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RESEARCH ARTICLE

Physiological Responses to Psychosocial Stress

Habitual fluid intake and hydration status influence cortisol reactivity to acute psychosocial stress

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Abstract

Shared pathways connect water regulation and cortisol release, and exaggerated cortisol reactivity to stress is associated with poor long-term health. This study investigated the unknown influence of habitual fluid intake and hydration status on saliva cortisol reactivity to psychosocial stress. After screening 62 healthy males and females and adopting low and high fluid intake quartiles from a national database, we identified 16 individuals with habitual low (LOW: 1.3 ± 0.4 L/day) and 16 with habitual high fluid intake (HIGH: 4.4 ± 1.2 L/day) who were comparable for factors likely to influence stress reactivity (e.g., trait anxiety). In pairs (one LOW and one HIGH), participants underwent 7 days of monitored habitual fluid intake. The following day, participants individually completed the Trier Social Stress Test (TSST). Hydration status was assessed in the days preceding and on the day of the TSST (urine osmolality and color; UOsm, UCol). Both UOsm and UCol were greater in LOW ($P < 0.001$). The TSST evoked similar increases in state anxiety and heart rate in LOW and HIGH, yet saliva cortisol increased significantly post-TSST only in LOW (group × time interaction, $P = 0.02$). Moreover, cortisol reactivity was greater in LOW (Δ, 6.2 ± 2.9 vs. HIGH: 4.0 ± 1.8 nmol/L; $P = 0.03$, Cohen's $d = 0.9$) and was associated with hydration status assessed as UOsm (Pearson $R = 0.7$, $P < 0.001$). These novel findings show greater cortisol reactivity to acute psychosocial stress in adults with habitual low fluid intake and suboptimal hydration, which may influence long-term health. Clinical Trial Registry Number: NCT05491122.

NEW & NOTEWORTHY Cortisol reactivity to acute stress predicts long-term health. Our novel findings show greater cortisol reactivity to acute stress in adults with habitual low fluid intake. Suboptimal hydration (e.g., darker morning urine) was associated with greater cortisol reactivity to acute stress. These findings provide one possible explanation for why habitual low fluid intake and suboptimal hydration are related to poor long-term health. Furthermore, researchers should account for hydration when assessing cortisol reactivity to acute stressors.

cortisol; fluid; hydration; stress; TSST

INTRODUCTION

Many adults routinely consume less than the recommended daily water intake as defined by American and European public health organizations [e.g., 2.5 L for men and 2 L for women as water in fluid and food (1–4)]. Individuals who consistently fall short of meeting the water intake recommendations typically present with suboptimal hydration marked by low urine output and high urine concentration (5–7). Although limited to the level of association, epidemiological studies have linked habitual low fluid intake with increased risk of renal, cardiovascular, and metabolic diseases (6–10). Chronic low water intake may increase morbidity risk by elevating key water-regulating hormones such as arginine vasopressin (AVP) and its surrogate copeptin, which modulate glucose regulation and renal function (5, 11, 12). In addition to its role in regulating body water homeostasis, AVP activates the hypothalamic-pituitary-adrenal (HPA) axis by stimulating adrenocorticotropic hormone (ACTH) secretion, in turn promoting stress hormone cortisol release (13). Besides its well-known responsivity to acute stress, cortisol shows a marked circadian rhythm characterized by the cortisol awakening response (CAR) and a gradual decline throughout the day. Although a moderate cortisol response to acute stress is associated with a healthy phenotype (14–16), blunted or exaggerated cortisol reactivity or dysregulated circadian cortisol rhythm are considered to have far-reaching negative implications for metabolism, immunity, and inflammation (17). For example, prospective cohort studies show that exaggerated cortisol reactivity to acute

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stress predicts poor health and disease outcomes (16, 18). The relationship between fluid intake, hydration, and cortisol remains unclear. Much of the empirical research on this relationship has observed increases in plasma cortisol after moderate-severe dehydration (3%–7% body mass loss) evoked by a combination of exercise, heat stress, and fluid restriction (19, 20). Since exercise independently raises circulating cortisol (21), it is difficult to isolate the effects of fluid intake and hydration. Moreover, the severity of the dehydration protocols is unlikely to be representative of daily fluctuations in water balance in the general population. By comparison, few studies have explored the relationship between fluid intake and cortisol in the general population under normal living conditions, where daily water balance varies primarily due to differences in fluid intake (22–24). Higher serum cortisol has been shown in individuals who consumed less than 1.2 L of fluid per day and presented with suboptimal hydration [e.g., elevated urine osmolality (UOsm) and plasma AVP] compared with those who consumed 2–4 L of fluid per day (22). Moreover, increasing total daily fluid intake by ∼1.5–2 L of fluid per day in participants with low habitual fluid intake reduced saliva cortisol in a single morning measurement (23). Overcoming the limitation of single cortisol measurements and the confounding influence of circadian variation, a recent study showed no effect of low daily fluid intake and poor hydration on the CAR, a measure of dynamic circadian cortisol variation (24). However, unlike measuring state cortisol reactivity to a standardized acute stressor, assessing the CAR captures a complex interaction between central circadian cortisol regulation and the state cortisol response to awakening (25). Standardized laboratory stressors, such as the commonly used and validated Trier Social Stress Test (26–28), afford the opportunity to assess robust HPA-axis reactivity under conditions that control for circadian cortisol regulation and external confounds (29).

Given the shared pathways linking whole-body water regulation and stress hormone cortisol release, the aim of this study was to test the hypothesis that individuals with habitual low fluid intake and suboptimal hydration exhibit greater cortisol reactivity to acute psychosocial stress. The findings of which may improve our understanding of how habitual low fluid intake and suboptimal hydration are associated with poor long-term health and highlight the importance of accounting for fluid intake and hydration status when assessing cortisol reactivity to acute psychosocial stress.

METHODS

This study received local ethical approval, and protocols were conducted in accordance with the Declaration of Helsinki (2013). The data presented herein were collected as part of a prospective intervention study investigating the influence of fluid intake on daily biological rhythm and mental performance in healthy young adults (Clinical Trial Registry Number: NCT05491122).

Participant Recruitment and Exclusion

Eligible participants were healthy, recreationally active adults, aged 18–35 yr, who were nonsmokers and free from any known sleep disorders and immune, cardiovascular, or metabolic diseases. Participants were not taking prescription medication, with the exception of prescribed oral combined contraception (OCC), which was an inclusion criterion for female participants. Before inclusion, volunteers were screened to identify individuals with habitual low fluid intake (LOW: males $<$ 1.6 L/day, females $<$ 1.5 L/day) and habitual high fluid intake (HIGH: males $\geq$ 2.9 L/day, females $\geq$ 2.5 L/day). Thresholds for low and high fluid intake were the lowest and highest quartiles from a national database (United Kingdom) matched for sex and age (2). Initial daily fluid intake estimates were obtained using items from the BEVQ-15 (30), with the format and terminology adapted for online use and for a UK population. The estimates of daily fluid intake were then verified during a 7-day total fluid intake screening phase, which required participants to fill out the Liq.In^7 record. This record has been validated against deuterium oxide disappearance and has been used in large cross-sectional studies characterizing fluid intake patterns across multiple continents (2, 31). Over the 7 days, participants recorded their daily fluid intake each day from all sources (water, hot drinks, sugar-sweetened drinks, milk, diet drinks, and alcohol). Males reporting fluid intake between 1.6 and 2.9 L/day, and females between 1.5 and 2.5 L/day during the screening phase, were excluded. In addition, as high caffeine intake and high alcohol intake can separately influence cortisol reactivity to acute stress (32, 33), individuals consuming $\geq$1.3 L/day of caffeinated beverages or $\geq$3 units/day of alcoholic beverages were also excluded. To ensure consistency in their daily drinking habits, any participant whose daily fluid intake volume varied by $>$25% across the 7-day screening phase was excluded from the study (34).

During the 7-day screening phase, participants were provided with two 3-L containers and asked to collect all urine produced between the hours of 1600 and 2000 across 2 days (1 weekday and 1 day during the weekend). Urine osmolality (UOsm) measured in urine samples collected between the hours of 1600 and 2000 has been shown to agree with UOsm measured in urine collected across 24 h (35). Study eligibility was confirmed when UOsm indicated suboptimal hydration in LOW ($\geq$500 mosmol/kgH_2O) and optimal hydration in HIGH ($<$500 mosmol/kgH_2O) (1).

Familiarization

Eligible participants attended a familiarization visit at the laboratory (visit length $\sim$ 1 h), where a member of the research team showed participants the laboratory suite, questionnaires, and the saliva sampling protocol. Considering the known influences of trait anxiety, recent life stress and sleep quality on psychobiological responses to acute stress (36–38), at the familiarization visit participants also completed the State-Trait anxiety inventory (STAI-T; 39), the perceived stress scale (40) and the Pittsburgh Sleep Quality Index (PSQI) (41) (Table 1). During the familiarization visit, participants were not explicitly informed about the TSST, only that they would complete a challenging mental performance task. After completing the study, participants attended a study debriefing where they were fully informed about the study aim (i.e., to induce an acute psychological stress response rather than to assess mental performance).

Table 1. *Baseline descriptives for study participants*

	Daily Fluid Intake		
	All	LOW	HIGH
	$n = 32$	$n = 16$	$n = 16$
Demographics and anthropometry			
Age, yr	23 ± 3	23 ± 4	23 ± 3
Sex: male, *n* (%)	22 (69)	11 (69)	11 (69)
Sex: female, *n* (%)	10 (31)	5 (31)	5 (31)
BMI, kg/m^2	24 ± 3	23 ± 3	25 ± 3
Trait anxiety			
Score (20–80)	37 ± 9	35 ± 7	38 ± 10
Score ≥ 40, *n* (%)	9 (28)	4 (25)	5 (31)
Sleep quality			
Score (0–21)	2 ± 2	2 ± 1	2 ± 2
Score ≥ 5, *n* (%)	3 (9)	1 (6)	2 (12)
Perceived stress			
Score (0–40)	13 ± 6	13 ± 5	13 ± 6

Values presented as means ± SD unless otherwise stated; n = no. of participants. Trait anxiety was assessed using the trait aspect of the State-Trait Anxiety Inventory, with a score of ≥40 considered high trait anxiety (39). Sleep quality in the month before study enrolment was assessed using the Pittsburgh Sleep Quality Index, with a score of ≥5 considered poor sleep (41). Perceived stress in the month before study enrolment was assessed using the Perceived Stress Scale (40). Higher scores indicate greater levels of trait anxiety and perceived stress and poorer sleep quality. All statistical comparisons were $P > 0.05$. BMI, body mass index.

Study Design and Procedures

This single-center prospective study was performed on two nonrandomized parallel groups, stratified by habitual LOW and HIGH fluid intake. To account for state-like confounding factors (e.g., an influence of changeable weather conditions on fluid intake and hydration) participants completed 7 days of monitored habitual fluid intake in pairs comprising one LOW and one HIGH (Fig. 1). Female OCC users completed the 7 days of monitored fluid intake during their 21-day pill cycle to maintain consistent sex hormone levels, mitigating natural sex hormone effects on stress responses (42). During the 7 days of monitored fluid intake, participants were instructed to attain an individualized daily fluid intake target based on their average daily consumption during the 7-day total fluid intake screening phase. A HidrateSpark smart bottle was given to participants to monitor compliance with the individualized daily fluid intake target; the bottle and application alerted participants as to when the daily fluid intake target had been attained. On *days 5* and *6* of the 7-day monitoring period, participants were asked to collect all urine produced between the hours of 1600 and 2000 in a container (in line with procedures carried out during the 7-day total fluid intake screening phase). Participants were asked to store the urine container in a fridge before bringing the container to the laboratory at the end of the week for the assessment of UOsm and urine color (UCol).

Laboratory Visits (Day 7 and Day 8)

Participants attended a morning laboratory visit (~1 h duration between 0830 and 1100), in a fasted state, on *day 7* of the 7 days of monitored fluid intake, the day before the TSST (Fig. 1). During this visit, participants were asked to complete the perceived stress scale and the PSQI to assess stress and sleep quality in the previous week. In addition, participants provided an on-the-spot urine sample for the assessment of hydration (i.e., UOsm and UCol). After a seated rest, a venous blood sample was collected from an antecubital vein for the assessment of plasma osmolality (POsm) and copeptin. These procedures were repeated the following morning (*day 8*), when participants were required to arrive at the laboratory within 30 min of the *day 7* arrival clock time. The perceived stress scale and PSQI were modified to assess stress and sleep quality experienced the previous day.

Acute Stress Test Protocol: Trier Social Stress Test

Participants returned to the laboratory on the afternoon of *day 8* to complete the Trier Social Stress test (Fig. 1). To minimize the impact of circadian variations in saliva cortisol (43), the Trier Social Stress Test was scheduled between 1200 and 1700 on *day 8* (visit length ~ 2 h). Participants were asked to avoid beverages containing caffeine and alcohol, food intake, and brushing their teeth in the 2 h before the visit. Upon arrival, participants completed a 30-min period of standardized seated rest (acclimation period) in a reception area. This acclimation period is necessary to mitigate against participants arriving at the laboratory already stressed (with elevated baseline saliva cortisol) due to recent events in the minutes before arrival (44). Following this, participants were exposed to the TSST, which comprised a preparation phase (5 min), followed by a mock job interview and a mental arithmetic task (5 min each) standing in front of two observers (1 male and 1 female, both unknown to the participant) and a sham video recording device (i.e., not recording), in line with procedures described elsewhere (29). Saliva samples, later analyzed for cortisol, were collected using a Salivette device

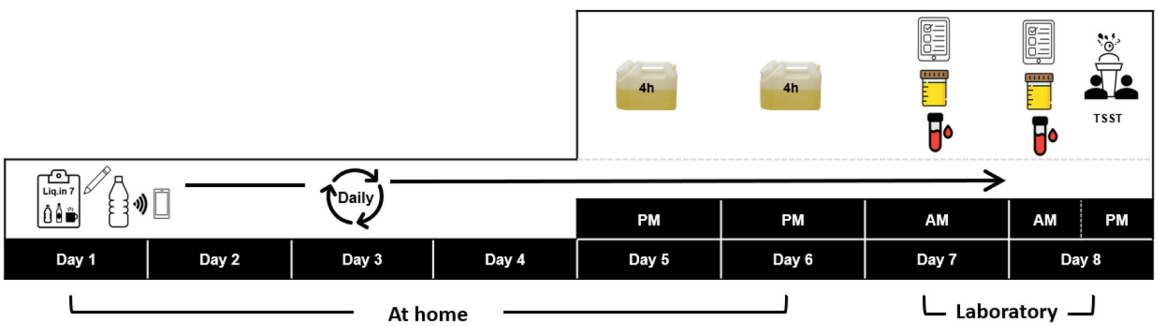

Figure 1. Schematic of the study protocol. TSST, Trier Social Stress Test.

(Salivette Cortisol, with synthetic swab, Sarstedt, Numbrecht, Germany), whereby participants were asked to chew the swab for 1 min. Saliva samples were obtained at "baseline" (−30 min), "pre" TSST (−5 min), immediately "post" and +10, +20, +30, +45, and +60 min post TSST. Questionnaires that assessed self-reported anxiety were administered to participants at −30 min (baseline), −5 min (pre), 0 (post), and +60 min post stress test. Heart rate was monitored continuously during the TSST protocol (Polar H10, Polar Electro, Kempele, Finland).

Study Measures

Daily fluid intake recording.

During the 7-day total fluid intake screening phase and 7 days of monitored habitual fluid intake, participants recorded their daily fluid intake from all sources, that is, water, hot beverages, juices, and sweetened drinks, using the Liq.In^7 record (2, 31).

Plasma hydration markers.

Whole blood samples were collected by venipuncture from an antecubital vein into vacutainer tubes containing lithium heparin (Becton-Dickinson, Oxford, UK). Subsequently, samples were centrifuged at 1,500 *g* for 10 min at 4°C. Plasma osmolality was assessed on fresh samples using freezing-point depression osmometry (Gonotec Osmomat). Samples were analyzed in duplicate, and if the values differed by greater than ±2 mosmol/kgH_2O, replicates were reanalyzed until two consecutive measurements were within ±2 mosmol/kgH_2O, in line with manufacturer instructions and local standard operating procedures. Remaining plasma was aliquoted into Eppendorf tubes and frozen at −80°C for later analysis. Plasma copeptin was measured in duplicate on stored samples, in an external laboratory (located at Jan Waldenströms gata 35, 91:12, Skåne University Hospital, Malmö, Sweden) using a commercially available chemiluminescence sandwich immunoassay copeptin ProAVP kit with a mean intra-assay coefficient of variation of <10% (B.R.A.H.M.S AG, Hennigsdorf, Germany) (34, 45).

Urine markers of hydration.

From each urine sample, 50-mL aliquots were dispensed into a plain screw-capped universal tube and analyzed immediately for UOsm and urine color. Urine osmolality was assessed using freezing-point depression osmometry (Gonotec Osmomat). Samples were analyzed in duplicate, and if the values from the two measurements differed by greater than ±2 mosmol/kgH_2O (if the value was <400 mosmol/kgH_2O) or greater than ±4 mosmol/kgH_2O (if the value was >401 mosmol/kgH_2O), replicates were reanalyzed until two consecutive measurements were within the acceptable error margin, in line with manufacturer instructions and local standard operating procedures. Urine color was assessed by a member of the investigating team who was blinded to the participant group (i.e., LOW or HIGH) using a urine color chart (1–8), as described (46). Urine osmolality and color were strongly correlated (Pearson $R = 0.9$, $P < 0.001$).

State anxiety.

Anxiety was measured using the state aspect of the state anxiety inventory (STAI-Y1) (39). The STAI questionnaire contains 20 items, and each item was rated on a four-point Likert scale (range from "1" = not at all to "4" = very much). The total score for this measure was obtained by summing the values assigned to each item, resulting in a range from 20 to 80, with higher total scores indicating more severe anxiety symptoms.

Saliva cortisol.

Saliva was extracted from Salivette swabs by centrifugation as per the manufacturer's instructions and frozen in multiple aliquots at −80°C until thawing for cortisol analysis. Saliva samples were analyzed in triplicate for free cortisol by enzyme-linked immunosorbent assay, with a mean intra-assay coefficient of variation of 5.7% (Salimetrics, State College, PA).

Statistical Analysis

All analyses were conducted using SPSS 29.0 (IBM, Armonk, NY) with statistical significance set at $P < 0.05$. Data were checked for normality and sphericity, and where sphericity was violated Greenhouse–Geisser correction was applied to the degrees of freedom. Participant demographic data, daily fluid intake, and hydration biomarkers are presented as means ± SD for continuous variables or absolute numbers and percentages for categorical variables; comparisons were made using independent *t* tests and chi-square analysis, where appropriate (Tables 1 and 2). On *day 8*, we assessed the proportion of LOW and HIGH presenting with suboptimal hydration using the following criteria: UOsm > 500 mosmol/kgH_2O (1), UCol ≥ 4 (46), and plasma copeptin > 4.3 pmol/L (34) (Supplemental Table S1).

Acknowledging that the findings presented herein were collected as part of a larger project with separate aims, outcomes, and for which a separate sample size estimate was performed, we are confident that the included sample size was sufficient for the present study aim. Sample size for the present study aim was estimated at 30 participants, where 15 LOW and 15 HIGH were required to detect a significant difference in saliva cortisol reactivity to the TSST, with alpha set at 0.05, power at 0.9, and Cohen's *d* effect size of 1.1. This effect size was calculated using published data from studies assessing the influence of a nutritional intervention on the saliva cortisol response to the TSST and the influence of water supplementation on morning saliva cortisol (23, 47). Group (LOW and HIGH) × time interactions for psychobiological responses to the TSST (state anxiety, heart rate, and saliva cortisol) were assessed using linear mixed-model analysis with post hoc Bonferroni pair-wise comparisons (Fig. 3, *A* and *B*). As saliva cortisol concentrations at each sampling time point pre- and post-TSST were positively skewed, data were log-transformed before linear mixed model analysis (Fig. 3*C*).

Robust HPA-axis reactivity was necessary to test the hypothesis that individuals with habitual low fluid intake and suboptimal hydration exhibit a greater magnitude of cortisol reactivity to acute stress. Acknowledging that the TSST does not elicit meaningful HPA-axis reactivity in all participants (29), an independent *t* test was used to compare saliva cortisol reactivity [calculated as the Δ pretest vs. peak post (48–51)] between LOW and HIGH participants who exhibited meaningful biological and subjective responses to

the TSST (Fig. 4). We defined meaningful stress test responders in two separate ways based upon the strength of *1*) the biological response (HPA-axis reactivity assessed as saliva cortisol) and *2*) the subjective response (state anxiety). The typical day-to-day variation in these parameters was established in an in-house pilot investigation as part of another study (49). A meaningful saliva cortisol response was determined when the delta change for the measure (pretest to peak post-TSST) exceeded the typical day-to-day intraindividual coefficient of variation (CVI = 34%) (52, 53). A meaningful state anxiety response was determined when the delta change in state anxiety (pretest to post-TSST) exceeded the day-to-day intraindividual coefficient of variation (CVI = 15%). As is common in the field, and to allow interlaboratory comparisons, we report the number and proportion of participants exhibiting a saliva cortisol response >2.5 nmol/L from pretest to peak post-TSST (54, 55). The magnitude of effect for *t* test comparisons was reported using Cohen's *d,* where 0.2, 0.5, and 0.8 represent small, medium, and large effects, respectively. Pearsons correlation was used to assess the association between hydration status (UOsm mean *day 5–8*) and saliva cortisol reactivity to the TSST in meaningful stress test responders (Fig. 5*A*). An independent *t* test was used to compare cortisol reactivity between "suboptimal" (UCol $\geq$ 4) and "hydrated" (UCol $\leq$ 3) participants on *day 8* before the TSST (Fig. 5*B*). One LOW individual who mounted a Δ saliva cortisol > 3 SDs above the mean was an outlier and removed from all saliva cortisol reactivity analyses.

RESULTS

Participant Flow and Descriptive Information

Participant flow, exclusion, and dropout before analysis are summarized in Fig. 2. Descriptive data for the n = 32 participants who commenced and completed the study are summarized in Table 1. There were no significant differences between LOW and HIGH for factors likely to influence stress reactivity, including sex, age, trait anxiety, perceived stress, and sleep quality in either the month before study enrolment or during the 7 days of monitored habitual fluid intake. Similar numbers of LOW and HIGH were categorized as high trait anxious [STAI-T score > 40, (39)] and reported poor sleep quality in the month before study enrolment [PSQI score > 5, (41), Table 1].

Fluid Intake and Hydration Markers

LOW consistently presented with higher UOsm, UCol, and plasma copeptin (all $P < 0.05$, Table 2). No statistical differences in thirst or POsm were observed between LOW and HIGH (all $P > 0.05$, Table 2). On the morning of the TSST (*day 8*), adopting a UOsm threshold of 500 mosmol/kgH_2O (1), 15 of 16 LOW were suboptimally hydrated, and 14 of 16 HIGH were hydrated. Suboptimal hydration status according to UOsm, UCol, and plasma copeptin is presented in Supplemental Table S1.

Anxiety and Heart Rate Responses to the TSST

Linear mixed model showed that the TSST elicited significant increases in state anxiety and heart rate [main effect of time, state anxiety: $F(3,90) = 69.6$, $P < 0.001$, Fig. 3*A*; heart rate: $F(6,180) = 142$, $P < 0.001$, Fig. 3*B*]. No significant interactions were observed for state anxiety and heart rate emphasizing the similar responses in LOW and HIGH [group × time interaction, state anxiety: $F(3,90) = 0.6$, $P = 0.6$, heart rate: $F(6,180) = 0.6$, $P = 0.7$]. A smaller proportion of individuals in LOW reported a meaningful increase in state anxiety in response to the TSST, exceeding typical day-to-day variation, compared with HIGH (overall 84%, where n = 11 of 15 LOW, n = 15 of 16 HIGH).

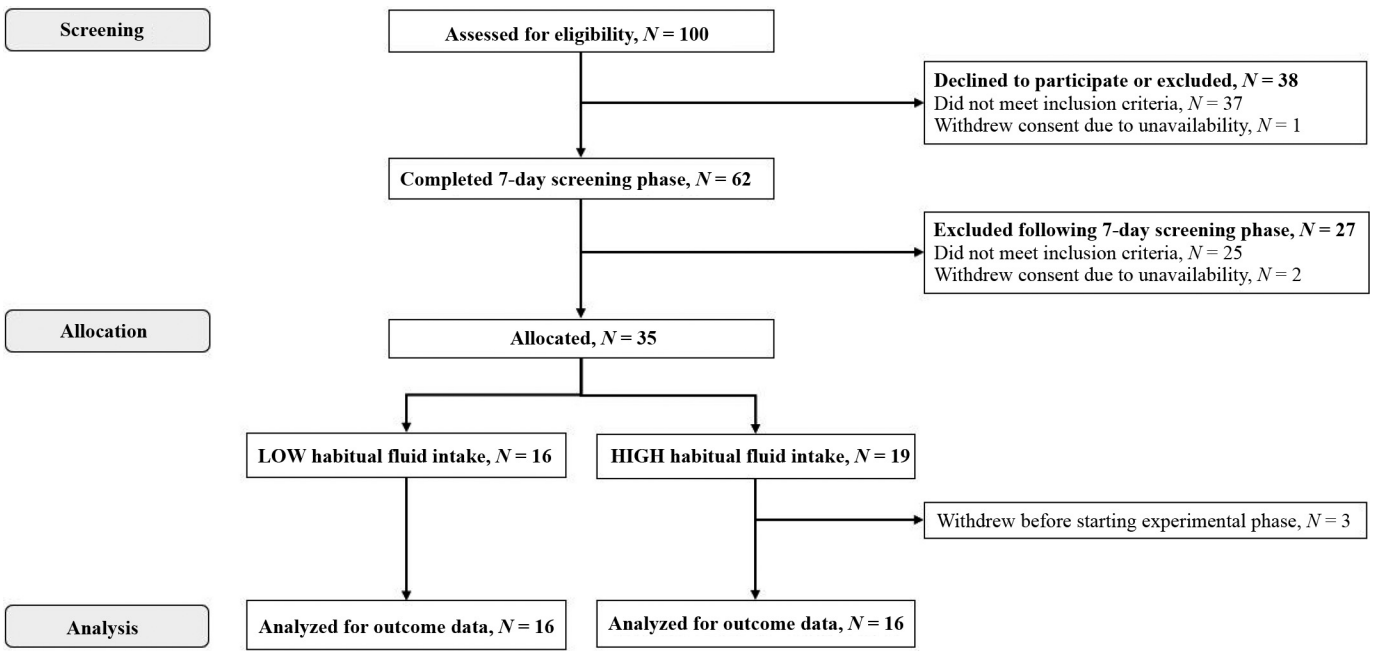

Figure 2. Flow diagram indicating the number of volunteers who registered an interest, were screened, allocated, and analyzed.

Table 2. *Daily fluid intake and hydration markers in participants with habitual low and high fluid intake*

	Daily Fluid Intake		
	All	LOW	HIGH
	$n = 32$	$n = 16$	$n = 16$
Liq.In[7] record, L/day			
Total fluid intake	2.9 ± 1.8	1.3 ± 0.4**	4.4 ± 1.2
Water	2.4 ± 1.8	0.9 ± 0.5**	3.7 ± 1.3
Hot caffeinated beverages	0.1 ± 0.2	0.1 ± 0.2	0.1 ± 0.1
Alcoholic beverages	0.0 ± 0.1	0.0 ± 0.1	0.1 ± 0.1
Urine osmolality, mosmol/kgH_2O			
Late afternoon: 1600–2000, *days 5* and *6*	430 ± 247	595 ± 210**	265 ± 153
Morning sample, *day 7*	578 ± 310	788 ± 241**	361 ± 266
Morning sample, *day 8*	511 ± 275	694 ± 228**	302 ± 160
Urine color (1–8)			
Late afternoon: 1600–2000, *days 5* and *6*	3 ± 1	5 ± 1**	2 ± 1
Morning sample, *day 7*	4 ± 2	5 ± 1**	2 ± 1
Morning sample, *day 8*	3 ± 1	4 ± 1	3 ± 1
Thirst			
Daily VAS (score: 0–100)	40 ± 16	45 ± 13	38 ± 17
Daily Likert (score: 1–7)	4 ± 1	4 ± 1	3 ± 1
Plasma osmolality, mosmol/kgH_2O[a]			
Morning sample, *day 7*	287 ± 7	287 ± 7	287 ± 8
Morning sample, *day 8*	287 ± 6	289 ± 8	286 ± 3
Plasma copeptin, pmol/L[a]			
Morning sample, *day 7*	4.4 ± 2.5	5.5 ± 3.2*	3.4 ± 1.3
Morning sample, *day 8*	5.5 ± 5.4	8.5 ± 6.8**	3.0 ± 1.5

Values presented as means ± SD; n = no. of participants. Urine color was assessed using the 1–8 urine color chart, with lighter shades (i.e., ≤3) indicating "hydrated" (46). Higher scores for the VAS and Likert indicate greater sensations of thirst. VAS, visual analogue scale. *Significantly different from "HIGH," $P < 0.05$; **significantly different from "HIGH," $P < 0.01$. [a]Blood samples available for $n = 29$, where 13 LOW and 16 HIGH.

Saliva Cortisol Responses to the TSST

Higher "baseline" saliva cortisol was observed in LOW compared with HIGH ($P = 0.04$, Fig. 3*C*), albeit the difference was small (Cohen's $d = 0.3$). After a standardized 25-min seated rest, saliva cortisol was similar in LOW and HIGH at "pre" (Fig. 3*C*). Linear mixed model, with "baseline cortisol" entered as a covariate, showed that the TSST elicited a significant increase in saliva cortisol [main effect of time: $F(6,156) = 10.7$, $P < 0.001$, Fig. 3*C*]. A paired t test showed a significant increase in saliva cortisol from "pre" to peak post-TSST (Δ saliva cortisol = 4.6 ± 4.4 nmol/L, $P < 0.01$, Cohen's $d = 1.0$). After the removal of one statistical outlier, the proportion of individuals who mounted a meaningful saliva cortisol response exceeding typical day-to-day variation was similar in LOW and HIGH (overall 81%, where $n = 12$ of 15 LOW, $n = 13$ of 16 HIGH). Likewise, the proportion of individuals who mounted a saliva cortisol response > 2.5 nmol/L was also similar in LOW and HIGH ($n = 9$ of 15 LOW, $n = 9$ of 16 HIGH). However, linear mixed model showed a group × time interaction ($P = 0.02$), whereby saliva cortisol was elevated compared with "pre" at +10, +20, and +30 min post-TSST in LOW ($P < 0.05$ for all comparisons, Fig. 3*C*), whereas saliva cortisol was not significantly increased above "pre" in HIGH. Similar group × time interactions were observed when linear mixed-model analyses were isolated to individuals who mounted meaningful biological reactivity ($P = 0.03$, Supplemental Fig. S1) and subjective reactivity ($P = 0.01$). Moreover, after the removal of one statistical outlier, the magnitude of saliva cortisol reactivity to the TSST was greater in LOW compared with HIGH when meaningful stress test responder status was determined by biological reactivity (between group difference, $P =$

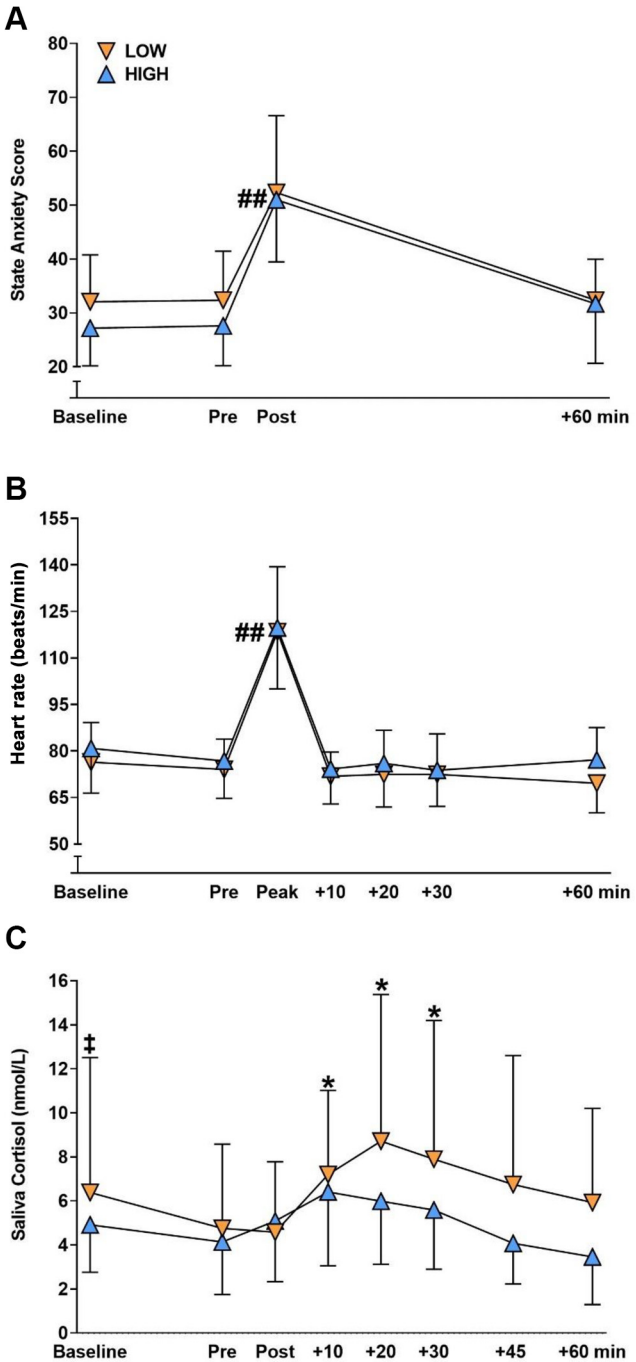

Figure 3. Psychobiological responses to the Trier Social Stress Test in all participants with habitual low (LOW: $n = 16$) and high fluid intake (HIGH: $n = 16$). State anxiety (*A*), heart rate (*B*), and saliva cortisol (*C*). Values are presented as means ± SD. Statistical comparisons for saliva cortisol (*C*) were conducted on log-transformed data to correct for positive skew; saliva cortisol is presented as absolute concentration (nmol/L) for illustrative purposes. ##Greater than "pre" main effect of time, $P < 0.01$. *Greater than "pre" group × time interaction, $P < 0.05$. ‡Greater than HIGH between-group difference, $P < 0.05$.

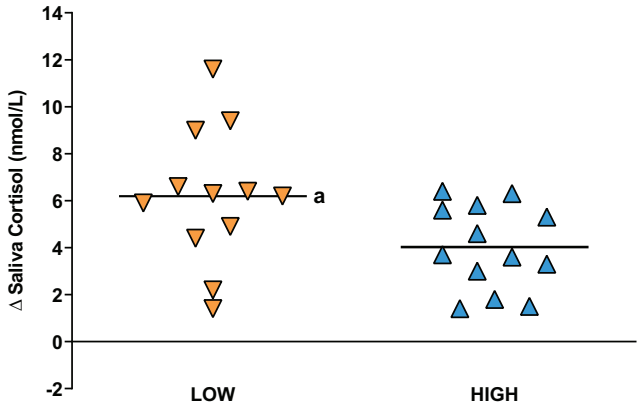

Figure 4. Influence of habitual low (LOW) and high (HIGH) fluid intake on saliva cortisol reactivity to the Trier Social Stress Test (TSST). Saliva cortisol reactivity was calculated as the change from pre to peak posttest (Δ). Data are shown in meaningful "saliva cortisol responders" (n = 25/31, 81%), who exhibited an increase in saliva cortisol in response to the TSST exceeding typical day-to-day variation (CVI 34%). One LOW individual who mounted a Δ saliva cortisol > 3 SDs above the mean was an outlier and removed. The unbroken horizontal line shows the mean change. [a]Greater than "HIGH," $P < 0.05$. CVI, coefficient of variation.

0.03, Cohen's $d = 0.9$, Fig. 4) and subjective reactivity (state anxiety; Supplemental Fig. S2).

Hydration Status and Saliva Cortisol Reactivity to the TSST

Correlation analysis demonstrated that hydration status, assessed as mean UOsm *day 5–8*, was positively associated with cortisol reactivity to the TSST. These findings are shown for biological stress test responders (Fig. 5*A*) and subjective stress test responders (Supplemental Fig. S3*A*). Similar associations were also observed between hydration status and cortisol reactivity to the TSST, when restricting hydration assessments to the 4-h late-afternoon urine samples collected on *days 5* and *6* (biological stress test responders, Pearson $R = 0.6$, $P < 0.001$; subjective stress test responders, $R = 0.6$, $P = 0.002$). From a practical standpoint, using a simple urine color assessment, individuals presenting with "suboptimal" hydration (UCol ≥ 4) on the morning of the TSST exhibited greater cortisol reactivity (biological stress test responders, $P = 0.02$, Cohen's $d = 1.0$, Fig. 5*B*; subjective stress test responders, $P < 0.001$, Cohen's $d = 1.5$, Supplemental Fig. S3*B*).

DISCUSSION

Prospective cohort studies show that blunted or exaggerated cortisol reactivity to acute stress predicts future health and disease outcomes (16, 18). Given the shared pathways linking whole-body water regulation and stress hormone cortisol release, testing the hypothesis that individuals with habitual low fluid intake and suboptimal hydration exhibit greater cortisol reactivity to acute psychosocial stress is an important knowledge gap. In accordance with our hypothesis, new and noteworthy findings were: *1*) despite the TSST eliciting similar increases in state anxiety and heart rate, saliva cortisol increased significantly post-TSST in LOW but not HIGH; *2*) the magnitude of saliva cortisol reactivity (Δ) was greater in LOW compared with HIGH; *3*) most notably, from a mechanistic standpoint, pre-TSST hydration status was associated with the magnitude of saliva cortisol reactivity (Pearson $R = 0.7$); and *4*) from a practical standpoint, individuals presenting with darker urine pre-TSST, exhibited greater saliva cortisol reactivity. These findings provide one

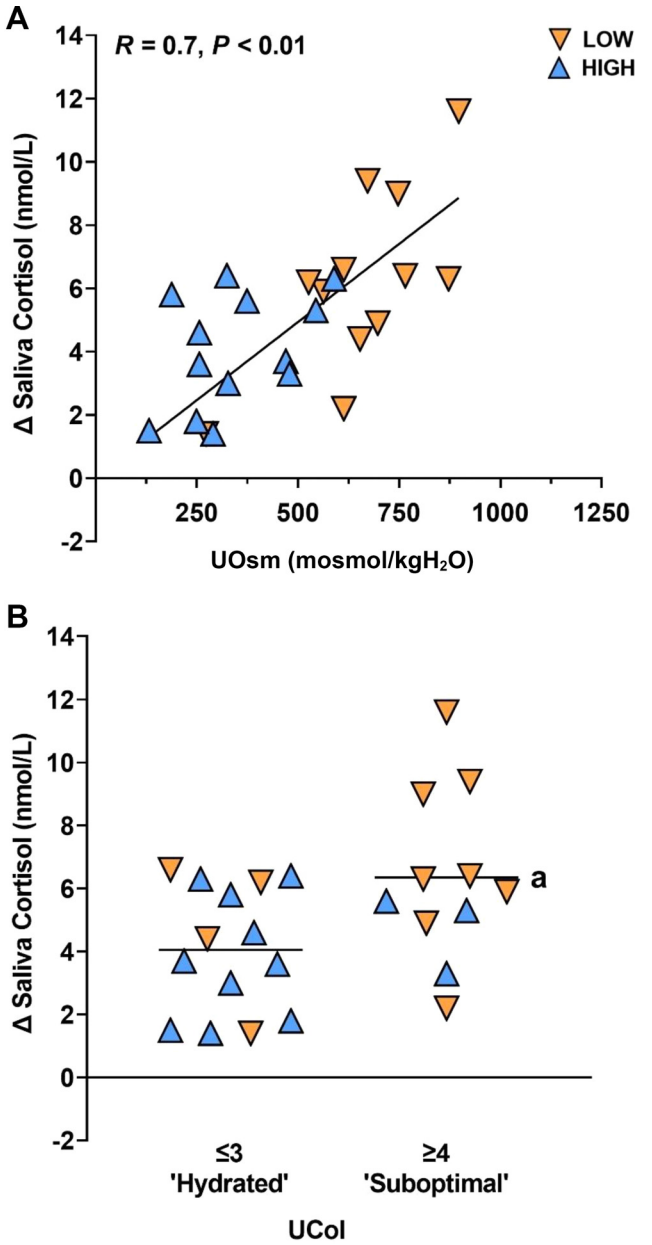

Figure 5. Prospectively assessed hydration status and saliva cortisol reactivity to the Trier Social Stress Test (TSST). In *A*, hydration status was assessed as urine osmolality (UOsm mean *days 5–8*). In *B*, for practical application, hydration status was assessed as urine color on the morning of the TSST (UCol *day 8*, 1–8 color chart). "Hydrated" participants presented with a UCol of ≤3, as described (46). Saliva cortisol reactivity was calculated as the change from pre to peak posttest (Δ). Data are shown in "saliva cortisol responders" (n = 25/31, 81%), who exhibited an increase in saliva cortisol in response to the TSST exceeding typical day-to-day variation (CVI 34%). One LOW individual who mounted a Δ saliva cortisol >3 SDs above the mean was an outlier and removed. The unbroken horizontal line shows the mean change. [a]Greater than "hydrated," $P < 0.05$. CVI, coefficient of variation.

possible explanation for why habitual low fluid intake and suboptimal hydration are related to poor long-term health (6–10). Furthermore, these results highlight the importance of accounting for fluid intake and hydration status when assessing cortisol reactivity to acute psychosocial stressors.

To the best of our knowledge, these are the first findings demonstrating that habitual low fluid intake and suboptimal hydration are associated with greater cortisol reactivity to acute psychosocial stress. By stratifying participants into low- and high-habitual fluid intake groups using a national database (2), this study effectively captured meaningful distinctions in habitual hydration status. A greater proportion of LOW exhibited suboptimal hydration, evidenced by consistently greater UOsm and UCol in the days leading up to and on the morning of the TSST. Despite higher POsm in LOW on the day of the TSST, this difference was not statistically significant. The observed dissociation between POsm and urine hydration markers is consistent with prior research (22, 23, 56, 57) and suggestive of compensatory renal mechanisms, mediated via AVP, to maintain osmotic homeostasis (22). Given the challenges in directly measuring plasma AVP, due to its known instability and rapid clearance from circulation, we instead measured copeptin, the C-terminal segment of the AVP prohormone, which is secreted in equimolar amounts and serves as a reliable surrogate marker of AVP release (45). Accordingly, we observed higher plasma copeptin in LOW and a greater proportion of LOW exhibited plasma copeptin >4.3 pmol/L; a threshold associated with suboptimal hydration (34). In addition to AVP's role in body water regulation, it moderates ACTH secretion from the pituitary gland, which in turn stimulates cortisol release from the adrenal cortex (58). This pathway provides a possible mechanism for our finding that habitual low fluid intake and suboptimal hydration were associated with greater cortisol reactivity to acute stress. In the present study, we did not correlate plasma copeptin and saliva cortisol reactivity to the TSST, as doing so would have required repeated venipuncture or invasive cannulation, procedures likely to limit participant recruitment, introduce bias, and induce nonspecific subjective stress and cortisol responses (54, 59). Acknowledging this limitation, a previous study reported a positive association between circulating copeptin and saliva cortisol reactivity to the TSST (60), albeit fluid intake and hydration status were not assessed.

Studies investigating the association between hydration status and cortisol have been limited to either moderate-severe dehydration elicited in multistressor scenarios (e.g., by combinations of exercise, heat, and fluid restriction) or have relied upon single cortisol measures at unstandardized timepoints (19, 20, 22, 23). A recent study overcame the limitation of single cortisol measurements at unstandardized timepoints by assessing the influence of fluid intake and hydration on the dynamic CAR (24). The absence of a significant effect in that study may be attributed to the CAR reflecting a complex interaction between central circadian cortisol regulation and the state cortisol response to awakening (25). Unlike the CAR, the TSST affords the opportunity to assess robust HPA-axis reactivity, in most individuals, under conditions that control for external confounds and circadian cortisol regulation (29). Robust HPA-axis reactivity was necessary to test the hypothesis that individuals with habitual low fluid intake and suboptimal hydration exhibit a greater magnitude of cortisol reactivity to acute stress. Acknowledging that the TSST does not elicit meaningful HPA-axis reactivity in all participants (29), we restricted cortisol reactivity analysis to responders (81%) whose saliva cortisol reactivity exceeded the typical day-to-day variation established in a separate pilot investigation (49). Although the proportion of meaningful saliva cortisol responders was similar in LOW (n = 12 of 15) and HIGH (n = 13 of 16), saliva cortisol reactivity was greater in LOW (Fig. 4). As HPA-axis reactivity and subjective reactivity to acute stress do not always align (61), we also separately restricted saliva cortisol reactivity analysis to participants who reported a meaningful increase in state anxiety during the TSST (84%, Supplemental Fig. S1). Despite a smaller proportion of meaningful state anxiety responders in LOW (n = 11 of 15) compared with HIGH (n = 15 of 16), saliva cortisol reactivity was greater in LOW. In state anxiety responders, we also separately showed that pre-TSST hydration status was associated with the magnitude of saliva cortisol reactivity (Supplemental Fig. S2). Together, these findings indicate that habitual low fluid intake and suboptimal hydration may increase HPA-axis reactivity to day-to-day challenges that increase anxiety, but this requires substantiation in a naturalistic setting.

We acknowledge that the present study has several limitations and that important knowledge gaps remain. Due to the cross-sectional design, the findings are limited to assessing group differences and associations, without establishing causation. Randomized controlled trials are required to investigate the influence of modifying habitual water intake, within safe limits, on saliva cortisol reactivity to acute psychosocial stress. From a design perspective, HPA-axis response habituation presents a limitation for researchers who plan to integrate repeated acute stress tests within a randomized controlled trial (62, 63). HPA-axis response habituation, characterized by blunted saliva cortisol upon repeat stressor exposure, makes it difficult to discern the true effects of any intervention. Although strategies such as extended washout periods between repeated TSST exposures (6 wk or more) and protocol modifications to preserve the unpredictability and uncontrollability of the repeated TSST may mitigate habituation (48), effectiveness is not guaranteed, and these modifications introduce substantial logistical and resource demands for investigators. As a result, most studies, including the present one, rely on cross-sectional comparisons of HPA-axis reactivity to the TSST. We also acknowledge that any long-term health implications of the present findings remain unknown. In this regard, abnormal HPA-axis function, marked by dysregulated cortisol secretion, is implicated in the pathophysiology and progression of cardiovascular and metabolic disease (18, 64, 65). Incidentally, habitual low fluid intake is also associated with cardiovascular and metabolic disease risk (7, 9, 10), but whether cortisol reactivity to psychosocial stress is involved remains to be determined. To this end, prospective longitudinal studies are required to investigate the association between habitual fluid intake, acute stress-induced cortisol reactivity, and health outcomes. Given the potential influence of postural changes on neuroendocrine reactivity, researchers should select acute stress protocols that standardize posture, such as the TSST (66). It is conceivable that increased orthostatic stress in suboptimally hydrated individuals may contribute, at least in part, to exaggerated cortisol reactivity to

acute stress involving postural changes (67), but this requires investigation. Another limitation is that despite balancing the number of males and females in LOW and HIGH, we did not investigate sex differences in stress reactivity (42). Also, male participants with a habitual fluid intake between 1.6 and 2.9 L/day and female participants consuming between 1.5 and 2.5 L/day were excluded by design, which may limit the generalizability of our findings. Notwithstanding, the participants included in this study represented the lowest and highest quartiles of habitual fluid intake using a national database (2), effectively capturing the fluid intake patterns of half the wider population of healthy adults aged 18–34 yr. The selected LOW and HIGH quartiles provided a broad distribution of hydration status across the normal clinical range [e.g., UOsm clinical range: 50–1,200 mosmol/kgH_2O (68), range of UOsm on the morning of the TSST: 60–1,013 mosmol/kgH_2O]. Finally, although the TSST is an established laboratory protocol for examining acute stress reactivity (26), experts raise challenges regarding ecological validity and argue that TSST responses offer limited insight into the cumulative effects of daily stress (69). Although the TSST has acknowledged limitations as a laboratory simulation, selling one's qualities in an interview scenario is a relevant life experience, enhancing its ecological validity (27), and saliva cortisol responses to the TSST have been shown to mirror responses in a naturalistic setting (28). Importantly, from a clinical perspective, the magnitude of stress reactivity to the TSST has been associated with poor future health and disease risk (16).

In conclusion, these findings show that adults with habitual low fluid intake exhibit greater cortisol reactivity to acute psychosocial stress. Suboptimal hydration status was associated with a greater magnitude of cortisol reactivity to acute psychosocial stress. Given cortisol's widely acknowledged role in health and disease across the lifespan, these findings provide a candidate mechanism to explain why habitual low fluid intake and suboptimal hydration are associated with poor long-term health. Furthermore, from a methodological standpoint, these findings highlight the importance of accounting for fluid intake and hydration status when assessing cortisol reactivity to acute psychosocial stress.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author, Neil P. Walsh, upon reasonable request.

SUPPLEMENTAL MATERIAL

Supplemental Table S1 and Supplemental Figs. S1–S3: https://doi.org/10.6084/m9.figshare.29654711.v1.

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DISCLOSURES

N.P.W. and J.P.E. report no conflicts of interest that are directly relevant to the completion of this work. Postdoctoral and research assistant salaries for D.S.K. and M.H. were paid by Danone Research & Innovation. J.Z. and J.L. are current full-time employees, and E.T.P. and A.D. were previous full-time employees of Danone Research & Innovation. A.-C.M. is a full-time employee of ATLANSTAT on behalf of Danone Research & Innovation. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

D.S.K., J.Z., E.T.P., A.D., and N.P.W. conceived and designed research; D.S.K., M.H., J.P.E., and N.P.W. performed experiments; D.S.K., M.H., J.P.E., and N.P.W. analyzed data; D.S.K., M.H., J.P.E., and N.P.W. interpreted results of experiments; D.S.K. and N.P.W. prepared figures; D.S.K. and N.P.W. drafted manuscript; D.S.K., J.Z., J.L., A.-C.M., E.T.P., and N.P.W. edited and revised manuscript; D.S.K., M.H., J.P.E., J.Z., J.L., A.-C.M., E.T.P., A.D., and N.P.W. approved final version of manuscript.

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