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Diurnal dynamics and psychobiological regulation of cell-free mitochondrial and nuclear DNA in human saliva

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A B S T R A C T

Cell-free mitochondrial DNA (cf-mtDNA) has emerged as a dynamic molecular signal responsive to psychological stress and a potential biomarker of stress-related physiological adaptations. While previous work has established the acute stress reactivity of cf-mtDNA in saliva, little is known about its diurnal regulation. In this intensive-sampling study, we characterized the diurnal dynamics of saliva cf-mtDNA across one weekday and one weekend day in healthy adults ($N = 25$, 52% female, 826 samples). Saliva was collected at awakening, during the first hour post-awakening, and at hourly intervals throughout the day. Using a quantitative PCR-based assay, we observed a robust cf-mtDNA awakening response, with concentrations peaking approximately 45 min after waking (2.5-fold change, Cohen's $d = 0.90$), followed by a second peak at 180 min post-awakening (5.7-fold change, $d = 1.70$) and relatively stable levels thereafter. Saliva cf-mtDNA closely tracked cell-free nuclear DNA (cf-nDNA) across time points ($r_S = 0.85$), suggesting shared release mechanisms. Diurnal cf-mtDNA showed limited correspondence with cortisol and other hormones in saliva. Psychosocial stress indicators—including daily hassles, lack of social support, negative emotional affect, trait anxiety, fatigue, and depressive symptoms—were associated with higher awakening cf-mtDNA levels, a diminished awakening response, and lower diurnal variability. These findings suggest that saliva cf-mtDNA exhibits a diurnal rhythm and is sensitive to psychosocial stress exposure. By establishing its diurnal patterns and individual-level variability, this study advances saliva cf-mtDNA as a promising non-invasive biomarker to dynamically capture stress-related mitochondrial signaling.

1. Introduction

Chronic psychosocial stress is a key risk factor for a range of adverse health outcomes, including accelerated aging, impaired immune function, and increased susceptibility to mental and physical disease (Chrousos, 2009; O'Connor et al., 2021). Although acute stress responses are adaptive, chronic and repeated stress exposure can alter physiological processes in complex, yet not fully understood ways (Chrousos, 2009; McEwen, 1998; Picard and McEwen, 2018). One promising molecular signal implicated in stress adaptation processes and stress pathophysiology is *cell-free mitochondrial DNA* (cf-mtDNA)—fragments or entire copies of the mitochondrial genome present in the circulation and extracellular space, either freely or encapsulated in vesicles, organelle-like structures, and intact cell-free mitochondria (Al Amir Dache and Thierry, 2023; Aucamp et al., 2018; Caicedo et al., 2024; Trumpff et al., 2025, 2021).

Cf-mtDNA levels in blood have been reported to rise within minutes after exposure to acute physical and psychosocial stressors (Hummel et al., 2018; Trumpff et al., 2019a; Limberg et al., 2025; Trumpff et al., 2025; Selleri et al., 2025; Herhaus et al., 2026). Although these studies vary in magnitude and timing of effects, they suggest that cf-mtDNA may act as a rapidly inducible biomarker of psychophysiological stress responses. Furthermore, blood cf-mtDNA levels are observed to fluctuate several-fold in the timescale of weeks, which has been linked in parts to stress exposure and related negative consequences, such as depressive symptoms (Behnke et al., 2023; Daniels et al., 2024; Hummel et al.,

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2018; Lindqvist et al., 2018, 2016; Trumpff et al., 2019a, 2019b).

These dynamics mirror the release patterns of established stress mediators, such as the steroid hormone cortisol, giving rise to the hypothesis that cf-mtDNA might serve as a valuable biomarker for monitoring acute and chronic stress, offering insights into stress-related adaptations at the nexus of energy metabolism and immune processes (Aucamp et al., 2018; Boyapati et al., 2017; Caicedo et al., 2024; Park et al., 2021; Trumpff et al., 2021; West and Shadel, 2017). Notably, cf-mtDNA can be accessed non-invasively in human saliva, which has sparked growing interest in saliva cf-mtDNA dynamics as an easy-to-access stress biomarker (Caicedo et al., 2024; Michelson et al., 2023). Recent findings indicate that saliva cf-mtDNA responds even more sensitively to a psychosocial stress paradigm than blood cf-mtDNA, emphasizing its potential utility in stress research (Limberg et al., 2025; Trumpff et al., 2025).

Additionally, recent studies indicated a rhythmic blood cf-mtDNA release pattern, similar to yet different from other stress biomarkers such as cortisol (Hummel et al., 2018; Trumpff et al., 2021). Importantly, in an initial study in two men performing four daily saliva collections over 53–60 consecutive days, we observed that saliva cf-mtDNA undergoes a pattern of diurnal rhythmicity, characterized by an awakening response, with marked 2- to 3-fold average increases in the first 30–45 min after waking (with large intra-individual, day-to-day differences), followed by rather stable levels throughout the day (Trumpff et al., 2022). These preliminary findings raise the possibility that saliva cf-mtDNA dynamics follow daily rhythms, conceivably influenced by both stress exposure (Trumpff et al., 2022) and other biological and lifestyle factors, such as age, sex, BMI, sleep patterns, and physical activity (Padilla-Sánchez et al., 2020; Trumpff et al., 2021, 2019b).

The present study aims to further investigate saliva cf-mtDNA as a potential stress biomarker through characterizing its diurnal dynamics in healthy individuals across multiple time points throughout two different days, assessing its awakening response, its relationship with psychosocial stress, and the potential impact of individual and lifestyle variables. We will compare the awakening responses of cf-mtDNA and cortisol in saliva (Fig. 1A), as cell-based experiments found cortisol stimulation to trigger mitochondrial DNA secretion from human fibroblasts (Trumpff et al., 2019a) and as our initial study has suggested an association between cf-mtDNA and cortisol diurnal dynamics in saliva (Trumpff et al., 2022). Exploring the psychobiological regulation of diurnal cf-mtDNA dynamics will advance the methodological consolidation of saliva cf-mtDNA assessments in stress research, and it will shed light on potential physiological adaptations to stress and related negative health conditions.

2. Materials and methods

2.1. Study cohort

A total of 25 healthy adults (52% female; age: $M = 31.1$, $SD = 8.0$ years; BMI: $M = 23.9$, $SD = 3.1$ kg/m^2; 96% non-smokers) participated in this study (see Table 1 for participant characteristics). Participants were recruited from the local Columbia University Irving Medical Center (CUIMC) community, using a stratification scheme to balance the cohort's diversity in terms of ethnicity and regional ancestry. Eligibility was determined based on inclusion/exclusion criteria (see Supplementary Material) and participants' informed consent to complete the study protocol, which included repeated saliva collections, use of wearable devices, and ecological momentary assessments (EMA) of mood, behavior, and physical activity. All procedures were approved by the New York State Institute Institutional Review Board (IRB No. 8149).

2.2. Study procedures

Recruitment, consent, and training. Participants were recruited through flyers posted within CUIMC buildings and through Columbia University's online recruitment platform, RecruitMe. Interested individuals completed an eligibility screening via REDCap. Eligible participants were contacted by the study coordinator to schedule a video call for informed consent, in which participants provided written informed consent using the REDCap platform. A subsequent video call served to review the study protocol in detail and ensure participants understood the procedures for saliva collection, storage, and shipping as well as the completion of surveys. The study coordinator demonstrated each procedure, answered participant questions, and shipped a study kit containing saliva collection tubes and instructional materials to the participants' home address.

Study framework. The study's data collection period spanned a maximum of 10 days, with the at-home data saliva collection and ecological momentary assessments conducted during day 3 and day 6, with a break of up to 4 days in between. Participants were randomly assigned to one of two timelines, with 'Timeline 1' having the first collection day on a weekday and the second collection on a weekend day, vs. 'Timeline 2' scheduled vice versa. Participants received a check-in phone call after the first collection to address any concerns and ensure continued compliance.

Initial questionnaires. On day 2, the day prior to the first at-home saliva collections, participants completed an initial set of questionnaires, administered through REDCap. The questionnaire battery assessed demographic, health-related, and psychosocial factors, requiring approximately 40–60 min to complete. These included: Self-perception of aging (Turner et al., 2021) and self-rated health (Idler and Benyamini, 1997); Bem's Sex-Role Inventory (Bem, 1974); the MacArthur Ladder (Adler et al., 2000) to assess the subjective social status; the Stanford Leisure-Time activity categorical (L-Cat) item to assess habitual physical activity (Kiernan et al., 2013); the Pittsburgh Sleep Quality Index (PSQI) (Buysse et al., 1989); the Morningness and Eveningness Questionnaire (MEQ) (Horne and Ostberg, 1976) to assess circadian rhythm; a 35-item version of the daily hassles list (DHL) (Kanner et al., 1981); modified Differential Emotions Scale (mDES) to assess the positive and negative affect (Fredrickson et al., 2003); Perceived Stress Scale 10-item version (PSS-10) (Cohen et al., 1983) to assess helplessness and lack of self-esteem; Perceived Social Support from Friends (PSS-Fr) (Procidano and Heller, 1983); Situation Fatigue Scale (SFS) (Yang and Wu, 2005); State and Trait Anxiety Inventory (STAI-Y1) (Spielberger et al., 1983); Beck Depression Inventory-II (BDI-II) (Beck et al., 1996).

At-home saliva collection. Participants collected saliva in salivettes (Sarstedt #511534500) at multiple timepoints on two separate days (weekday vs. weekend day). On each collection day, saliva samples were taken upon awakening, at 30, 45, and 60 min after awakening, and then every hour until bedtime, resulting in a total of up to 20 saliva samples per day (Fig. 1A). A mobile device app based on REDCap alerted participants to collect saliva, and participants were requested to protocol the timing and conditions of collections in contemporary REDCap surveys on their app. Participants were instructed to refrain from food/beverage intake and oral hygiene procedures until all samplings within the first hour of awakening were completed. Along with each sampling, participants completed EMA surveys via REDCap to assess their physical activity, and intake of food, beverages, and substances.

Participants stored saliva samples in their home freezers immediately after collection, and after completing both collection days, returned them to the laboratory in insulated containers with ice packs. All shipments were received with ice packs still frozen. Upon arrival, samples were stored at −80°C until preprocessing, and subsequently at −80°C until cell-free DNA and hormone assays. A total number 828 saliva samples were collected and further processed (see Fig. S1A for data and specimen availability and timing across sampling time points). The delay between saliva collection and preprocessing was $Mdn = 22$ days (Q1–Q3 = 15–90, range = 8–247).

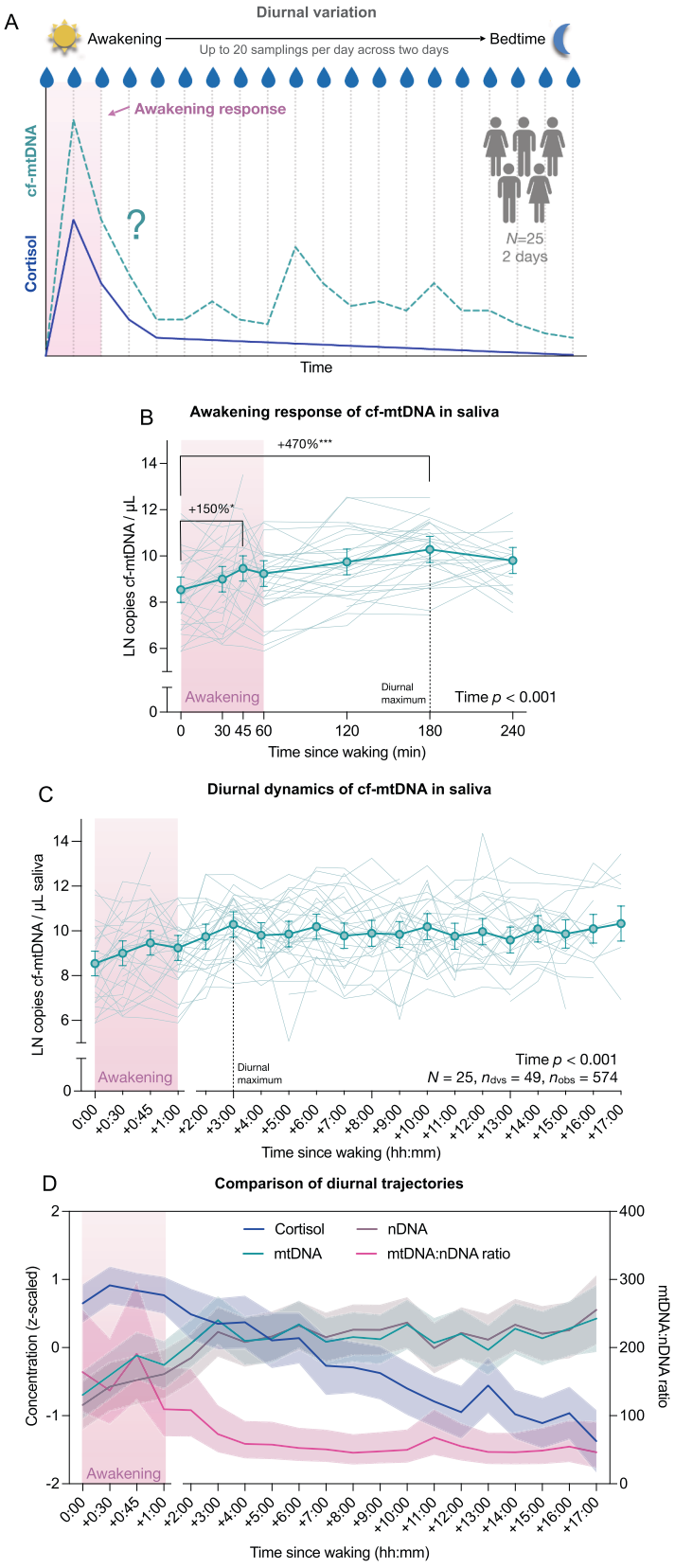

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Fig. 1. Saliva cf-mtDNA diurnal variation and awakening response. **(A)** Overview of the experimental design. Healthy participants ($N = 25$) collected up to 20 saliva samples over the course of one weekday and one weekend day for saliva cell-free DNA and steroid hormones (including cortisol) measurements. **(B)** Diurnal saliva cell-free mitochondrial DNA (cf-mtDNA) trajectories for the awakening response, and **(C)** for all time points. **(D)** Diurnal trajectories of cortisol (blue), cell-free mitochondrial DNA (cf-mtDNA, green), and cell-free nuclear DNA (cf-nDNA, violet), as well as the mtDNA-to-nDNA ratio (pink) are compared. Values are presented as (B,C) marginal means from linear mixed-effects models fitted to log-transformed concentrations with 95% confidence intervals (bold opaque lines and symbols) and individual trajectories (thin transparent lines), (D, left y-axis) *z*-scaled natural log-transformed marginal mean concentrations of cortisol, mtDNA, and nDNA with 95% confidence intervals, and (D, right y-axis) mtDNA-to-nDNA ratio, with confidence interval. (B,C) Effect sizes and *p*-values from linear mixed-effects models and Tukey's *post hoc* tests are reported, * $p_{Tukey} < 0.050$, *** $p_{Tukey} < 0.001$, two-tailed. For the awakening response, cf-mtDNA levels increased relative to awakening (baseline) by 2.5-fold (+150%) at 45 min post-awakening (Cohen's $d = 0.90$, $p_{adj} = 0.033$, moderate-to-large effect) and reached a diurnal maximum at 180 min post-awakening (5.7-fold increase, +470%, $d = 1.70$, $p_{adj} < 0.001$, large effect). For clarity, only the post hoc comparisons showing the greatest changes have been plotted.

2.3. Saliva processing

Salivettes were thawed and centrifuged at 1,000g × 5 min at room temperature. Saliva was collected from the bottom of the outer salivette tubes by pipette. Caution was taken to avoid pipetting any cell pellets that may have precipitated. Saliva was then transferred to 1.5 mL tubes and centrifuged at 5,000g × 10 min at room temperature. The resulting supernatants were transferred to clean 1.5 mL tubes, again taking caution to avoid pipetting precipitates, and stored at −80°C until assay.

2.4. Cell-free mitochondrial DNA assay

Mitochondrial and nuclear cell-free DNA in saliva supernatants were quantified using previously described methods (Michelson et al., 2023) with a few modifications. Briefly, cell-free saliva supernatants were thermolyzed overnight in duplicate. Lysates were analyzed in triplicate using TaqMan chemistry-based real-time quantitative polymerase chain reactions (qPCR) targeting the mitochondrial gene ND1 and the nuclear gene B2M. The medians of triplicate cycle threshold (C_T) values of samples were compared to those of a serial dilution series of a synthetic DNA standard (ranging from 197,542 to 12 copies of ND1 and B2M per reaction) to calculate absolute copy numbers of target genes. The lower limit of detection (LOD) was 24 copies/µl undiluted saliva. Average PCR efficiencies for ND1 and B2M were 91.4% and 91.8%, respectively. The average coefficients of variation of natural log-transformed ND1 and B2M copy number between replicates were 1.2% and 4.7%. Copy numbers were adjusted by correction factors calculated from plate-specific and experiment-wide measurements of reference standards to correct for batch effects. Not detectable B2M values (13.4%) were replaced with the LOD/$\sqrt{2}$ (see Table S2 for details on detection frequencies and missing samples). Additional information is available in the Supplemental material. The delay between sample preprocessing and DNA assays was *Mdn* = 197 days (Q1–Q3 = 110–223, range = 37–271).

2.5. Hormones in saliva

Salivary concentrations of cortisol, cortisone, corticosterone, dehydroepiandrosterone (DHEA), testosterone, progesterone, and estradiol were measured in duplicate for 306 samples using a validated high-throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (Gao et al., 2015). The lower limits of detection (LOD) and quantification (LOQ) were as follows: cortisol (LOD = 1.62 pg/ml, LOQ = 5.66 pg/ml), cortisone (LOD = 1.42 pg/ml, LOQ = 4.34 pg/ml), corticosterone (LOD = 1.49 pg/ml, LOQ = 4.90 pg/ml), DHEA (LOD = 10.47 pg/ml, LOQ = 35.55 pg/ml), testosterone (LOD = 2.10 pg/ml, LOQ = 6.86 pg/ml), progesterone (LOD = 1.03 pg/ml, LOQ = 3.38 pg/ml), and estradiol (LOD = 1.26 pg/ml, LOQ = 4.35 pg/ml) (Dresden LABservice GmbH, 2025). Values below LOD were substituted with the respective LOD/$\sqrt{2}$. Detailed information on detection frequencies and missing samples for all analytes is provided in Table S2. The delay between saliva preprocessing and hormone measurements was *Mdn* = 227 days (Q1–Q3 = 147–255, range = 67–310).

2.6. Data processing

Timing of Samplings. In accordance with established quality control guidelines for cortisol awakening responses (Stalder et al., 2016), we excluded saliva samples that were not timed from the statistical analyses. A tolerance of ±5 min was allowed for the first four samples (from awakening to 60 min post-awakening), and a tolerance of ±10 min was permitted for subsequent samples throughout the day. This resulted in the exclusion of 209 untimed samples, leaving $n = 619$ timed samples for further analysis (see Fig. S1A and S1B for data and specimen availability and timing across sampling time points, and Table S2 for frequencies of available biomarker measurements in timed samples). All available time-stamped values were used when calculating associations between biomarkers.

Potential covariates. Bivariate correlations indicated small associations between analytes and storage intervals (Table S3). Potential short-term influences (recent intake of food/beverages, nicotine, alcohol, oral hygiene, and physical activity) were assessed via EMA surveys paralleling each saliva collection. Given limited temporal resolution and to ensure model stability, variables were operationalized parsimoniously (details in Supplementary Material), and included in sensitivity analyses to evaluate the robustness of diurnal biomarker trajectories.

Although all participants reported being non-smokers during the onboarding interview, one individual indicated occasional smoking in the baseline survey. As this individual reported no smoking during the two sampling days in the EMA surveys, their data were retained to preserve statistical power.

Quantification of Diurnal Biomarker Activity. We originally intended to quantify the diurnal dynamics of saliva biomarkers using area under the curve (AUC) measures that integrate all samples collected throughout the day (Hellhammer et al., 2007; Khoury et al., 2015; Pruessner et al., 2003). However, the incidence of missing, and especially, mistimed samples resulted in too many missing values (≥48%; Fig. S1B), limiting the feasibility of this approach. Instead, we characterized diurnal biomarker activity using five complementary metrics, each capturing a distinct dimension of physiological reactivity while reducing missing values: (a) *Awakening level* (S1) — representing the baseline concentration at the start of the day; (b) *Awakening response* (AR) — estimated using the fold change from awakening to the major diurnal peak of the respective biomarker; (c) *Daily average* (AV) — reflecting overall biomarker levels across the day, integrating both stability and fluctuation; (d) *Diurnal variability* (%CV) — the coefficient of variation expressed as a percentage (100 × *SD* / mean), quantifying within-day variability relative to the individual's mean level, with lower values indicating less diurnal fluctuation range (i.e., a flatter profile); and (e) *Diurnal AUC above baseline* (AUCi) — estimated using a simplified three-point trapezoidal method based on awakening, group-level peak, and bedtime samples, to approximate the cumulative biomarker output above baseline across the day. Detailed formulas and visualizations in two example trajectories are provided in the supplement (Fig. S2). Together, these metrics allow for a multidimensional assessment of biomarker dynamics, capturing not only absolute levels but also the shape, variability, and regulatory characteristics of the diurnal profile. Due to missing and mistimed samplings, the derived metrics could not

Table 1
Characteristics of the study cohort ($N = 25$).

Variable	*M* (*SD*) or count (%)	*Mdn* (*IQR*)	Range
Sociodemography			
Age [years] [a]	31.1 (8.0)	28.5 (13.25)	[21, 47]
Sex, assigned	Female: 13 (52%) Male: 12 (48%)		
Gender	Woman: 13 (52%) Man: 10 (40%) Diverse: 2 (8%)		
Ethnic identity	African American/Black: 6 (24%) Asian: 6 (24%) Hispanic/Latino: 7 (28%) Mixed: 1 (4%) White: 5 (20%)		
MacArthur Ladder	5.8 (2.0)	6.0 (2.0)	[1, 10]
Lifestyle variables			
BMI [kg/m^2]	23.9 (3.1)	23.6 (4.8)	[18.5, 29.5]
Daily Activity	2.4 (1.2)	2.0 (1.0)	[0, 5]
Sleeping quality (PSQI)	27.6 (12.4)	24.0 (13.0)	[12, 57]
Morningness (MEQ)	49.9 (6.7)	49.9 (10.6)	[39.3, 60.5]
Occasional smoking	1 [b] (4%)		
Alcohol consume	Never: 10 (40%) 1–2/week: 7 (28%) 1–2/month: 8 (32%)		
Menstrual cycle status [c]			
At Sampling Day A	Menstrual: 0 (0%) Follicular: 1 (4%) Early luteal: 3 (12%) Later luteal: 9 (36%) None/Male: 12 (48%)		
At Sampling Day B	Menstrual: 2 (8%) Follicular: 1 (4%) Early luteal: 0 (0%) Later luteal: 10 (40%) None/Male: 12 (48%)		
Stress and mood perception			
State Anxiety (STAI-S)	26.0 (10.4)	23.0 (17.0)	[14, 49]
Negative emotional states (mDES)	6.3 (6.1)	5.0 (7.0)	[0, 24]
Positive emotional states (mDES)	21.4 (9.1)	19.0 (12.0)	[1, 35]
Daily Hassles [per week] (DHL)	26.4 (15.8)	25.0 (26.0)	[0, 55]
Social support from friends (PSS-Fr)	41.0 (8.3)	39.0 (10.0)	[28, 59]
Trait Anxiety (STAI-T)	32.0 (10.9)	32.0 (15.0)	[17, 62]
Physical fatigue (SFS)	12.0 (7.6)	10.0 (11.0)	[0, 26]
Mental fatigue (SFS)	10.9 (8.9)	9.0 (11.0)	[0, 29]
Perceived Helplessness (PSS-10)	9.2 (5.6)	8.0 (5.0)	[0, 24]
Lack of Self-esteem (PSS-10)	10.4 (3.4)	10.0 (5.0)	[5, 16]
Depressive symptoms (BDI-II)	7.9 (10.9)	4.0 (9.0)	[0, 51]
Self-perception of Aging (SPA)	13.8 (3.6)	14.0 (6.0)	[6, 18]

Note. [a] One missing value. [b] This individual reported no smoking during the two sampling days. [c] Cycle phases were estimated using the reported first day of the last menstrual period and typical bleeding duration, assuming a 28-day cycle. Phases were defined as: menstrual (days 1–bleeding duration), follicular (post-menstrual to day 13), ovulatory (days 14–15), early luteal (days 16–21), and late luteal (days 22–28).

be computed for a different number of diurnal trajectories (AV: 8%; S1: 18%; %CV: 20%; AR: 38%; AUCi: 44%), depending on the respective formula.

2.7. Statistical analysis

Statistical analyses were conducted using R, version 4.4.2 (R Core Team, 2024). Diurnal trajectories were modeled using linear mixed-effects models using the *lme4* package (Bates et al., 2015). Outcome variables exhibiting strong right skew were natural log-transformed. All models included random intercepts to account for repeated measures within individuals, nested within day type (weekday vs. weekend) to account for potential differences across sampling days.

Time since awakening was modeled as a fixed effect to estimate mean diurnal trajectories. Moderating effects of covariates (e.g., sex, menstrual cycle phase) were tested via interactions with time. Potential time-varying influences (e.g., food/beverage intake, physical activity) and collection-to-assay storage duration were included as covariates in sensitivity analyses. Continuous predictors were mean-centered, and categorical predictors were sum-contrast coded. *Post hoc* linear contrasts were conducted using the *emmeans* package (Lenth, 2025), with *p*-values adjusted for multiple comparisons.

Spearman's rank correlations (r_s) were used to examine associations between psychosocial, biological, and lifestyle variables and diurnal biomarker metrics. All statistical tests were two-tailed, with an α level of 0.05 considered statistically significant.

3. Results

3.1. Diurnal dynamics and the awakening response of salivary cell-free DNA

Consistent with our prior findings (Trumpff et al., 2022), salivary cf-mtDNA exhibited a pronounced awakening response. On average across all participants and days, cf-mtDNA levels rose following awakening, reaching a local peak at 45 min (2.5-fold increase, Cohen's $d = 0.90$, $p_{adj} = 0.033$) and a diurnal maximum at 180 min post-awakening (5.7-fold increase, $d = 1.70$, $p_{adj} < 0.001$) (Fig. 1B). Thereafter, cf-mtDNA levels remained relatively stable, fluctuating between 0.51- and 1.04-fold relative to the 180-minute peak (all $p_{adj} \geq 0.546$) (Fig. 1C; *post hoc* tests are detailed in the supplement). Time as a fixed factor explained a small portion of the variance ($R^2_{marg} = 7.8\%$), while the full model ($R^2_{cond} = 58.8\%$, ICC = 0.553) indicated moderate between-person differences and considerable within-person fluctuations, often diverging from the cohort's averaged pattern (Fig. 1B,C).

Blood cf-mtDNA is thought to be released into circulation via both active (e.g., vesicular transport) and passive (e.g., cell death) mechanisms. To assess the contribution of cell-death-related DNA release, we also quantified salivary cell-free nuclear DNA (cf-nDNA)—another type of cell-free DNA predominantly released during cell death. Like cf-mtDNA, cf-nDNA showed an awakening response with a diurnal peak at 180 min post-awakening (7.2-fold increase, $d = 1.60$, $p_{adj} < 0.001$; Fig. 1D, cf. Fig. S3A for additional details), followed by relative stability throughout the day (0.65 to 1.81-fold relative to peak, all $p_{adj} \geq 0.999$) (Fig. S3B; *post hoc* tests are detailed in the supplement). The average trajectory accounted for 11.8% (R^2_{marg}) of the variance, with moderate between-person differences and considerable within-person fluctuation across the day ($R^2_{cond} = 59.0\%$, ICC = 0.535).

Cf-mtDNA and cf-nDNA levels were strongly correlated across all diurnal samples ($r_S = 0.85$, $p < 0.001$; Fig. 2A), with weaker correlations at awakening (S1: $r_S = 0.49$, $p = 0.001$; Fig. 2B) and for the awakening response (AR: $r_S = 0.47$, $p = 0.019$; Fig. 2C). The cell-free mitochondrial-to-nuclear DNA ratio significantly decreased across the day (Fig. 1D), with *post-hoc* comparisons indicating a progressive decline of up to 2-fold between morning and 240 min post-awakening ($p_{adj} \leq 0.026$; Fig. S3C), and relative stability thereafter (Fig. S3D; *post hoc* tests are

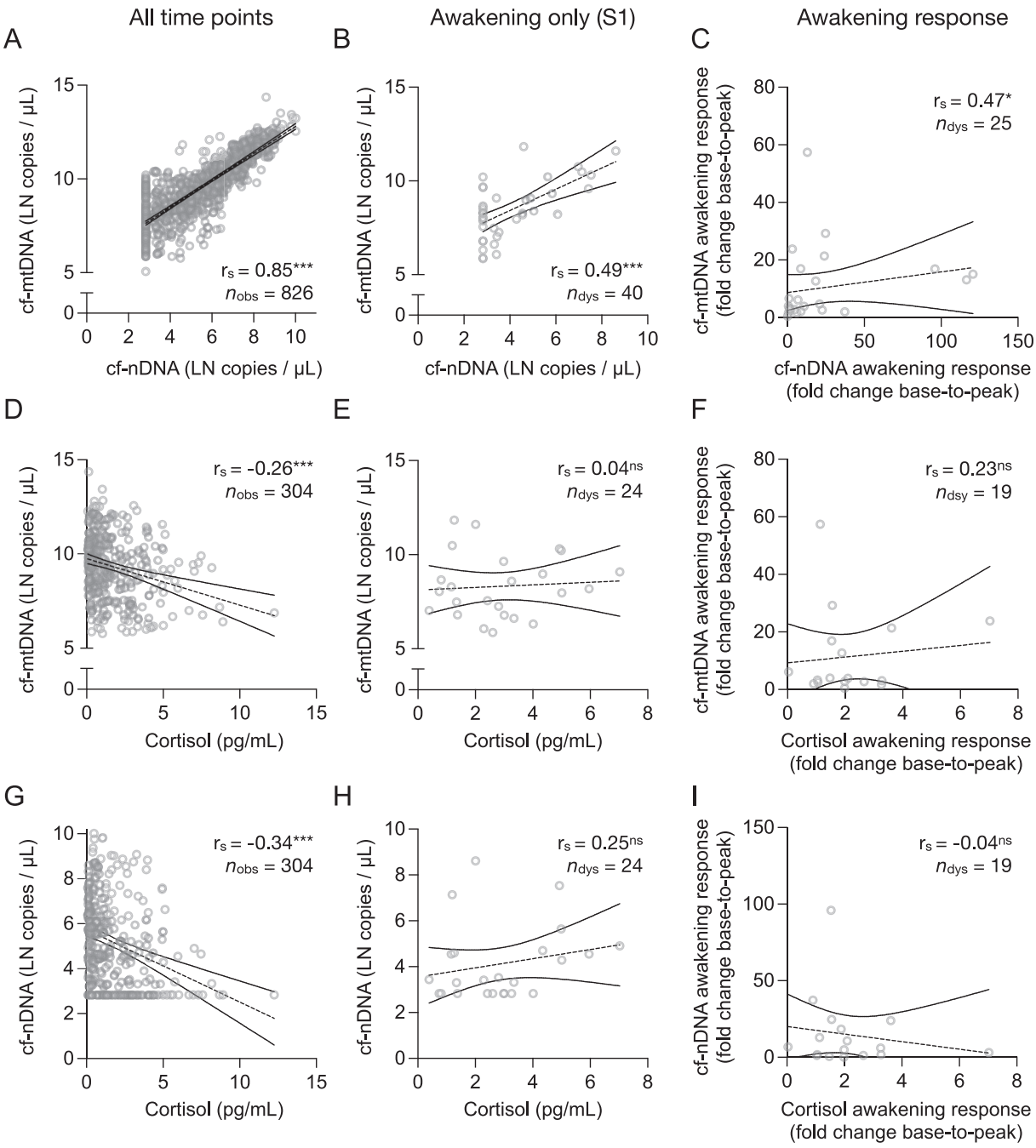

Fig. 2. Associations between cell-free DNAs and cortisol in saliva. Scatter plots illustrating the correlation between cell-free DNAs and cortisol, with the association of salivary cell-free nuclear DNA (cf-mtDNA) and cell-free mitochondrial DNA (cf-mtDNA) **(A)** over all time points, **(B)** at awakening, and **(C)** awakening responses (fold change from awakening to +180 min). Same **(D-F)** for the association of saliva cf-mtDNA and cortisol **(D)** over all time points, **(E)** at awakening, and **(F)** awakening response (cortisol awakening response computed as fold change from awakening to the peak at +30 or +45 min), and **(G-I)** the association of saliva cf-nDNA and cortisol **(G)** over all time points, **(H)** at awakening, and **(I)** awakening response. Linear trend lines and confidence intervals serve illustrative purposes. Effect sizes and two-tailed *p*-values from (A-I) Spearman rank correlations, ^{ns} $p \geq 0.050$, * $p < 0.050$, ** $p < 0.010$, *** $p < 0.001$.

detailed in the supplement). The average trajectory accounted for modest variance (R^2_{marg} = 8.0%), with substantial inter- and intra-individual variability in the diurnal change of the cell-free DNA ratio (R^2_{cond} = 47.5%, ICC = 0.429). These findings suggest a transient decoupling of cf-mtDNA and cf-nDNA dynamics in early hours post-awakening (awakening to ~180 min), followed by a stable co-regulation later in the day, likely reflecting shared mechanisms of DNA-to-saliva release.

Cell-free DNA dynamics could not be modelled more accurately when distinguishing weekday and weekend samples as fixed main effect and interaction with time (*p*'s $\geq$ 0.495, $\eta^2_p \leq 0.04$; Table S2; Figs. S4A). Consistent with our pilot data, we observed moderate within-subject correlations in cf-mtDNA and cf-nDNA concentrations across days (cf-mtDNA: whole-day $r = 0.55$, $p < 0.001$; at awakening $r = 0.51$, $p = 0.039$; cf-nDNA: whole-day $r = 0.53$, $p < 0.001$; at awakening $r = 0.61$, $p = 0.009$; Fig. S4B–E). These findings indicate a degree of within-individual stability in cell-free DNA levels, while also reflecting temporal fluctuations driven by daily physiological processes. Covariates capturing recent physical activity and intake of food/beverages prior to saliva samplings and pre-analytical storage duration were included in

sensitivity analyses to assess robustness of diurnal trajectories. Covariates were not significant predictors and did not influence the diurnal patterns of cell-free DNA levels (Table S5−S6).

3.2. Cf-mtDNA and cortisol

Our prior report suggested a moderate positive association between the salivary trajectories of cf-mtDNA and cortisol (Trumpff et al., 2022). In the current study, cortisol displayed its typical diurnal pattern, peaking approximately 30 min after awakening, followed by a gradual decline throughout the day (Fig. S5A). In line with this, the dynamics of cf-mtDNA and cortisol in saliva showed only modest correlation across matched time points (Fig. 2A). While there was a negative correlation between contemporaneous cortisol and cf-mtDNA levels over the entire day ($r_S = -0.26$, $p < 0.001$, Fig. 2D), no significant correlation was found in the awakening sample (S1: $r_S = 0.04$, $p = 0.866$, Fig. 2E), nor in the awakening response (AR: $r_S = 0.23$, $p = 0.340$, Fig. 2F). A similar pattern was observed for cf-nDNA (whole day: $r_S = -0.34$, $p < 0.001$, Fig. 2G; S1: $r_S = 0.25$, $p = 0.242$, Fig. 2H; AR: $r_S = -0.04$, $p = 0.836$, Fig. 2I). Importantly, the reliability of these statistical associations is limited by the high rate of missing cortisol data (57% across all samples and individuals; 38% of AR values, see Table S1−S2).

Additionally, we measured the diurnal concentrations of other hormones in saliva, including cortisone, corticosterone, testosterone, estradiol, progesterone, and DHEA (with 57% missing values and up to 28.5% non-detectable values, as detailed in Tables S1−S2). Cortisone, testosterone, and DHEA concentrations exhibited significant changes across the day (see Figs. S5A−E and Table S4−S5 for statistical results). Sensitivity analyses indicated higher concentrations of cortisol and cortisone after infrequent vigorous activity, small differences associated with recent food/beverage intake in progesterone, and a minor positive association of storage duration with testosterone (Table S6−S7). Importantly, inclusion of covariates did not meaningfully change the diurnal patterns across hormonal markers, supporting the robustness of the main findings.

We found negative associations of cell-free DNA levels with the levels of cortisone and corticosterone, resembling the results for cortisol. In addition, cf-mtDNA was positively associated with testosterone levels and weakly negatively associated with progesterone and estradiol levels across all available samplings (Table S8−9). In sum, both the observed associations and the lack thereof should be interpreted cautiously. Due to the substantial proportion of missing/non-detectable hormone data, these analyses are exploratory and insufficient to draw definitive conclusions regarding co-dynamics of cell-free DNA and hormones in saliva.

3.3. Psychosocial influences on cell-free DNA in saliva

Similar to other neuroendocrine stress biomarkers, we recently showed that acute psychological stress increases saliva cf-mtDNA levels (Trumpff et al., 2025). Here, we extended these results by investigating the relationship between psychosocial stress and diurnal cell-free DNA dynamics. We investigated the relationships between current mood states (e.g., negative emotions, state anxiety), stress exposure (e.g., 2-week daily hassles), and integrated measures of stress perception and mood disorder symptoms (e.g., trait anxiety, depression) and several metrics to represent complementary aspects of the diurnal cell-free DNA dynamics (see supplementary material for all correlations).

As shown in Fig. 3A, our findings reveal that higher awakening levels (S1), a blunted awakening response (AR), and lower diurnal variability (%CV) of cf-mtDNA were each significantly associated with indicators reflecting greater psychosocial stress, including negative emotional states, anxiety, fatigue, depressive symptoms, and lower perceived social support.

Specifically, higher awakening levels (S1) of cf-mtDNA were observed among individuals reporting elevated state anxiety (STAI-S: $r_S = 0.34$, $p = 0.045$), more negative emotional states (mDES: $r_S = 0.43$, $p = 0.009$), increased physical and mental fatigue (SFS physical: $r_S = 0.44$, $p = 0.009$; SFS mental: $r_S = 0.34$, $p = 0.047$), and lower perceived social support from friends (PSS-Fr: $r_S = -0.37$, $p = 0.030$).

Conversely, diminished awakening responses of cf-mtDNA (i.e., lower rise from awakening to diurnal peak at 180 min) were associated with elevated state anxiety (STAI-S: $r_S = -0.54$, $p = 0.005$), negative emotional states (mDES: $r_S = -0.40$, $p = 0.046$), higher trait anxiety (STAI-T: $r_S = -0.52$, $p = 0.007$), and more perceived helplessness (PSS-10: $r_S = -0.43$, $p = 0.034$).

Following a similar pattern, the diurnal variability (%CV) of cf-mtDNA was lower in individuals reporting more negative emotional states (mDES: $r_S = -0.50$, $p = 0.003$), higher trait anxiety (STAI-T: $r_S = -0.39$, $p = 0.021$), more (subclinical) depressive symptoms (BDI: $r_S = -0.39$, $p = 0.023$), more perceived helplessness (PSS-10: $r_S = -0.33$, $p = 0.055$), more fatigue symptoms (SFS physical: $r_S = -0.40$, $p = 0.018$; SFS mental: $r_S = -0.41$, $p = 0.017$), greater exposure to daily hassles (DHL: $r_S = -0.38$, $p = 0.028$), and lower social support from friends (PSS-F: $r_S = 0.43$, $p = 0.010$) (see Fig. 3B for scatter plots).

A similar association pattern was present for cf-nDNA (Fig. 3A), although the associations were mostly not statistically significant.

We also investigated the relationship between the parameters of diurnal dynamics of cell-free DNAs and biological and lifestyle factors, including age, BMI, assigned sex, social gender, alcohol consumption, chronotype, and habitual sleep quality. We found no significant statistical association between these factors and the diurnal dynamics of cell-free DNAs in saliva. However, there was a trend for a greater diurnal variability (%CV: $r_S = 0.32$, $p = 0.064$), higher mean concentrations (AV: $r_S = 0.31$, $p = 0.051$), and higher cumulative output over baseline (AUCi: $r_S = 0.54$, $p = 0.009$) of cf-mtDNA among individuals reporting more habitual physical activity (Fig. 3A, Fig. S6).

4. Discussion

This study provides a detailed characterization of the diurnal saliva cf-mtDNA dynamics under naturalistic conditions across two separate days in healthy individuals. Three main findings emerge: First, saliva cf-mtDNA exhibits a robust post-awakening increase followed by stabilization toward a plateau at mid-morning (180 min). Second, cf-mtDNA and cf-nDNA display highly parallel diurnal trajectories and are strongly correlated across the day, indicating that their levels are influenced by largely shared physiological mechanisms. Cell-free DNA dynamics showed limited correspondence with salivary cortisol and other hormones in the available data. However, given the substantial proportion of non-available hormone measurements, we cannot draw conclusions on the presence or absence of coordinated regulatory mechanisms. Third, psychosocial stress-related variables, such as daily hassles, emotional states, fatigue, and depressive symptoms, are associated with higher awakening cf-mtDNA levels, diminished awakening response, and lower diurnal variability, pointing to an interaction between psychological state and mitochondrial signaling in saliva.

Consistent with our initial observation (Trumpff et al., 2022), cf-mtDNA follows a biological rhythmicity characterized by increase after awakening and reaching a cohort-level maximum approximately 180 min post-awakening before stabilizing. Importantly, substantial inter- and intra-individual heterogeneity characterized diurnal trajectories. Consequently, the apparent 180-minute “peak” emerged from cohort-level averaging and was not uniformly observed at identical time points across individuals or days. Accordingly, the more robust feature of the data appears to be a general post-awakening rise followed by stabilization at mid-morning toward a diurnal plateau, rather than a sharply timed circadian maximum.

A central observation of this study is the strong coupling between cf-mtDNA and cf-nDNA ($r_S = 0.85$). Both markers exhibited pronounced and highly similar post-awakening increases, and time-of-day effects accounted for comparable proportions of variance. Across most of the day, the mitochondrial-to-nuclear DNA ratio remained relatively stable.

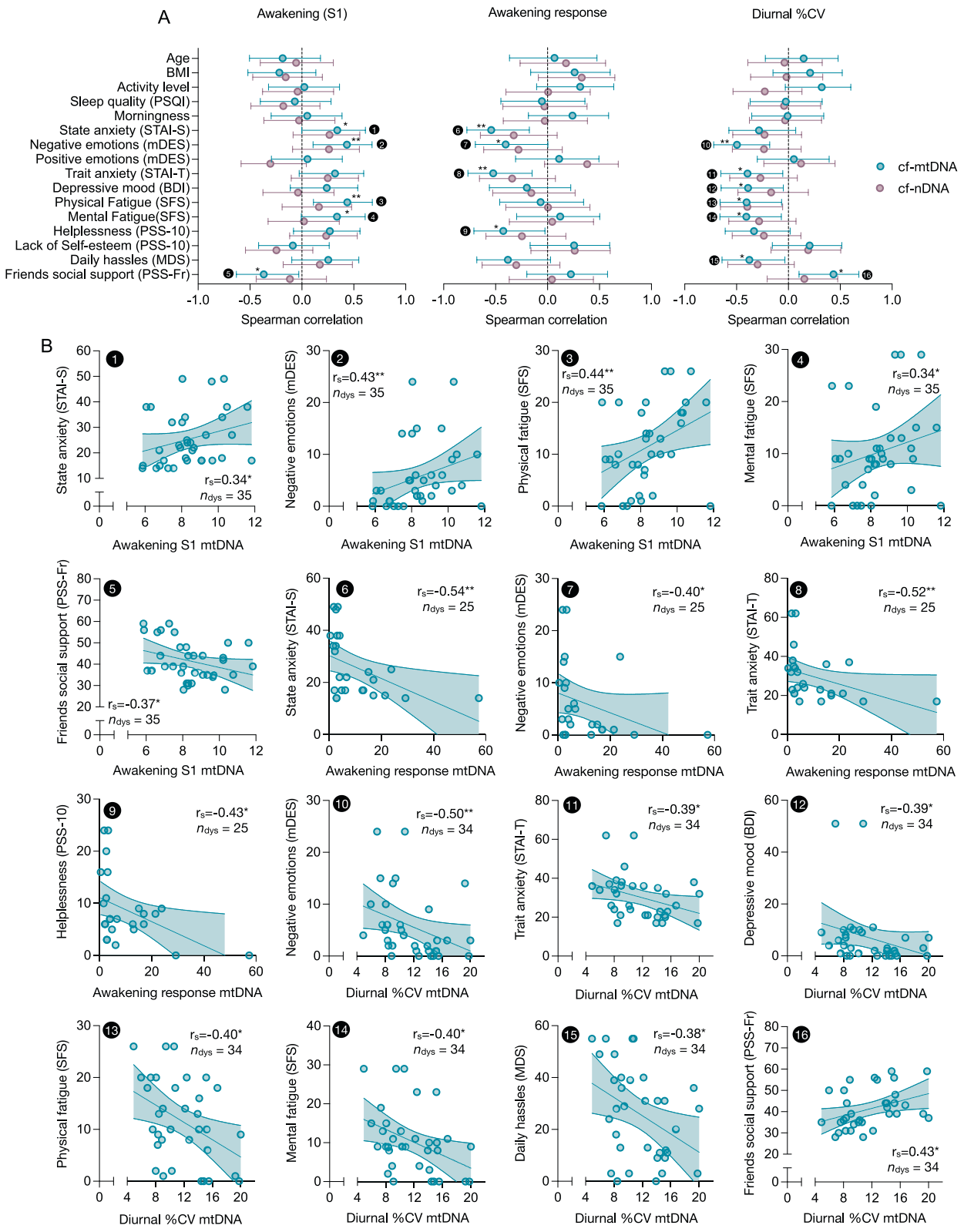

Fig. 3. Correlations of saliva cell-free DNA measurements with psychobiological parameters. **(A)** Forest plots illustrating associations between participants' cell-free mitochondrial DNA (cf-mtDNA, green) and cell-free nuclear DNA (cf-nDNA, purple) diurnal metrics (value at awakening, S1; awakening response (fold change from awakening to +180 min); and diurnal percent coefficient of variance, %CV) and psychobiological characteristics. %CV represents the within-day coefficient of variation of cf-mtDNA concentrations; lower values indicate a flatter diurnal profile with reduced dynamic range. Dots and error bars indicate effect size (as Spearman's ρ) and 95% confidence interval. Encircled numbers indicate significant correlations illustrated as **(B)** scatter plots. Linear trend lines and confidence intervals serve illustrative purposes. Effect sizes and two-tailed *p*-values from Spearman rank correlations, * $p < 0.050$, ** $p < 0.010$, *** $p < 0.001$.

These findings suggest that salivary cell-free DNA dynamics are predominantly governed by shared sources or coordinated release mechanisms rather than mitochondrial DNA-specific regulation.

Several, not mutually exclusive, biological models may account for this coupling. *First*, cf-mtDNA and cf-nDNA may arise from common cellular sources within the oral cavity, including epithelial turnover, mechanical disruption, or immune cell activity, resulting in parallel passive release into saliva. *Second*, salivary cell-free DNA may partially reflect plasma-derived DNA entering the oral cavity via gingival crevicular fluid or salivary gland secretion. In this scenario, saliva would partially reflect systemic cell-free DNA dynamics, although previous studies could not demonstrate consistent contemporaneous or lagged saliva–blood associations (Limberg et al., 2025; Trumpff et al., 2025). *Third*, membrane-enclosed vesicles or mitochondria-containing particles may co-transport mitochondrial and nuclear DNA fragments, structurally linking their abundance. In the circulation, most cf-mtDNA is membrane-enclosed, including within intact cell-free mitochondria and mitochondria-derived extracellular vesicles (Al Amir Dache and Thierry, 2023; Pisareva et al., 2023), and similar mitochondria-related structures have been observed in saliva (Trumpff et al., 2025).

Against this background, our analytical approach quantified total amplifiable human mitochondrial DNA in cleared saliva supernatant following two centrifugation steps (up to 5,000 g) and membrane lysis. These steps remove intact cells and larger debris while retaining smaller extracellular components, including vesicles, cell-free mitochondria, mitochondria-derived vesicles, particle-bound DNA, and free (fragmented) mitochondrial DNA. The subsequent lysis step enables recovery of DNA from membrane-enclosed structures. Thus, our measurement captures a broader spectrum of cf-mtDNA carriers involved in diverse physiological functions, including intercellular signaling and inflammation, as primarily characterized in the circulation (Al Amir Dache and Thierry, 2023). Accordingly, methodological differences in specimen processing can shift the relative representation of these DNA carriers and thereby influence absolute cf-mtDNA concentrations as well as their biological interpretation (Pisareva et al., 2023).

Within the overall coupling of cell-free DNA dynamics, we observed a modest early-morning shift in the relative contribution of mitochondrial and nuclear DNA. Both cf-mtDNA and cf-nDNA increased substantially after awakening; in relative terms, cf-nDNA exhibited a slightly stronger proportional rise, resulting in a gradual reduction of the cell-free DNA ratio from awakening to approximately 240 min post-awakening. These changes reflect proportional redistribution within parallel trajectories rather than divergent dynamics. Importantly, the magnitude of this shift was small compared to the pronounced deviation of cell-free mitochondrial and nuclear DNA as well as their ratio reported in pathological conditions such as sepsis, hypoxia, or cancer (e.g., Meddeb et al., 2019; Otandault et al., 2020; Timmermans et al., 2016). Thus, the observed early-morning ratio decline does not resemble disease-associated dysregulation but instead suggests subtle time-dependent variation in the relative contribution of shared DNA sources.

Awakening and the early post-awakening period involve complex physiological and behavioral transitions, including changes in posture, light exposure, autonomic activation, behavioral engagement, and metabolic state. Although speculative, the observed rise and subsequent stabilization of salivary cell-free DNA may reflect temporally restricted adjustments in saliva flow, local cellular turnover, mitochondrial signaling, or autonomic regulation during this transition from rest to sustained daytime activity. The relative contribution and precise timing of these influences remain to be determined.

In fact, the regulation of cf-mtDNA and cf-nDNA release in biofluids remains poorly understood, particularly regarding the influence of psychosocial and behavioral factors. Previous research has predominantly focused on the acute reactivity of cf-mtDNA in blood and saliva in response to different experimental stressors, revealing inconsistent response patterns (Herhaus et al., 2026; Hummel et al., 2018; Limberg et al., 2025; Trumpff et al., 2025, 2019a, 2019b). Our study extends this literature by showing that psychosocial stressors and related psychological states—including daily hassles, reduced social support, emotional states, fatigue, and depressive symptoms—were associated with higher awakening cf-mtDNA levels and reduced diurnal variability (%CV). A complementary analysis with hourly ecological momentary affect assessments within the same cohort provides further context for interpretation (Petri et al., 2026): momentary negative emotional states—including stress and frustration—were associated with short-term reductions in salivary cf-mtDNA, whereas positive affect predicted transient increases. Importantly, this association was context-dependent: positive affect was linked to higher cf-mtDNA primarily when negative affect was low, whereas elevated negative affect attenuated this relationship.

Thus, repeated stress exposure and sustained negative affect may blunt transient positive-affect–related increases, such that aggregating samples across the day results in an overall reduction of diurnal variability (%CV). In this sense, reduced variability may reflect a compression of dynamic range under greater psychosocial strain rather than a simple tonic shift in cf-mtDNA levels. Accordingly, cf-mtDNA variability may index adaptive capacity in daily life—capturing biobehavioral flexibility in response to changing affective states and stress (Petri et al., 2026). Although this interpretation requires confirmation, it conceptually parallels findings in heart rate variability, where reduced variability is widely interpreted as diminished regulatory flexibility under stress and pathological conditions (Kim et al., 2018).

The observed pattern of diurnal change in cf-mtDNA raises a broader question: its biological function in saliva and its potential role as a stress-related marker. Cf-mtDNA release into the circulation has been proposed to serve various functions, including modulation of inflammatory responses and cell-to-cell communication (Al Amir Dache and Thierry, 2023; Boyapati et al., 2017; Trumpff et al., 2021). In saliva, the observed diurnal rhythms, sensitivity to psychosocial stress, and transient decoupling from cf-nDNA dynamics motivate the consideration of at least two hypothesized functional roles for cf-mtDNA: one reflecting basal physiological turnover under endogenous control—possibly linked to circadian and/or behavioral cues—and another reflecting acute physiological adaptation to early-day activation and psychosocial or physical stress exposure.

Although not testable in the present design, we have speculated previously that the transient surge in salivary cf-mtDNA content might reflect an anticipatory response that facilitates tissue repair during stressful situations (Trumpff et al., 2025). Notably, application of mitochondria-rich platelets to injured tissue has been shown to promote wound healing in mouse models, where the release and uptake of free mitochondria by stromal cells was necessary (Levoux et al., 2021). This represents a form of cell-to-cell mitochondrial transfer with reparative effects (Brestoff et al., 2025). In this context, the act of licking wounds could apply mitochondria-rich saliva to injured tissue, potentially stimulating local repair mechanisms such as immune activation and epithelial regeneration. While direct evidence in humans is lacking, we speculate that stress-induced elevations in salivary mitochondrial content, measurable as increased cf-mtDNA levels, might reflect an evolutionarily conserved mechanism to enhance tissue readiness and repair capacity in anticipation of injury (see Trumpff et al., 2025 for details). The post-awakening rise in saliva cf-mtDNA might accordingly reflect a biological shift toward increased readiness for environmental engagement and potential stress exposure.

This evolutionary interpretation is clearly hypothesis-generating and intends to stimulate future mechanistic studies to resolve several key questions. To advance this line of research, we outline directions for future investigation: *First*, what are the precise cellular sources and release pathways for cf-mtDNA into saliva? Advanced proteomic and vesicle-isolation studies are needed to identify whether these molecules stem from epithelial cells, immune cells, filtered plasma, or cell-free mitochondria. *Second*, it remains unclear whether the cf-mtDNA in saliva possesses signaling properties or is merely a byproduct of other

processes. The application of mitochondrial membrane-specific dyes, electron microscopy, and single-vesicle profiling may help address this question. *Third*, the potential for cf-mtDNA to serve as a stress-related biomarker in mental health research warrants further investigation. Our findings highlight that diurnal variability of cf-mtDNA is sensitive to emotional states, trait anxiety, depressive symptoms, and daily stressors, opening up the possibility of using this metric in ecologically valid, repeated-measure assessments of stress vulnerability or resilience. Lastly, given the non-invasive nature of saliva sampling, cf-mtDNA may be a feasible tool for large-scale or vulnerable population studies where blood collection is impractical.

4.1. Limitations

Despite its contributions, our study has limitations. The sample size was modest but consistent with previous intensive-sampling protocols and in line with recommendations for covering weekday-weekend differences (Stalder et al., 2016). Although we observed moderate to strong within-day and within-subject consistency, additional sampling days are preferred when determining the test–retest reliability of cell-free DNA dynamics and when drawing conclusions about trait-like differences in cell-free DNA (Hellhammer et al., 2007; Stalder et al., 2016).

Missing data, particularly hormone quantifications (~57% missing), limited our ability to evaluate hormonal co-dynamics of cell-free DNA. Future studies with more complete hormonal measurements are needed to determine whether salivary hormones and cell-free DNA dynamics exhibit coordinated diurnal regulation. Of note, recent studies could not demonstrate a contemporaneous coupling of glucocorticoids and cell-free DNA release in saliva upon acute stress exposure (Limberg et al., 2025).

Diurnal patterns of sleep, physical activity, dietary intake, and oral hygiene are likely to modulate saliva composition and, consequently, cell-free DNA dynamics, underscoring the value of objective behavioral monitoring and experimentally controlled designs in future studies (Rodrigues et al., 2025). Sensitivity analyses indicated the observed temporal patterns of cell-free DNA dynamics were robust to the inclusion of EMA-based covariates reflecting recent food/beverage intake and physical activity. However, statistical power was limited to model these influences at higher temporal and behavioral granularity.

We relied on a well-established protocol to measure cell-free DNA in saliva (Michelson et al., 2023), and our findings were robust to variation in collection-to-assay delays. Nevertheless, pre-analytical conditions (e.g., temperature conditions during at-home storage and shipping) can be expected to have introduced unsystematic noise, and technical differences between our and others' protocols (e.g., centrifugation, lysis, qPCR targets) influence the detectable DNA fractions and limit cross-protocol comparability (Daubermann et al., 2025; Limberg et al., 2025; Pisareva et al., 2023; Trumpff et al., 2025, 2021). Notably, hormone concentrations appeared more sensitive to storage conditions than cell-free DNA, highlighting challenges for future at-home intensive sampling protocols.

Finally, the study cohort consisted of healthy, predominantly non-smoking, middle-aged adults, warranting further research to generalize the findings to other age groups and clinical populations.

5. Conclusions

This study expands our understanding of cf-mtDNA dynamics in human saliva. By establishing a reproducible diurnal rhythm, a robust awakening response, and associations with self-reported measures of psychosocial stress, our findings provide the foundation for future work exploring the psychobiological regulation of saliva cf-mtDNA. Beyond its mechanistic interest, cf-mtDNA emerges as a promising, non-invasive marker of mitochondrial stress signaling, with potential applications in mental health and behavioral medicine. Moving forward, studies integrating physiological monitoring, broader biomarker panels, and mechanistic cellular assays will be needed to clarify the biological role and diagnostic utility of saliva cf-mtDNA in human health.

Author contributions

C.T. and M.P. designed the study, with A.G. assisting in organizing the study setup and obtaining legal and ethical approvals. S.L. coordinated data and sample collection from participants. D.S. and A.P. conducted the cell-free DNA assays. C.K. provided the methodology for steroid hormone measurement. A.B. performed the statistical analyses and drafted the manuscript with support from C.T. and D.S. All authors reviewed and approved the final version of the manuscript.

CRediT authorship contribution statement

Alexander Behnke: Writing – review & editing, Writing – original draft, Visualization, Funding acquisition, Formal analysis, Data curation. **David Shire:** Writing – review & editing, Visualization, Validation, Methodology, Investigation. **Samantha Leonard:** Investigation. **Aidan Park:** Methodology, Investigation. **Lauren Petri:** Writing – review & editing. **Anjali Goyal:** Writing – review & editing, Project administration, Methodology. **Clemens Kirschbaum:** Methodology. **Martin Picard:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Caroline Trumpff:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of Competing Interest

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

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Appendix A. Supplementary material

The supplementary material provides additional details about methods and analyses (File 1), supplementary figures (File 2), post hoc test statistics (File 3), and correlation test statistics (File 4). Supplementary material to this article can be found online at https://doi.org/10.1016/j.bbi.2026.106825.

Data availability

Data will be made available on request.

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