able to measure DNAm in children ages 3 to 5 years within 6 months of exposure and again at approximately a year post-exposure, observing hypermethylation within 6 months of traumatic events and then hypomethylation a year after. Based on these findings, Parent and colleagues hypothesize that mixed findings of hyper- vs hypomethylation reported in previous studies may reflect an acute hypermethylation response to early childhood stressors but hypomethylation in response to continued chronic or severe stress throughout development. As noted in the Introduction, Vukojevic et al. (2014) point to the role of DNAm and gene expression in memory enhancement and suppression. This may partly explain individual variation in coping with painful experiences. Hypomethylation may suggest enhanced glucocorticoid negative feedback in individuals experiencing trauma (Morris, 2012).

As a proof of concept, our study was limited by small sample size, brief duration in each phase (sandwiched between data collection for a large-scale, different study), and piloting of the LEC in this population. Additionally, we were unable to correct for cellular heterogeneity because this is a candidate-gene study, and reference-free methods are designed for epigenome-wide studies. Nevertheless, the DNAm changes in several of our significant comparisons were above the range of error and some of our findings survived adjustment for multiple testing at either 0.05 or 0.1. A full-scale study is called for to attempt to replicate and deepen the findings.

5 | CONCLUSION

This article presented findings of our study that examined NR3C1 methylation in a group of Kenyan pastoralist young men in relation to culturally relevant difficult experiences, including coalitional lethal gun violence, and timing of exposure to that violence. Our findings contributed both to the well-studied exon 1_F of NR3C1 and to the less well examined exon 1_D.

Our results are consistent with findings in previous studies of NR3C1, pointing toward DNA methylation changes in response to cumulative stressors across the life course, and toward the importance of stressor timing. Our findings concerning DNAm in relation to stressor timing point to complexities in understanding the direction of methylation and the need to examine individual CpG sites. Our study may be of particular interest in its focus on an understudied population for DNA methylation research—young men in a small-scale, low-income society who are also ethnic marginals. The strong association between DNAm changes and participating in raids that resulted in enemy deaths is particularly noteworthy.

ACKNOWLEDGMENTS

In Kenya, the authors would like to thank Kenya's National Commission for Science, Technology and Innovation (NACOSTI) for permission to conduct research, and the Samburu participants and their families for supporting and participating in the research. The research was supported by intramural grants from the Western Michigan University Office of the Vice President for Research and College of Arts and Sciences. Background research on Samburu warriors and coalitional gun violence was supported by National Science Foundation Senior Grant 0413431. Appreciation is extended to Charles Hilton and to anonymous reviewers for incisive comments that helped the authors improve this article.

AUTHOR CONTRIBUTIONS

Bilinda Straight: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing-original draft; writing-review and editing. **Georgiana Fisher:** Formal analysis; software; validation; writing-review and editing. **Belinda L. Needham:** Conceptualization; formal analysis; methodology; validation; writing-review and editing. **Amy Naugle:** Conceptualization; formal analysis; methodology; validation; writing-review and editing. **Charles Olungah:** Data curation; investigation; project administration; writing-review and editing. **Puntipa Wanitjirattikal:** Formal analysis; writing-review and editing. **Cecilia Root:** Conceptualization; investigation; writing-review and editing. **Jen Farman:** Conceptualization; investigation; writing-review and editing. **Todd Barkman:** Formal analysis; validation; writing-review and editing. **Claudia Lalancette:** Formal analysis; software; validation; writing-review and editing.

CONFLICT OF INTEREST

None of the authors has a conflict of interest.

DATA AVAILABILITY STATEMENT

The data collected for this article includes sensitive clandestine economic activity data and DNA methylation data from an indigenous population. Study participants did not give consent for DNA methylation data to be deposited into an online repository or shared. Ethical responsibility with respect to this type of data is high, and policies at the Kenyan national and Samburu local levels are still developing.

ORCID

Bilinda Straight https://orcid.org/0000-0002-1387-5514

REFERENCES

Bollig, M., & Oesterle, M. (2008). "We turned our enemies into baboons": Warfare, ritual and pastoral identity among the Pokot of northern Kenya. In M. Bollig, A. Rao, & M. Bock (Eds.), *The practice of war: Production, reproduction and communication of armed violence* (pp. 23–51). New York: Berghahn Books.

Buffa, G., Dahan, S., Sinclair, I., St-Pierre, M., Roofigari, N., Mutran, D., ... Dancause, K. N. (2018). Prenatal stress and child development: A scoping review of research in low- and middle-income countries. *PLoS One*, *13*(12), e0207235. https://doi.org/10.1371/journal.pone.0207235

Cicchetti, D., & Handley, E. D. (2017). Methylation of the glucocorticoid receptor gene (NR3C1) in maltreated and nonmaltreated children: Associations with behavioral undercontrol, emotional lability/negativity, and externalizing and internalizing symptoms. *Development and Psychopathology*, *29*(5), 1795–1806. https://doi.org/10.1017/S0954579417001407

Cook, K. H., & Vizy, E. K. (2012). Impact of climate change on mid-twenty-first century growing seasons in Africa. *Climate Dynamics*, *39*(2012), 2937–2955. https://doi.org/10.1007/s00382-012-1324-1

Cook, K. H., & Vizy, E. K. (2013). Projected changes in East African rainy seasons. *Journal of Climate*, *26*, 5931–5948. https://doi.org/10.1175/JCLI-D-12-00455.1

Efstathopoulous, P., Andersson, F., Melas, P. A., Yang, L. L., Villaescusa, C., Rüegg, J., ... Lavebratt, C. (2018). NR3C1 hypermethylation in depressed and bullied adolescents. *Translational Psychiatry*, *8*, 121. https://doi.org/10.1038/s41398-018-0169-8

Feise, R. (2002). Do multiple outcome measures require p-value adjustment? *BMC Medical Research Methodology*, *2*, 8. https://doi.org/10.1186/1471-2288-2-8

Funk, C. (2011). We thought trouble was coming. *World View*, *476*, 7. go.nature.com/uoe7at

Greiner, C. (2013). Guns, land, and votes: Cattle rustling and the politics of boundary (re)making in northern Kenya. *African Affairs*, *112*(447), 216–237. https://doi.org/10.1093/afraf/adt003

Herba, C. M., Glover, V., Ramchandani, P. G., & Rondon, M. B. (2016). Maternal depression and mental health in early childhood: An examination of underlying mechanisms in low-income and middle-income countries. *Lancet Psychiatry*, *2016*(3), 983–992. https://doi.org/10.1016/S2215-0366(16)30148-1

Ho, V., Ashbury, J. E., Taylor, S., Vanner, S., & King, W. D. (2016). Quantification of gene-specific methylation of DNMT3B and MTHFR using Sequenom EpiTYPER. *Data in Brief*, *6*, 39–46.

Holtzman, J. (2016). *Killing your neighbors: Friendship and violence in northern Kenya an beyond*. Berkeley: University of California press. https://www.ucpress.edu/book/9780520291928/killing-your-neighbors

Houseman, E., Molitor, J., & Marsit, C. J. (2014). Reference-free cell mixture adjustments in analysis of DNA methylation data. *Bioinformatics*, *30*(10), 1431–1439. https://doi.org/10.1093/bioinformatics/btu029

Kasfir, S. L. (2007). *African art and the colonial encounter: Inventing a global commodity*. Bloomington: Indiana University Press. http://www.iupress.indiana.edu/product_info.php?products_id=41627

Langie, S. A. S., vel Szic, K. S., Declerck, K., Traen, S., Koppen, G., Van Camp, G., … De Boever, P. (2016). Whole-genome saliva and blood DNA methylation profiling in individuals with a respiratory allergy. *PLoS One*, *11*(3), e0151109. https://doi.org/10.1371/journal.pone.0151109

Leenen, F. A. D., Muller, C. P., & Turner, J. D. (2016). DNA methylation: Conducting the orchestra from exposure to phenotype? *Clinical Epigenetics*, *8*(2016), 92. https://doi.org/10.1186/s13148-016-0256-8

Lorenzo-Seva, U., & Ferrando, P. J. (2006). FACTOR: A computer program to fit the exploratory factor analysis model. *Behavior Research Methods*, *38*(1), 88–91. https://doi.org/10.3758/BF03192753

McDade, T. W., Ryan, C., Jones, M. J., MacIsaac, J. L., Morin, A. M., Meyer, J. M., … Kuzawa, C. W. (2017). Social and physical environments early in development predict DNA methylation of inflammatory genes in young adulthood. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(29), 7611–7616. www.pnas.org/cgi/doi/10.1073/pnas.1620661114

Melas, P., Wei, Y., Wong, C. C. Y., Sjöholm, L. K., Åberg, E., Mill, J., … Lavebratt, C. (2013). Genetic and epigenetic associations of MAOA and NR3C1 with depression and childhood adversities. *International Journal of Neuropsychopharmacology*, *16*, 1513–1528. https://doi.org/10.1017/S1461145713000102

Mislevy, R. J., & Bock, R. D. (1990). *BILOG 3 Item analysis and test scoring with binary logistic models*. Mooresville: Scientific Software.

Morris, M. (2012). Relations among posttraumatic stress disorder, comorbid major depression, and HPA function: A systematic review and meta-analysis. *Clinical Psychology Review*, *32*(4), 301–315. https://doi.org/10.1016/j.cpr.2012.02.002

Mulligan, C., D'Errico, N., Stees, J., & Hughes, D. (2012). Methylation changes at NR3C1 in newborns associate with maternal prenatal stress exposure and newborn birth weight. *Epigenetics*, *7*(8), 853–857.

Opiyo, F., Wasonga, O., Nyangito, M., Schilling, J., & Munang, R. (2015). Drought adaptation and coping strategies among the Turkana pastoralists of northern Kenya. *International Journal of Disaster Risk Science*, *6*, 295–309. https://doi.org/10.1007/s13753-015-0063-4

Palma-Gudiel, H., Córdova-Palomera, A., Leza, J. C., & Fañanás, L. (2015). Glucocorticoid receptor gene (NR3C1) methylation processes as mediators of early adversity in stress-related disorders causality: A critical review. *Neuroscience and Biobehavioral Reviews*, *55*, 520–535. https://doi.org/10.1016/j.neubiorev.2015.05.016

Palma-Gudiel, H., Córdova-Palomera, A., Tornador, C., Falcón, C., Bargalló, N., Deco, G., & Fañanás, L. (2018). Increased methylation at an unexplored glucocorticoid responsive element within exon 1_D of NR3C1 gene is related to anxious-depressive disorders and decreased hippocampal connectivity. *European Neuropsychopharmacology*, *28*(2018), 579–588. https://doi.org/10.1016/j.euroneuro.2018.03.015

Parent, J., Parade, S. H., Laumann, L. E., Ridout, K. K., Yang, B.-Z., Marsit, C. J., … Tyrka, A. R. (2017). Dynamic stress-related epigenetic regulation of the glucocorticoid receptor gene promoter during early development: The role of child maltreatment. *Development and Psychopathology*, *29*(2017), 1635–1648. https://doi.org/10.1017/S0954579417001298

Romens, S. E., McDonald, J., Svaren, J., & Pollak, S. D. (2015). Associations between early life stress and gene methylation in children. *Child Development*, *86*(1), 303–309. https://doi.org/10.1111/cdev.12270

Shumay, E., Logan, J., Volkow, N. D., & Fowler, J. S. (2012). Evidence that the methylation state of the monoamine oxidase a (MAOA) gene predicts brain activity of MAOA enzyme in healthy men. *Epigenetics*, *7*(10), 1151–1160. https://doi.org/10.4161/epi.21976

Smith, A. K., Kilaru, V., Klengel, T., Mercer, K. B., Bradley, B., Conneely, K. N., … Binder, E. B. (2015). DNA extracted from saliva for methylation studies of psychiatric traits: Evidence tissue specificity and relatedness to brain. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics*, *168B*(1), 36–44. https://doi.org/10.1002/ajmg.b.32278

Straight, B. (2005). In the belly of history: Memory, forgetting, and the hazards of reproduction among Samburu of northern Kenya. *Africa*, *75*(1), 83–104. https://doi.org/10.3366/afr.2005.75.1.83

Straight, B. (2007). *Miracles and extraordinary experience in Northern Kenya*. Philadelphia, PA: University of Pennsylvania Press.

Straight, B. (2009). Making sense of violence in the badlands of Kenya. *Anthropology and Humanism*, *34*(1), 21–30. https://doi.org/10.1111/j.1548-1409.2009.01020.x

Straight, B. (2019). Unanchored deaths: Grieving the unplaceable in Samburu. In A. Panagiotopoulous & D. Espirito Santo (Eds.), *Articulate necrographies: Comparative perspectives on the voices and silences of the dead* (pp. 125–140). New York: Berghahn Press.

Straight, B., Lane, P., Hilton, C., & Letua, M. (2016). Dust people: Samburu perspectives on disaster, identity, and resilience. *Journal of East African Studies*, *10*(1), 168–188. https://doi.org/10.1080/17531055.2016.1138638

Straight, B., Naugle, A., Farman, J., Root, C., Lekalgitele, S., & Olungah, C. (2020). Killing, mercy, and empathic emotions: The emotional lives of East African warriors. Chapter 8. In W. Kiblinger (Ed.), *Analyzing and learning from human conflict*. Berlin: Springer.

Straight, B., Needham, B. L., Oniescu (Fisher), G., Wanitjirattikal, P., Barkman, T., Root, C., … Lekalgitele, S. (2019). Prosocial emotion, adolescence, and warfare: DNA methylation associates with culturally salient combat variables. *Human Nature*, *30*, 192–216. https://doi.org/10.1007/s12110-019-09344-6

Straight, B., Pike, I., Hilton, C., & Österle, M. (2015). Suicide in three East African pastoralist communities and the role of researcher outsiders for positive transformations: A case study. *Culture, Medicine, & Psychiatry*, *39*(3), 557–578. https://doi.org/10.1007/s11013-014-9417-4

Thayer, Z. M., & Non, A. L. (2015). Anthropology meets epigenetics: Current and future directions. *American Anthropologist*, *117*(4), 722–735. https://doi.org/10.1111/aman.12351

Tierney, J. E., Ummenhofer, C. C., & deMenocal, P. B. (2015). Past and future rainfall in the horn of Africa. *Science Advances (Climatology)*, *1*(9), e1500682. https://doi.org/10.1126/sciadv.1500682

Turecki, G., & Meany, M. J. (2016). Effects of the social environment and stress on glucocorticoid receptor gene methylation: A systematic review. *Biological Psychiatry*, *79*(2), 87–96. https://doi.org/10.1016/j.biopsych.2014.11.022

Tyrka, A. R., Parade, S. H., Eslinger, N. M., Marsit, C. J., Lesseur, C., Armstrong, D. A., ... Seifer, R. (2015). Methylation of exons 1_D, 1_F, and 1_H of the glucocorticoid receptor gene promoter and exposure to adversity in preschool-aged children. *Development and Psychopathology*, *27*(2015), 577–585. https://doi.org/10.1017/S0954579415000176

Tyrka, A. R., Price, L. H., Marsit, C., Walters, O. C., & Carpenter, L. L. (2012). Childhood adversity and epigenetic modulation of the leukocyte glucocorticoid receptor: Preliminary findings in healthy adults. *PLoS One*, *7*(1), e30148. https://doi.org/10.1371/journal.pone.0030148

van der Knapp, L. J., Riese, H., Hudziak, J. J., Verbiest, M. M. P. J., Verhulst, F. C., Oldehinkel, A. J., & van Oort, F. V. A. (2014). Glucocorticoid receptor gene (NR3C1) methylation following stressful events between birth and adolescence: The TRAILS study. *Translational Psychiatry*, *4*, e381. https://doi.org/10.1038/tp.2014.22

Vukojevic, V., Kolassa, I.-T., Fastenrath, M., Gschwind, L., Spalek, K., Milnik, A., ... de Quervain, D. J.-F. (2014). Epigenetic modification of the glucocorticoid receptor gene is linked to traumatic memory and post-traumatic stress disorder risk in genocide survivors. *The Journal of Neuroscience*, *34*(31), 10274–10284. https://doi.org/10.1523/JNEUROSCI.1526-14.2014

Watkeys, O. J., Kremerskothen, K., Quidé, Y., Fullerton, J. M., & Green, M. J. (2018). Glucocorticoid receptor gene (NR3C1) DNA methylation in association with trauma, psychopathology, transcript expression, or genotypic variation: A systematic review. *Neuroscience and Biobehavioral Reviews*, *95*(2018, 85–122. https://doi.org/10.1016/j.neubiorev.2018.08.017

Weathers, F. W., Blake, D. D., Schnurr, P. P., Kaloupek, D. G., Marx, B. P., & Keane, T. M. (2013). *The life events checklist for DSM-5 (LEC-5)*. Instrument available from the National Center for PTSD at www.ptsd.va.gov.

Weaver, I. C., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Secki, J. R., ... Meaney, M. J. (2004). Epigenetic Programming by Maternal Behavior. *Nature Neuroscience*, *7*, 847–854.

Yehuda, R., Daskalakis, N. P., Desarnaud, F., Makotkine, I., Lehrner, A. L., Koch, E., ... Bierer, L. M. (2013). Epigenetic biomarkers as predictors and correlates of symptom improvement following psychotherapy in combat veterans with PTSD. *Frontiers in Psychiatry*, *4*, 118. https://doi.org/10.3389/fpsyt.2013.00118

Zefferman, M., & Mathew, S. (2020). Combat stress in warriors of a small-scale society highlight evolutionary roots of post-traumatic stress disorder and moral injury in war. OSF Preprints. January 9. https://doi.org/10.31219/osf.io/wtc8d.

How to cite this article: Straight B, Fisher G, Needham BL, et al. Lifetime stress and war exposure timing may predict methylation changes at NR3C1 based on a pilot study in a warrior cohort in a small-scale society in Kenya. *Am J Hum Biol*. 2021;33:e23515. https://doi.org/10.1002/ajhb.23515