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Effects of psychological, sensory, and metabolic energy prime manipulation on the acute endocrine stress response in fasted women

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ABSTRACT

The stress response supports survival through energy mobilization. Paradoxically, a low blood glucose level dampens the endocrine stress response, and sugar consumption prior to stress restores it. Thus, energy availability may play a causal role in the endocrine stress response. Yet, it has never been tested whether sweet taste or expectations towards a drink content modulate the stress response. We investigated the potential role of sweetness, energy load and expectations towards energy load of a drink consumed prior to stress in restoring stress reactivity after fasting. $N = 152$ women ($mean_{age}$=21.53, sd_{age}=2.61) participated in the Trier Social Stress Test for groups in the morning after an overnight fast. Prior to stress induction, participants consumed a drink containing saccharose (*sugar*, $n = 51$), an equally sweet drink containing non-caloric sweetener (*sweetener*, $n = 46$), or *water* ($n = 56$). Additionally, participants in the sugar and sweetener group ($n = 97$) were informed whether or not their drink contained any calories (*energy prime*), which was deceptive in 50% of the cases. Eight salivary cortisol (−30, −20, −10, 0, +12, +25, +35, +45 min) and three blood glucose samples (−30, 0, +25 min) were assessed throughout the experiment. The effects of the experimental manipulations on cortisol trajectories were tested using multilevel mixed models. We found that compared with water, sugar and sweetener both significantly increased cortisol stress reactivity and with comparable intensity. However, our sensitivity analysis revealed a significant effect of sugar on cortisol trajectories compared to water and to sweetener. Drink-induced changes in blood glucose concentration were not associated with increases in cortisol. The *energy prime* did not affect the stress response. Overall, we could replicate the boosting effect of sugar consumption in a female sample after 8 h of fasting. The specific contribution of sweet taste and metabolic hormones to this boosting effect should be tested more rigorously in sex-balanced designs in the future.

1. Introduction

Exposure to acute stress triggers psychophysiological processes involving the activation of central limbic structures, the autonomic nervous system (ANS), and the hypothalamic-pituitary adrenal (HPA) axis (Hermans et al., 2014; Pruessner et al., 2008; Ulrich-Lai and Herman, 2009). These processes support survival by triggering adrenaline and cortisol release, which mobilize glucose from body storages. As a consequence, blood glucose levels rise (*hyper*glycemia) facilitating energy availability in the periphery and the brain. This tight link between the HPA axis and glucose metabolism is illustrated by the nomenclature of the HPA axis' major compound class: *gluco*corticoids (McEwen and Akil, 2020).

Paradoxically, the endocrine stress response seems to depend on energy availability. This was proposed by a study that showed that men with low blood glucose levels after an 8 h overnight fast showed no cortisol response to acute stress (Kirschbaum et al., 1997). While glucose consumption prior to stress restored the cortisol response, glucose consumption by itself, in absence of stress, was not sufficient to trigger a cortisol increase (although there is mention of a cortisol lunch peak, suggesting that glucose intake can activate the HPA axis (Quigley and Yen, 1979)). In this small, yet well-controlled study (Kirschbaum et al., 1997), the restoring effect of glucose was attributed to the blood glucose rise (in the following referred to as *energy load*). A follow-up study

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supported the energy load hypothesis by showing that neither fat, nor complex carbohydrate, nor protein consumption prior to stress had similar effects (Gonzalez-Bono et al., 2002). In sum, a sugar-induced rise in blood glucose levels seems to increase the cortisol stress response after long fasting intervals in men.

These earlier studies (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997) focused on metabolic characteristics of glucose; other possible aspects of glucose load were neither examined, nor controlled for. Besides its caloric content, the prominent sweet taste is one distinct feature of glucose. It is perceived whenever compounds such as caloric sweeteners (sugar, e.g. glucose, saccharose), or non-caloric sweeteners (e.g. aspartame, stevia) activate type 1 taste receptors (T1R2/T1R3) in the oral cavity (Behrens and Meyerhof, 2019; Lee and Owyang, 2017; Meyers and Brewer, 2008). Although T1R2/T1R3 activation at sites outside of the mouth is not accompanied by sensation of sweet taste, it is nevertheless related to physiological changes (Tucker and Tan, 2017). For example, in the gastrointestinal tract, T1R2/T1R3 play a major role in the sensation of nutrients, and thus in the regulation of food intake and glucose homeostasis (Lee and Owyang, 2017). Interestingly, endocrine signals, e.g., circulating hormones such as adrenaline, can modulate taste perception (Foster et al., 2014). In turn, it seems plausible that T1R2/T1R3 activation could indirectly modulate endocrine stress responses, e.g. by stimulating mesolimbic, reward related pathways (Ulrich-Lai and Ryan, 2014). Moreover, the effects of glucose and non-nutritive sweetener load on behavior and physiological responses have been investigated in other fields of neuroscience, e.g., in studies on cognitive control (Dang, 2016; Vadillo et al., 2016) or ostracism (e.g. Miller et al., 2014). There, the role of energy load as a buffer against ego depletion or ostracism was questioned, yet effects of sweetness on motivation have been discussed in a similar fashion (Dang, 2016). If sweet drinks, regardless of their caloric content, can modulate stress responses after long fasting intervals, this would question the energy load hypothesis, and a linear relationship between blood glucose levels and cortisol stress responses.

First evidence supporting this notion stems from two studies investigating the effect of sweetener load on the cortisol stress response after short fasting periods of 3–4 h (von Dawans et al., 2020; Zänkert et al., 2020). A study in men and women compared the effect of glucose, grape juice (frequently used in research investigating the acute stress response due to it having the highest sugar content among natural fruit juices, Zänkert et al., 2020), and maltodextrin (a polysaccharide which has a similar caloric load, but is perceived far less sweet as compared with glucose) prior to stress to a control group, which did not receive any drink, after 3 h of fasting (Zänkert et al., 2020). Although blood glucose levels were not measured objectively, results indicated that sweet drinks with differing caloric load (32 g of sugar in the grape juice, 75 g in the glucose condition) led to comparable increases in cortisol stress responses in comparison to the control group. Interestingly, cortisol stress trajectories of the group consuming maltodextrin (75 g) lay between the control group (from which it did not differ significantly), and the glucose and grape juice groups. These results imply that energy load is not the sole factor driving the restoration of the cortisol stress response after short fasting intervals. In line with this finding, in a study in which male subjects drank either sugar, sweetener, or water before stress after 4 h of fasting (von Dawans et al., 2020) there was no linear relationship between blood glucose and cortisol stress responses. Again, this speaks against the earlier proposed energy load hypothesis (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997). Here, it is noteworthy that only sugar, but not sweetener increased cortisol levels in comparison to the water control group (von Dawans et al., 2020). Since the fasting period was rather short in these studies (von Dawans et al., 2020; Zänkert et al., 2020), it is at this point unclear, whether the taste-related or the metabolic property of glucose, or a combination of the two, or any other factor related to glucose uptake caused the restoring effect of glucose on cortisol stress reactivity after long fasting periods of at least 8 h (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997). Further, the association has never been studied in female participants who fasted for longer than 4 h.

Besides the energy load and the sweet taste perception provided by glucose uptake, there are several other factors that could explain the restoring effect of glucose on the cortisol stress response after fasting. In a natural environment, we try to infer the drink's content prior to consumption e.g., based on its color, or verbal descriptions, both of which have been shown to affect subsequent taste ratings (Verhagen and Engelen, 2006; Wansink et al., 2006). Such cues could lead to implicit or explicit expectations towards drink content, which in turn may trigger various anticipatory responses. The verbal information of whether a drink is caloric vs. non-caloric independent of its actual energy load (in the following referred to as *energy prime*) might therefore influence physiological responses, for example, by influencing brain circuits regulating energy homeostasis (Veldhuizen et al., 2013). Taken together, there are several different factors that could explain why glucose intake prior to stress enhances the cortisol stress response after long fasting intervals.

Aim of this study was to test three plausible mechanisms: First, we wanted to test whether *energy load* affects the cortisol stress response after long fasting periods, as had been suggested by prior studies (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997). Second, we wanted to investigate the effect of sweet taste independent of caloric load. Third, we were interested in whether an *energy prime* would affect the cortisol stress response after long fasting periods. To this end, we conducted the following experiment as part of a larger research project: In the morning after an overnight fast, participants received written information on whether they would consume a drink containing calories vs. no calories (*energy prime*) which was deceptive in 50% of the cases. Independent of the information presented, a sugar-sweetened, caloric drink or a drink containing non-caloric sweetener was consumed (*energy load*). A control group drank plain *water* and received neither energy prime nor energy load. After that, participants were exposed to a modified version of the Trier Social Stress Test for groups (von Dawans et al., 2011), a well-established and standardized paradigm to induce psychosocial stress in a group setting. Physiological and subjective stress measures were assessed at eight, and blood glucose levels were assessed at three predefined timepoints.

Prior to the statistical analysis of the data, we preregistered our statistical analysis plan on the Open Science Framework platform (see https://osf.io/pfxe8/; date of registration: January 30, 2020): We set out to test the differences between (a) groups consuming sweet drinks vs. water (effect of *sweetness*), (b) groups consuming sugar vs. non-caloric drinks (effect of *energy load*), and (c) groups receiving the information that the drink contains calories vs. no calories (effect of *energy prime*). Further, we planned to explore the combined effect of *sweetness*, *energy load* of drinks, and *energy prime* in an interaction model. These hypotheses were formulated in a non-directional manner, since the studies on effects of glucose and sweetener administration on the cortisol stress response after short fasting periods (von Dawans et al., 2020; Zänkert et al., 2020) were not published at the time of registration. Taking the results of recent studies (von Dawans et al., 2020; Zänkert et al., 2020) into account, we would have expected that *sugar* load prior to stress increases the cortisol stress response after long fasting periods compared to *water* or *sweetener* load.

Lastly, although not preregistered, we decided to test the relationship between blood glucose levels and cortisol stress reactivity. While some studies found a positive relationship between the two (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997), others found evidence that speaks against the proposed energy load hypothesis (von Dawans et al., 2020; Zänkert et al., 2020).

2. Materials and methods

To answer our research questions, we combined data of two experiments collected within a larger research project in which we

investigated metabolic aspects of the endocrine stress system. The project was approved by the Ethics Committee of the University of Constance and was carried out in accordance with the Declaration of Helsinki. The two experiments were equal in their temporal and procedural sequence, and in the key psychometric and physiological markers. A complete list of variables that were assessed during both experiments can be obtained from the Open Science Framework project associated with this work (https://osf.io/5vzwu/). In the first wave experiment (n = 122), participants with varying degrees of perceived maternal care during childhood were quasi-randomly assigned to consume either *grape juice* or *water* before psychosocial stress exposure (Bentele et al., 2021). For results on the grape juice group, please see (Bentele et al., 2021). In the second wave experiment (n = 105), fasted participants received an *energy prime* (either "The drink you consume is caloric and contains energy" indicated by '+', or "The drink you consume is non-caloric and does not contain energy" indicated by '-') and consumed a sweet drink containing either *sugar* or a non-caloric *sweetener* before psychosocial stress exposure, resulting in a 2 × 2 design. Thus, participants of the second wave were randomly assigned to one of four experimental conditions: *sugar+* , *sugar-*, *sweetener+* , *sweetener-*. For further information on the blinding procedure, see supplemental information, S1. For financial and human resource reasons, the *water* group of experiment 1 constituted the convenience control group in the current analysis, since procedures were identical.

2.1. Participants, procedure and sample size

Recruitment for both experiments took place in two waves via flyers and online advertisements at the University of Constance between June 2017 and February 2019 (first wave, experiment 1) and between February 2019 until December 2019 (second wave, experiment 2). We had originally planned to implement a sex-balanced design in the project. Yet, due to a very small number of recruited male participants after six months of testing in the first wave (despite of extensive advertisement), we had to drop the recruitment of males and decided to focus on female participants. In addition, there is still a lack of research on this topic including female participants. In each wave, an online screening took place for the following exclusion criteria: (1) age< 18, or> 35 years, (2) current pregnancy, (3) symptoms of moderate to severe depression (indicated by Beck's Depression Inventory II sum score<19) (Kühner et al., 2007), (4) being underweight or obese (indicated by a body mass index<17.5, or>30), (5) smoking> 5 cigarettes per day, (6) working night-shifts, (7) current drug or medication intake affecting the autonomous, endocrine or central nervous system (e.g. antihistamines), (8) lack of German language skills. Furthermore, participants with sugar or sweetener intolerance or allergy, or participants deliberately avoiding sugar in their diet were excluded during the recruitment of the second wave.

Eligible participants were invited to a 90 min laboratory session in groups of up to four. Prior to the experimental session, participants were asked to fast for at least 8 h, and refrain from smoking 1 h prior to testing. To make fasting easier for the participants, we invited them to the laboratory in the morning, at 0800 h or 1000 h. First, they gave written informed consent and provided demographic data (10 min). Participants then received an energy prime and consumed a sweet drink, while the control group received no prime and drank water. Participants were then exposed to the TSST-G (35 min). In the following recovery period (30 min), participants completed questionnaires. Throughout the experiment, participants provided eight saliva samples and subjective stress ratings at −30, −20, −10, 0, +12, +25, +35, +45 min in respect to the start of the TSST-G. Further, we measured blood glucose levels at three timepoints, at −30, 0 and + 25 min. At the end, participants were thanked, debriefed, and compensated (€25). The full study procedure is depicted in Fig. 1.

The sample size determination for both projects was based on feasibility considerations regarding financial and personnel resources. Prior to conducting the second wave assessment, we decided to assess a total of n = 100 participants, with n = 25 participants in each experimental condition (*sugar+, sugar-, sweetener+, sweetener-*) which is comparable to the sample size of a recent study in this context (von Dawans et al., 2020). To account for dropouts and potential exclusions, we tested n = 105 participants in the second wave.

By adding the additional *water* group (n = 61), data of N = 166 women of the two waves were considered for this analysis. From this sample, n = 4 were excluded due to increased fasting blood glucose levels (>110 mg/dl), n = 9 were excluded due to non-compliance to the instruction (e.g., reported to be in a non-fasted state), or due to exposure to the TSST within the past six weeks, and n = 1 was excluded due to

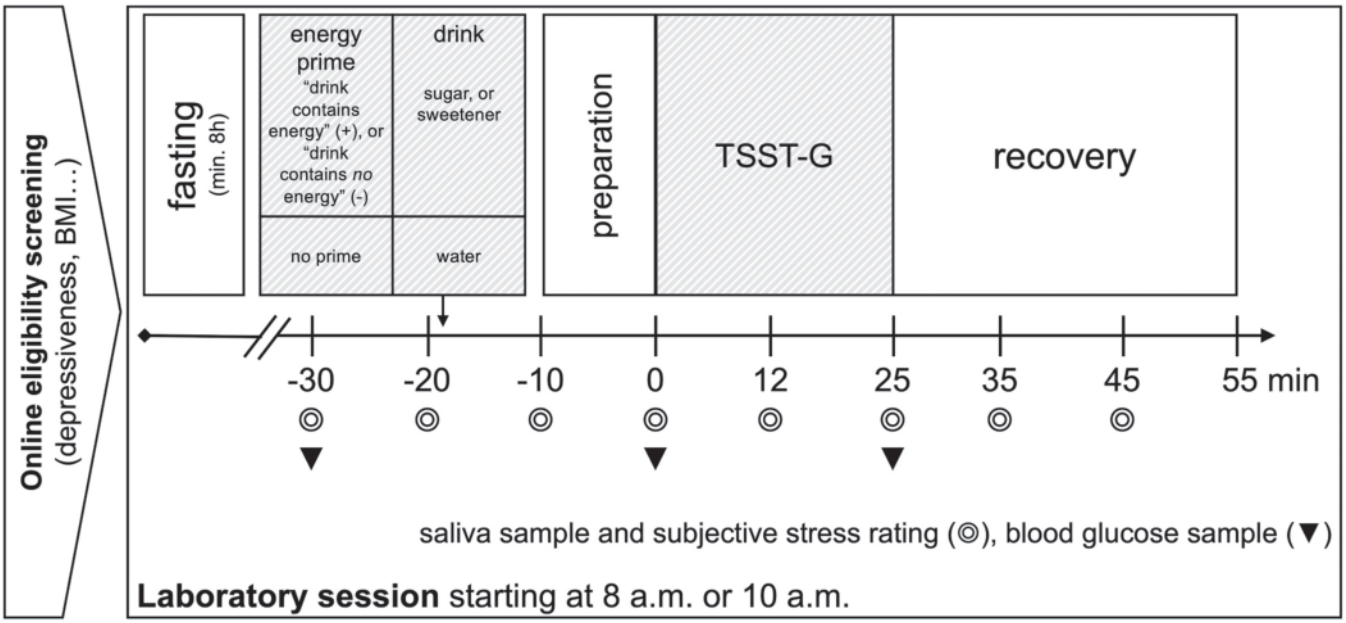

Fig. 1. Overview of the study procedure. After baseline measurements, eligible, fasted participants received the energy prime, and a drink containing caloric, or non-caloric sweetener. The control group consumed water. Later, participants were exposed to a modified Trier-Social-Stress-Test for groups (TSST-G). During recovery, participants completed questionnaires. BMI=body mass index.

insufficient amount of saliva provided in the samples.

2.2. Experimental manipulation

2.2.1. Energy prime and consumed drinks

2.2.1.1. Energy prime. After obtaining two cortisol and subjective stress baseline measurements (−30 and −20 min), participants consuming *sugar* or *sweetener* either received the written information "The drink you consume is caloric and contains energy" (indicated by '+'), or "The drink you consume is non-caloric and does not contain energy" (indicated by '-'). The presented information did not depend on the actual energy load of the drink (see below). Thus, roughly 50% of participants were deceived (they received the information that they would consume a non-caloric drink although the drink contained calories, and vice versa), while 50% of the information matched the actual drink content. The information was blinded for experimenters; participants were asked not to disclose it to others. The water group did not receive an energy prime.

2.2.1.2. Drinks. Participants consumed a drink containing either 25 g of saccharose (*sugar*), or 25 g of non-caloric sweetener (*sweetener*), dissolved in water. The non-caloric sweetener we used was 'borchers bff Stevia Kristall' (mix of erythrite E968 and stevioglycoside E960), which replaces the sweetness of saccharose in a 1:1-ratio. This allowed us to blind experimenters and participants to drink content. Erroneously, either 200 or 400 ml of water were used to dissolve the crystals; volumes were noted on the testing protocol and its effect was tested in the course of the statistical analysis (Table 1 lists the number of 200 and 400 ml water doses used per experimental group). The control group received non-sparkling, mineral water (400 ml). All drinks were consumed at room temperature.

2.2.2. Stress induction

The Trier Social Stress Test for groups (TSST-G) (von Dawans et al., 2011) was applied as an economic, standardized laboratory procedure that reliably induces acute psychosocial stress in a group of six people. It combines high levels of uncontrollability and social-evaluative threat (Dickerson and Kemeny, 2004). The TSST-G consists of a preparation period (10 min) and a fictive, videotaped job interview in which participants perform a free speech (2 min for each participant, 12 min overall) and an arithmetic task (80 s for each participant, 8 min overall) in front of a two-member, mixed-sex committee wearing white laboratory coats. During the free speech and the arithmetic task, the committee calls participants in random order. For feasibility reasons, we modified the temporal sequence of the TSST-G (preparation period: 5 min; free speech task: 3 min for each participant, 12 min overall; arithmetic task: 3 min for each participant, 12 min overall), which resulted in a slightly longer arithmetic problem solving period for each participant in our protocol compared with the original protocol. In case of individual cancellations, the overall duration was divided equally between participants. If only two participants were present, individual speaking time in each task was set to 5 min for each participant, which is comparable to the original TSST protocol for a single participant (Kirschbaum et al., 1993), with 1 min breaks between speakers. We did not change other parts of the procedure. Using this modified version of the TSST-G, our group had previously induced robust cortisol stress responses (Meier et al., 2021; Popovic et al., 2020).

2.3. Measures

2.3.1. Biomarker assessment

2.3.1.1. Cortisol. Saliva samples for free cortisol (nmol/l) analysis were collected at eight prescheduled timepoints (see Fig. 1) using Salivettes (Sarstedt, Nümbrecht, Germany) (Gröschl et al., 2008). Samples were stored at − 20 °C until biochemical analysis which took place within half a year after collection of the samples. Average storage duration across both waves was 42 days, with a range of 0–105 days. A statistical comparison of storage duration showed that it was significantly shorter than the recommended 6 months (or 183 days) across all study groups (all $p < .001$ in *t*-tests comparing mean storage time per group to 183

Table 1
Descriptive statistics of the experimental conditions.

	Sugar+ ($n = 24$)	Sugar- ($n = 28$)	Sweetener+ ($n = 25$)	Sweetener- ($n = 21$)	Water ($n = 54$)	*inferential statistics*	*p-value*	*effect size*
age	22.67 ± 3.10	20.21 ± 1.89	21.60 ± 2.77	20.71 ± 2.05	21.98 ± 2.51	$F(4, 147) = 4.17$	$p = .003$	$eta^2_{partial} = 0.10$
BMI[a]	22.31 ± 2.38	21.45 ± 2.18	22.27 ± 1.97	22.14 ± 2.92	21.63 ± 2.18	$F(4, 147) = 0.87$	$p = .485$	$eta^2_{partial} = 0.02$
depressiveness[b]	4.42 ± 4.41	4.96 ± 4.46	4.60 ± 4.95	4.43 ± 3.88	5.94 ± 5.45	$F(4, 147) = 0.70$	$p = .592$	$eta^2_{partial} = 0.02$
childhood trauma[c]	1.17 ± 0.82	1.14 ± 0.76	1.48 ± 1.33	1.24 ± 0.89	1.38 ± 1.04	$F(4, 146) = 0.58$	$p = .675$	$eta^2_{partial} = 0.02$
cortisol baseline[d]	5.16 ± 3.75	5.32 ± 3.10	4.82 ± 3.38	4.59 ± 3.54	9.88 ± 6.56	$F(4, 147) = 9.09$	$p < .001$	$eta^2_{partial} = 0.20$
fasting blood glucose	93.25 ± 7.24	93.50 ± 9.72	89.28 ± 7.41	89.48 ± 9.21	87.76 ± 9.78	$F(4, 147) = 2.74$	$p = .031$	$eta^2_{partial} = 0.07$
hormonal status[e,f] (follicular/ luteal/OC)	7/6/10	9/8/11	4/4/17	6/7/7	17/21/16	$X^2(8) = 11.39$	$p = .180$	Cramer's V= 0.16
session start[e] (0800 h/ 1000 h)	5/19	0/28	6/19	4/17	35/19	$X^2(4) = 42.95$	$p < .001$	Cramer's V= 0.53
drink volume[e] (200 ml/ 400 ml)	20/4	16/12	21/4	16/5	0/54	$X^2(4) = 82.45$	$p < .001$	Cramer's V= 74

Note. If not otherwise specified, a one-way Analysis of Variance by experimental condition was calculated to test whether groups differed in respect to the listed variables. In these cases, data is expressed as *mean* ± *standard deviation*.

[a] BMI=body mass index,

[b] indexed by Beck's Depression Inventory II sum score,

[c] indexed by Childhood Trauma Questionnaire sum score (Bernstein et al., 2003),

[d] average of the first two measurements,

[e] Pearson's Chi-squared test was calculated to test whether groups differed in respect to the listed variable,

[f] $n = 150$ due to missings. OC=oral contraceptive use. Hormonal status was determined as described by Benz and colleagues (Benz et al., 2019). Results of post-hoc *t*-tests are reported in Section 3.1. Preliminary analyses.

days).

Samples of the first wave were analyzed at the biochemical laboratory of the University of Trier using a fluorescence immunoassay with proven reliability and validity (Dressendörfer et al., 1992) (lower detection limit: 0.43 nM, inter-assay coefficient of variation (CV) below 9.0% and intra-assay CV below 6.7% according to manufacturer). Thawed samples were centrifuged at 3000 rpm for 6 min. Samples of the second wave were analyzed in the biochemical laboratory of the Department of Neuropsychology of the University of Constance using a commercially available competitive enzyme immunosorbent assay (Cortisol Saliva ELISA, RE-52611, IBL International GmbH, Hamburg, Germany; lower detection limit: 0.030 µg/dL, inter-assay CV below 9.3% and intra-assay CV below 7.3% according to manufacturer). Thawed samples were centrifuged at 2500 g for 10 min. No values below the lower, or over the upper detection limit were observed.

2.3.1.2. Blood glucose. Blood glucose concentrations (mg/dl) were measured at three scheduled timepoints (see Fig. 1) in capillary blood of the fingertip using disposable lancets (Roche Diabetes Care, Mannheim, Germany) and glucometer (A. Menarini diagnostics, Berlin, Germany).

2.3.2. Self-report measures

2.3.2.1. Subjective stress. Subjective stress was assessed along the dimensions pleasure and arousal using the Affect Grid (Russell et al., 1989). The Affect Grid assesses pleasure and arousal on a single item scale; the scores on each dimension range from 1 (low arousal, and low pleasure resp.) to 9 (high arousal, and high pleasure resp.). Arousal and (inverted) pleasure scores were multiplied to receive a single-item score, with higher scores indicating higher levels of *subjective stress* (range 1–81).

2.3.2.2. Potential covariates. We used the Beck's Depression Inventory II to measure *depressiveness* (Kühner et al., 2007) and the Childhood Trauma Questionnaire (Bernstein et al., 2003) to estimate the overall exposure to childhood trauma. For both scales, we computed a total sum score, which were tested as potential covariates in the subsequent statistical analysis.

Further, self-reported information on the last menstrual cycle, usual menstrual cycle duration and oral contraceptive use was assessed to estimate women's hormonal status using a formula described previously (Benz et al., 2019).

2.3.2.3. Energy prime manipulation check. At the end of the experiment, participants consuming sweet drinks were asked to rate whether they thought the drink they consumed contained more or less sugar compared to the same amount of Coke® (which contains approximately 10 g sugar per 100 ml; answer format: 5-point Likert scale ranging from 1 ="My drink contained considerably less sugar compared to Coke®." to 5 ="My drink contained considerably more sugar compared to Coke®."). Further, they reported what they thought they had consumed (sweetener, sugar, or water).

A complete list of variables that were assessed during the project but were not included within the presented statistical analysis can be found on the Open Science Framework project associated with this work (https://osf.io/qmcgz/).

2.4. Data processing

First, raw cortisol values were investigated for plausibility. Since cortisol responsiveness has been shown to be reduced after fasting intervals of 8–11 h (Kirschbaum et al., 1997), as a result of which blood glucose levels usually range between 70 and 110 dg/ml (American Diabetes Association, 2001), we considered an increase criterion for cortisol non-responder detection (Miller et al., 2013a) was inadequate. Instead, individual cortisol trajectories were screened visually for plausibility and non-responsiveness due to very high initial cortisol concentration (>20nmol/l of sample −30 and −20 min, both taken prior to the experimental manipulations). Such high concentrations were potentially caused by an ongoing cortisol awakening response (Miller et al., 2016). Subsequent analyses were conducted both including and excluding non-responders ($n = 20$).

Second, since absolute values determined by different immunoassays are not readily comparable, raw cortisol values were converted into cortisol factor scores for statistical analyses (Miller et al., 2013b), an approach that has already been successfully applied in other studies (e.g. Miller et al., 2016; Reyes et al., 2015).

Third, cortisol, blood glucose, and subjective stress values were winsorized across experimental groups, so that values that exceeded the mean of the experimental group by more than 3 *SDs* were replaced with *3 SD* to decrease the impact of statistical outliers (cortisol: 2.14% of datapoints>3 *SD*; blood glucose: 0.44% of datapoints>3 *SD*; subjective stress: 0.82% of datapoints>3*SD*).

Fourth, cortisol, blood glucose, and subjective stress values were screened for missing values. Missing data at the first or last assessment were replaced by the mean of the respective experimental group at that timepoint (cortisol: 0%; blood glucose: 0%; subjective stress: 0.08%). Missing values at other timepoints were imputed linearly by inserting the mean of the individual's value prior to the missing value and the individual's value after the missing value (cortisol: 0.08%; blood glucose: 0.22%; subjective stress: 0.16% missing values).

After that, *cortisol baseline* and *subjective stress baseline* were calculated by averaging the first two measurements. *Cortisol stress reactivity* was operationalized using the area under the cortisol curve with respect to increase ($AUCi_{cort}$) (Pruessner et al., 2003) from stressor onset (0 min) to end of recovery (45 min) and calculated using the winsorized cortisol factor scores. *Blood glucose increase* in response to the drink was operationalized by subtracting the second blood glucose value from the fasting level. *Subjective stress increase* in response to the stressor was operationalized by subtracting the *subjective stress baseline* from the measurement after cessation of the stressor (+25 min). Last, to enhance interpretability of the statistical models, cortisol factor scores were z-transformed.

2.5. Statistical analysis

Analyses were conducted using *R* version 3.5.3 (R Core Team, 2019), *RStudio* version 1.1.463 (RStudio Team, 2016), and *nlme* (Pinheiro et al., 2018). Graphs were created using *ggplot2* (Wickham, 2016) and *patchwork* (Pedersen, 2019). The level of significance was set to $\alpha = 0.05$. Parts of this analysis were preregistered at the Open Science Framework (https://osf.io/pfxe8/), however, we have in some parts deviated from this preregistration. The main preregistered analysis included the outcome variables cortisol, alpha amylase, high-frequency heart rate variability, and subjective pleasure and arousal. Due to the closure of our biochemical laboratories during the corona pandemic, salivary alpha amylase has not been analyzed yet and is thus not included in this work. Furthermore, since we were focusing on the effect of sweetness, we decided to not include HF-HRV, as these data are not available for the *water* group. Thus, in the presented analysis, we included the variables cortisol, and subjective pleasure and arousal (which was summarized to subjective stress). Originally, we had preregistered that we expected significant group differences in terms of subjective mood measures (pleasure and arousal) dependent on the different experimental manipulations. Given the complexity of the current set of findings as it stands, we decided to not conduct the subjective mood effects analyses.

In a first step, we examined the influence of potential person-related covariates that might have influenced the main outcomes of our study. One-way Analysis of Variances (ANOVAs) with *experimental condition* (five levels: *sugar+*, *sugar-*, *sweetener+*, *sweetener-*, and *water*) as independent variable and *age*, *body mass index* (BMI), *depressiveness* (Beck's

Depression Inventory II sum score), *childhood trauma* (Childhood Trauma Questionnaire sum score), *cortisol baseline*, and *fasting blood glucose* as dependent variables were used to detect potential covariates associated with *experimental condition*. Pearson's Chi-squared test was used to test whether *hormonal status* (follicular/luteal/oral contraceptive use), *session start* (0800 h /1000 h), and *drink volume* (200 ml/400 ml) were equally distributed across *experimental conditions*. Variables that were not equally distributed across the groups were considered as potential covariates and their effect was evaluated in subsequent analyses.

In a second step, we tested the influence of design-related factors on cortisol concentration at baseline and on cortisol reactivity. A Welch two-sample *t*-test was used to test the effect of *session start* on *cortisol baseline*. Using an Analysis of Covariance (ANCOVA), we tested whether *session start* had an influence on *cortisol stress reactivity* while controlling for the influence of *experimental condition*. Using the same approach, we tested whether *cortisol baseline* affected *cortisol stress reactivity*. In two ANCOVAs, we tested whether *drink volume* (200 ml and 400 ml) and *hormonal status* (follicular/luteal/oral contraceptive use) influenced *cortisol stress reactivity*, while controlling for the influence of *experimental condition*. To indirectly get a sense of whether the different volumes affected taste perception, we further tested whether *drink volume* and drink content (*sugar* or *sweetener*), or an interaction of both variables affected participant's rating of the drink's estimated amount of sugar as compared to Coke® using a multiple regression model.

In a third step, we ran two manipulation checks: Two ANOVAs were used to test whether *experimental condition* had an influence on *blood glucose increase* in response to the drink consumption, and on *subjective stress increase* in response to the stressor.

In a fourth step, we tested our hypothesis following the models we preregistered at the Open Science Framework (https://osf.io/pfxe8/): We modeled multiple growth curves to test whether (A) *sweetness* (dummy variable: *sugar*+=1, *sugar*-=1, *sweetener*+=1, *sweetener*-=1, and *water*=0), (B) *energy load* (dummy variable: *sugar*+=1, *sugar*-=1, *sweetener*+=0, *sweetener*-=0, and *water*=0), and (C) *energy prime* (dummy variable: *sugar*+=1, *sugar*-=0, *sweetener*+=1, *sweetener*-=0; *water* was dropped since no prime was applied) influenced cortisol trajectories, while accounting for interindividual variability in cortisol responses (random effects). The models were built hierarchically: fixed intercept model (cortisol predicted by intercept), random intercepts across individuals, fixed slopes across time, random slopes across time, and a linear, quadratic, and cubic trend of time as orthogonal predictors (time, time^2, and time^3 model). Then, the interaction of time trend and the respective independent variable was included. Resulting changes in overall model fit by means of log-likelihood ratio were compared using an ANOVA and the final model was evaluated. Lastly, we planned to model a growth curve including all three independent variables (*sweetness*, *energy load*, and *energy prime*) to evaluate their combined effect on cortisol trajectories. Since *energy prime* did not significantly change cortisol trajectories, we subsequently did not include it in the interaction model to enhance model parsimony. Due to model convergence issues when *sweetness* and *energy load* were entered as separate dummy variables, we used the variable *drink* (numeric variable with three levels: *sugar*=2, *sweetener*=1, *water*=0) to evaluate the hypothesis.

In a last step, we computed Pearson's correlation coefficients of *blood glucose increase, second blood glucose sample, third blood glucose sample* and *cortisol stress reactivity* to explore the relationship between those measures analogously to the computational approach of previous studies (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997). This analysis was not preregistered.

To test the robustness of the observed effects, we ran a sensitivity analysis in a subset of the sample by excluding cortisol non-responders and participants tested at 0800 h.

3. Results

3.1. Preliminary analyses

The final sample consisted of N=152 healthy women (mean$_{age}$=21.53, sd$_{age}$=2.61) from the experimental conditions *sugar*+ (n=24), *sugar*- (n=28), *sweetener*+(n=25), *sweetener*- (n=1), and *water* (n=54). Descriptive statistics of the groups are summarized in Table 1.

From overall n=98 participants receiving an *energy prime,* 87% (n=84 of 97; n=1 did not answer that question) believed the information. Further, 83% of the deceived participants (groups *sugar-, sweetener+; n*=44 of 53) believed the information. More information can be found in the Supplemental information, S2.

The results of the analyses conducted to identify covariates associated with *experimental condition* are summarized in Table 1. Following up on significant group differences, Bonferroni corrected post-hoc *t*-test revealed that *sugar-* was significantly younger than *sugar+*(p=.006), and *water* (p=.029). Although the omnibus test comparing group differences in respect to *fasting blood glucose* was significant, Bonferroni corrected post-hoc *t*-tests revealed no significant differences between *experimental conditions* (all p>.05).

Cortisol baseline of *water* was significantly higher in comparison to all other groups (*sugar+, p*=.001; *sugar-, p*=.001; *sweetener+, p* < .001; *sweetener-, p* < .001). This could be related to the facts that (A) *water* was tested predominantly at 0800 h, while all other conditions were tested more frequently at 1000 h (variable *session start*) and (B) *cortisol baseline* was significantly higher in participants tested at 0800 h (n=50, *mean*=10.43, *SD*=6.41) vs. 1000 h (n=102, *mean*=4.92, *SD*=3.44), t(160.58)=−5.99, p<.001, d=−0.69. In turn, *cortisol baseline* had a significant effect on *cortisol stress reactivity*, F(1, 146)= 31.78, p<.001, eta$^2_{partial}$= 0.18, when controlling for the influence of *experimental condition*, F(4, 146)=0.44, p=.778, eta$^2_{partial}$= 0.01.

In accordance with these finding, we found that *session start* had a significant effect on *cortisol stress reactivity*, F(1, 146)= 12.80, p<.001, eta$^2_{partial}$= 0.08, when controlling for the influence of *experimental condition*, F(4, 146)=0.50, p=.739, eta$^2_{partial}$= 0.01. Following up on this main effect using five independent Welch two-sample *t*-tests showed that *cortisol stress reactivity* was however neither significantly related to *session start* in the *water* condition, t(53.00)=−0.77, p= .444, d=−0.15, nor in the *sugar+*, t(23.00)=1.19, p=.248, d=0.34, *sweetener+*, t(24.00)=1.69, p= .105, d=0.48, nor in the *sweetener-* condition, t(20.00)=1.84, p=.081, d=0.57.

To minimize the influence of the significantly different cortisol baseline values on our analyses (because we were not interested in baseline differences, but the stress response), we decided to exclude cortisol baseline measurements taken at −20 min and −30 min and focus on the time during and after the stressor (from 0 min to +45 min). To account for potential influences of higher *cortisol baseline* levels on cortisol stress responses in participants tested at 0800 h and in the water group, we subsequently decided to use the variables *session start* and *cortisol baseline* as covariates in our analysis. To reduce multicollinearity (Pearson's correlation between *session start* and *cortisol baseline*: r=−0.49, p<.001), we decided to include only one of the variables as covariate in the models. Since the interpretation and significance of the results was independent of whether we used *session start* or *cortisol baseline* in our analyses, we decided to report the analyses using *session start*. The results comprising the variable *cortisol baseline* can be obtained from the RMarkdown analysis scripts provided at the Open Science Framework project associated with this work.

Cortisol stress reactivity was neither significantly affected by *drink volume*, F(1, 146)=0.01, p=.930, eta$^2_{partial}$<0.01, while controlling for the influence of *experimental condition, F*(4, 146)=4.00, p=.004, eta$^2_{partial}$=0.10, not by *hormonal status*, F(2, 143)=2.66, p=.073, eta$^2_{partial}$=0.04, while controlling for the influence of *experimental condition, F*(4, 143)=2.82, p=.027, eta$^2_{partial}$=0.07. Participant's rating of the drink's estimated amount of sugar as compared to Coke® was

neither significantly related to *drink volume,* b=0.01, T=.13, p=.896, *drink content* (*sugar* or *sweetener*), b=0.19, T=.24, p=.810, nor an interaction between the two, b=−0.01, T=-.74, p=.462 (adjusted R^2<0.01, F(3, 93)= 0.86, p=.465).

To sum up, we included *age* and *session start* as covariates in our main analyses.

3.2. Blood glucose trajectories

Blood glucose increase differed significantly across *experimental condition* (five levels: *sugar+*, *sugar-*, *sweetener+*, *sweetener-*, *water*), F(4, 147)= 61.60, p < .001, $eta^2_{partial}$= 0.63. Bonferroni corrected t-tests showed that *blood glucose increase* was significantly higher in both *sugar* (*mean*=31.92, *SD*=15.09), compared with both *sweetener* groups (*mean*=1.11, *SD*=8.67), t(83.07) = 12.57, p < .001, d= 2.47, or the *water* group (*mean*=2.40, *SD*=8.36), t(78.95) = 12.40, p < .001, d= 2.43, without a significant difference between *sweetener* and *water*, t(94.27) = 0.75, p = .453, d= 0.15. *Sugar+* did not significantly differ from *sugar-*, t(46.67) = −0.18, p = .855, d= −0.05; neither did *sweetener+* significantly differ from *sweetener-*, t(43.82) = −1.13, p = .264, d= −0.33. Including *age,* F(1, 145)= 0.65, p = .420, $eta^2_{partial}$< 0.01, and *session start,* F(1, 145)= 0.62, p = .431, $eta^2_{partial}$< 0.01, did not change the significance of *experimental condition,* F(4, 145)= 61.30, p < .001, $eta^2_{partial}$= 0.63. Blood glucose results per group are depicted in Fig. 2A.

3.3. Subjective stress trajectories

There was no significant effect of *experimental condition*, F(4, 147)= 0.94, p = .441, $eta^2_{partial}$= 0.03, on *subjective stress increase*. Including *age,* F(1, 145)= 0.26, p = .612, $eta^2_{partial}$< 0.01, and *session start,* F(1, 145)= 1.41, p = .238, $eta^2_{partial}$< 0.01, did not change the significance of *experimental condition,* F(4, 145)= 0.94, p = .442, $eta^2_{partial}$= 0.03. *Subjective stress increase* differed significantly from zero across all groups, t(151) = 10.16, p < .001, d= 0.82. Subjective stress results per group are depicted in Fig. 2B.

3.4. Growth curve models

In all models, incorporation of random intercepts, random slopes, and linear, quadratic, and cubic trends of time led to significant increases in model fit by means of the log-likelihood ratio (for details of the results, see the respective tables in the Supplemental information, which are linked in the following paragraphs).

3.5. Planned contrasts: Effects of sweetness, energy load, and energy prime

Evaluating the effects of *sweetness*, *energy load* and *energy prime*, we found (A) a significant difference in cortisol trajectories after sweet vs. non-sweet drinks (best explained by the interaction between *sweetness* and a cubic effect of time; see Supplemental information, S3), (B) a

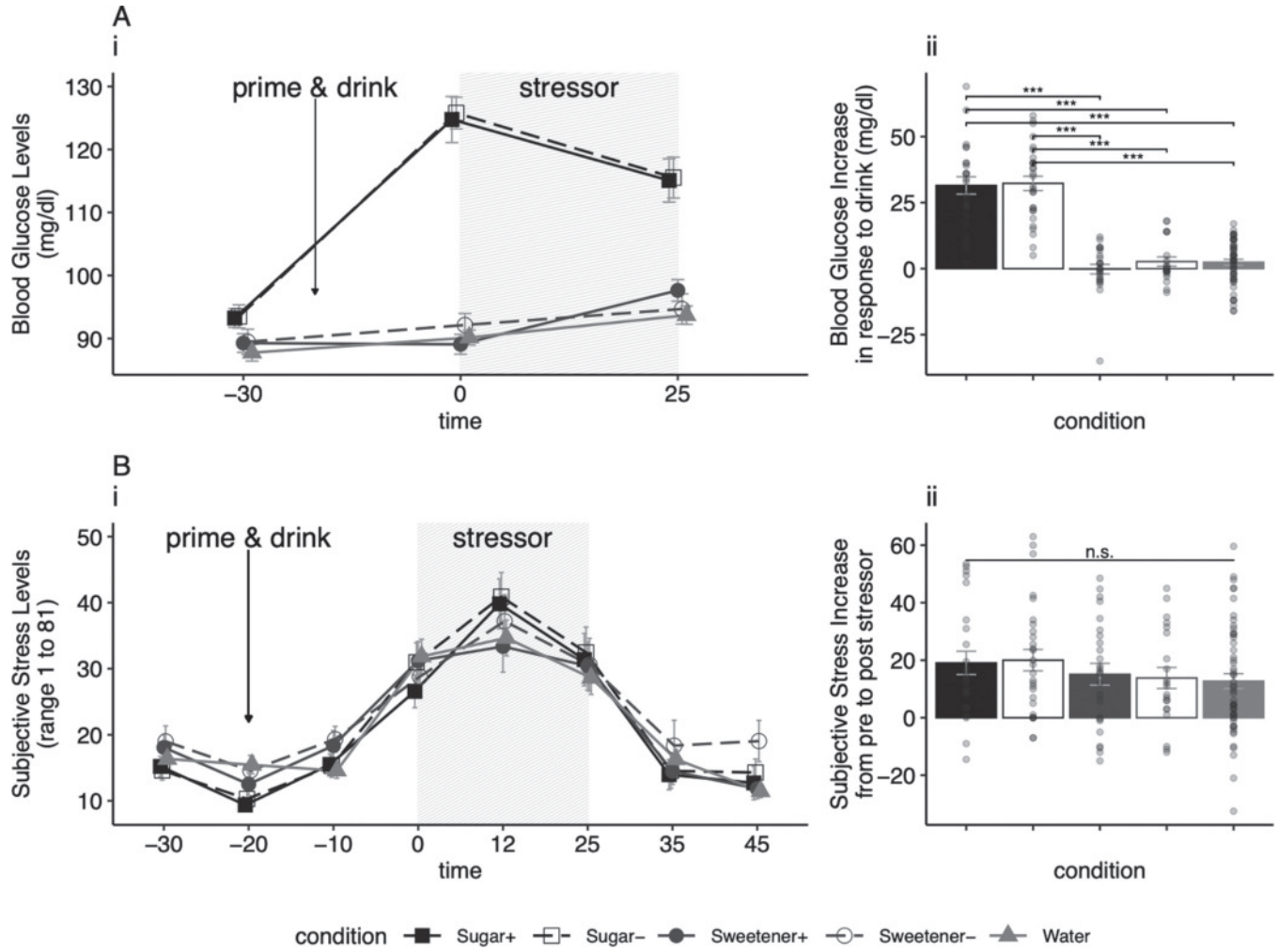

Fig. 2. Changes in blood glucose (A), subjective stress (B), over time (i), and in response to the experimental manipulations (ii) per experimental condition. Values are depicted as *mean* ± *SE*.

significant difference in cortisol trajectories after caloric vs. non-caloric drinks (best explained by the interaction between *energy load* and a quadratic effect of time; see Supplemental information, S4), and (C) no significant difference in cortisol trajectories after energy prime + vs. – (see Supplemental information, S5). The incorporation of significant covariates did not change the results of these analyses.

3.6. Interaction model

Both, *drink* and the interaction terms of different trends of *time x drink* significantly improved model fit (see Supplemental information, S6). Evaluation of the final model (Table 2) showed that cortisol trajectories differed significantly dependent on consumed drink (*time*^{3} *x drink*). Incorporating *age*, *session start*, and *session start x time* did not change the results (see Supplemental information, S6).

Following up on this effect, we used the same growth curve approach as described above to contrast *water* against *sweetener* (*water*=0, *sweetener*=1), *water* against *sugar* (*water*=0, *sugar*=1), and *sweetener* against *sugar* (*sweetener*=0, *sugar*=1). Here, cortisol trajectories of (a) *sweetener* differed significantly from *water* (*time*^{3} *x drink* significant; see Supplemental information, S7), (b) *sugar* differed significantly from *water* (*time*^{3} *x drink* significant; see Supplemental information, S8), and (c) *sweetener* did not significantly differ from *sugar* (no significant interaction of drink with any time trend; see Supplemental information, S9).

Testing the effects of *drink* on *cortisol stress reactivity* using an ANOVA, this effect was again reflected in a significant omnibus effect of *drink*, $F(2, 149) = 3.90$, $p = .022$, $\text{eta}^2_{\text{partial}} = 0.05$. Bonferroni corrected post-hoc *t*-tests showed a significant difference between *sugar* and *water*, $p = .041$; yet, there was neither a significant difference between *sweetener* and *water*, $p = .070$, nor between *sugar* and *sweetener*, $p > .99$. Including *age*, $F(1, 145) = 0.33$, $p = .567$, $\text{eta}^2_{\text{partial}} < 0.01$, and *session start*, $F(1, 147) = 6.21$, $p = .014$, $\text{eta}^2_{\text{partial}} = 0.04$, did not change the significance of *drink*, $F(2, 147) = 4.02$, $p = .020$, $\text{eta}^2_{\text{partial}} = 0.05$.

Cortisol results for the groups consuming different drinks are depicted in Fig. 3A.

3.7. Exploratory analysis: relationship between blood glucose levels and cortisol stress reactivity

While *cortisol stress reactivity* was neither associated with the *second blood glucose sample*, $r(150) = 0.11$, $p = .187$, nor with *blood glucose increase*, $r(150) = 0.08$, $p = .338$ (Fig. 3B), it was positively related to the *third blood glucose sample*, $r(150) = 0.24$, $p = .002$.

Table 2
Model parameters of the final model contrasting the groups consuming different drinks.

	coefficient	*SE*	*df*	*inferential statistics*	*p-value*	*effect size*
(Intercept)	0.28	0.11	602	2.60	$p = .010$	$d = 0.21$
time	0.10	1.38	602	0.07	$p = .942$	$d = 0.01$
time^2	-0.31	0.44	602	-0.70	$p = .482$	$d = -0.06$
time^3	-0.93	0.44	602	-2.11	$p = .035$	$d = -0.17$
drink	-0.29	0.08	150	-3.41	$p < .001$	$d = -0.56$
time x drink	1.84	1.07	602	1.72	$p = .086$	$d = 0.14$
time^2 *x drink*	-1.02	0.34	602	-3.01	$p = .003$	$d = -0.25$
time^3 *x drink*	-0.74	0.34	602	-2.19	$p = .029$	$d = -0.18$

Note. Time represents the linear, *Time*^{2} represents the quadratic, and *Time*^{3} represents the cubic effect of time. *Drink* is a numeric variable (three levels: *sugar*=2, *sweetener*=1, *water*=0). *Time x drink* represents the interaction between the respective trend of time and drink. 'x' represents an interaction of the respective effects.

3.8. Sensitivity analysis (n = 95 participants)

After excluding cortisol non-responders (n=20; n=13 tested at 0800 h) and participants that were tested at 0800 h (n=37), the sensitivity analysis was run on n=95 participants (*sugar+*: n=26; *sugar-: n=18*; *sweetener+: n*=15; *sweetener-: n*=18; *water*: n=18). In this analysis, all results remained stable except for the following: We found no significant difference in cortisol trajectories after sweet vs. non-sweet drinks. The post-hoc contrasts revealed no significant difference in cortisol trajectories between the groups *water* and *sweetener*, but a significant difference between the groups *sugar* and *sweetener* (*drink* x *time*^{2} significant). Testing the effect of drinks on *cortisol reactivity* (using the $\text{AUCi}_{\text{cort}}$) showed no significant main effect of *drink*.

All results can be obtained from the *RMarkdown* script provided at the Open Science Framework project associated with this work.

4. Discussion

Our aim was to investigate mechanisms behind the restoring effect of glucose on the cortisol stress response after long fasting periods (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997). We experimentally manipulated women's expectations of caloric content (*energy prime*) and the caloric content (*energy load*) of sweet drinks before psychosocial stress exposure and compared the effects to a water control group. Our manipulation checks showed that blood glucose increased only after *sugar*, but not after non-caloric *sweetener* or *water* load. Further, we successfully induced an increase in subjective stress using a modified version of the Trier Social Stress Test for groups. In our main analysis, we found that *sugar* and *sweetener* load increased the cortisol stress response in comparison to *water* consumption. The cortisol response after the ingestion of *sweetener* and *sugar* was not significantly different. These findings could however not be confirmed in our sensitivity analysis that focused on a subsample that was tested at 1000 h: Although it showed a significantly stronger cortisol stress response after *sugar* consumption in comparison to *water*, *sweetener* did not lead do significantly higher cortisol stress responses in comparison to the *water* group. Further, the group *sugar* displayed significantly higher cortisol stress responses in comparison to *sweetener*. This was paralleled by the finding, that sweet drinks in general did not lead to higher cortisol responses compared to water in the sensitivity analysis. Overall, our results implicate that sugar intake increases the cortisol stress response after long fasting periods in women. Concerning the effect of *sweetener*, our results overall point to an effect on cortisol responses but are less conclusive. Interestingly however, drink-induced blood glucose increase was not related to cortisol stress reactivity in both analyses. Also, the *energy prime* had no effect on cortisol reactivity.

The finding that *sugar* load increased cortisol reactivity compared to *water* is in line with previous studies comprising long (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997), and short fasting intervals (von Dawans et al., 2020; Zänkert et al., 2020). Further, this result expands the findings of studies in males comprising long fasting periods (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997), on the one hand by studying a female sample, and on the other hand by adding a group consuming non-caloric *sweetener*. Although the boosting effect of *sugar* on cortisol stress responses has been reported repeatedly by now and seems to be robust, the underlying mechanism of the effect remain unclear.

While it has been suggested that the effect is driven by the increase in blood glucose that sugar uptake triggers (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997), recent findings do not support this hypothesis (von Dawans et al., 2020; Zänkert et al., 2020). As in the analysis by von Dawans and colleagues (von Dawans et al., 2020), drink-induced blood glucose changes were not significantly associated with stress-induced cortisol increases in our analyses. These findings are paralleled by evidence from a study in which sweet drinks with differing caloric content (grape juice with 32 g of sugar and a glucose drink with 75 g of sugar)

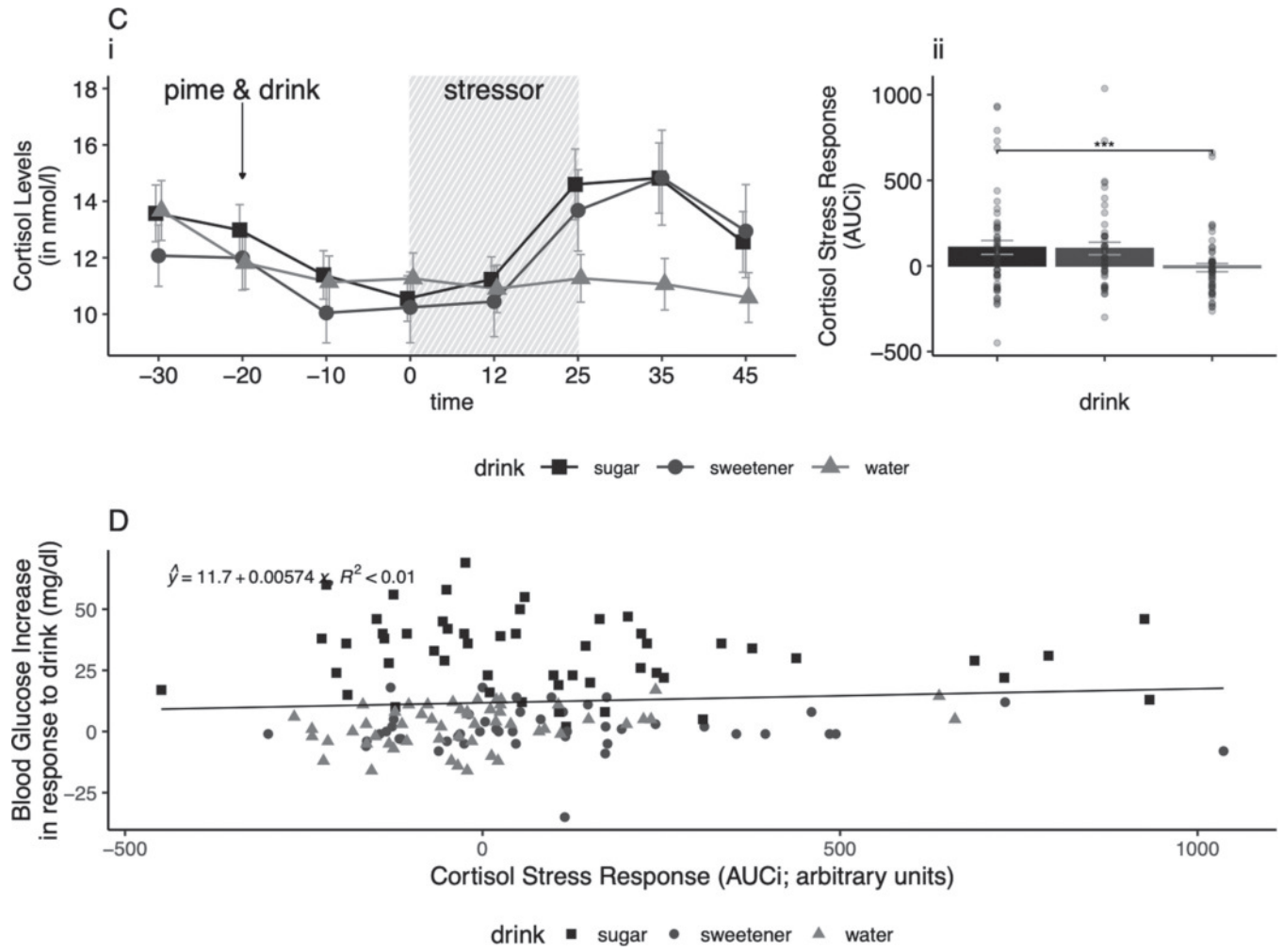

Fig. 3. Results concerning the endocrine stress response. (A) shows changes in salivary cortisol levels over time (i) and cortisol stress reactivity in response to the stressor (ii) for the groups consuming different drinks. Values are depicted as *mean* ± *SE*. (B) shows scatterplot between *blood glucose increase* and *cortisol stress reactivity*. AUCi=Area under the curve in respect to the increase.

led to comparably augmented cortisol stress responses after 3 h of fasting (Zänkert et al., 2020). Yet, a non-sweet, but caloric drink (maltodextrin, which has a similar glycemic index as compared to sugar; hence also triggers a rapid rise in blood glucose levels) did not boost the cortisol stress response as strongly as sweet *and* caloric drinks (glucose and grape juice) (Zänkert et al., 2020). Taken together, these (von Dawans et al., 2020; Zänkert et al., 2020) and our results call the proposed linear relationship between drink-induced blood glucose increase and cortisol stress reactivity (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997) into question. While recommendations regarding the control of glucose levels prior to stress tests might remain unaffected (Labuschagne et al., 2019; von Dawans et al., 2020; Zänkert et al., 2020), the assumed linear correlation between glucose availability and cortisol stress responses in normal physiological functioning should be questioned and examined more rigorously.

As far as alternative explanations of the boosting effect of sugar on cortisol stress responses are concerned, we are aware of only one study that has looked at the effects of sweet taste independent of caloric input by providing *sweetener* prior to stress induction (von Dawans et al., 2020). This study was conducted in male participants who fasted for a short fasting period of 4 h. The findings of this study indicated that only *sugar*, but not *sweetener* increased the cortisol stress response in comparison with *water* (von Dawans et al., 2020). When analyzing our sample of women who fasted for 8 h, in respect to the effects of *sweetener* our findings in the full sample are contrasting this finding, while the findings of the sensitivity analysis are in line with the results by von Dawans and colleagues. Currently, it is impossible to determine where these differences stem from, because several methodological factors which could affect the results differ between the studies (e.g., duration of fasting, daytime of fasting and testing, lag between drink consumption and stressor, participants' sex, etc.). To sum up, our results on the effect of *sugar* are in line with previous results, but the findings in respect to the effects of *sweetener* are inconclusive and should be interpreted with caution.

Although the results by von Dawans and colleagues question the role of sweetness alone, we think that investigating the effect of sweeteners further could provide meaningful insights in this context, because both, non-caloric and caloric sweeteners activate T1R2/T1R3 receptors (Behrens and Meyerhof, 2019; Lee and Owyang, 2017), and T1R2/T1R3 activation has lately been discussed as a modulator of neuroendocrine processes (Behrens and Meyerhof, 2019; Rother et al., 2018). At the same time, the role of metabolic agents (like insulin, ghrelin, glucagon) has not been studied yet and should be examined in future studies (e.g., also discussed in von Dawans et al., 2020). Lastly, since carbohydrate reward is regulated by sweet taste and metabolic load of drinks (Veldhuizen et al., 2017), and it seems that the combination of sweet taste and caloric load leads to the greatest effect on the cortisol stress response, one could also speculate that mesolimbic pathways might play a mediating role here. To be able to disentangle the effects of sweet taste from the effects of caloric load, future studies could aim at implementing a

fully balanced design by independently manipulating the sweetness and energy load of drinks prior to stress exposure.

The energy prime neither altered participants' physiological response to drinks (glucose trajectories), nor to the stressor (cortisol trajectories). Although 87% of the participants believed the information, the prime in its current format might not be strong enough to elicit detectable effects, or other manipulations might have masked its effect. Overall, the low number of (deceived) participants who did not believe the *energy prime* did not make a subsequent comparison of believers and non-believers meaningful. Still, the results suggest that expectations and psychological effects related to the consumption of sweet drinks might play a rather subordinate role in this context.

At this point, some limitations should be kept in mind when interpreting our results. First, the generalizability of our results is limited due to restrictions in study population heterogeneity in terms of sex (females only), age (young adults), ethnicity (predominantly Caucasian background) and educational status (university students). While former studies have focused on men, feasibility restrictions prohibited us to implement a sex-balanced design. As such, sex-specific effects could explain differences in findings between our and former results (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997; von Dawans et al., 2020); and indeed, sex-specific effects have been reported recently in this context (Zänkert et al., 2020). This raises the question of whether the effects of sweetener consumption are comparable in men and women. To clarify this, future studies that focus on longer fasting periods of at least 8 h should aim to again test the effects of non-caloric sweeteners in a sex-balanced design. Second, we tried to control for circadian influences on cortisol reactivity by restricting testing to the morning hours (Miller et al., 2016). This however led to some participants showing very high initial cortisol levels (>20nmol/l). In healthy individuals, such high values are typically only reached during the cortisol awakening response (CAR) (Pruessner et al., 1997). However, we did neither assess, nor control for awakening time, or instruct participants to wake up at least 1.5 h prior to the session. We thus suspect that in some subjects, an ongoing CAR might have prevented a cortisol stress response. We tried to account for this by conducting a sensitivity analysis, but the findings of our main and sensitivity analysis are contradictory. While we have greater statistical power in the complete sample when measured purely in terms of the number of subjects, it is important to keep in mind that sample size is not the only determinant of statistical power in a study. For example, the reliability of the measured constructs also plays a role: the more reliable the constructs are measured, the better the signal-to-noise ratio and the higher the power to detect a real effect. Thus, after excluding subjects whose stress reactivity was potentially dampened by the ongoing cortisol awakening response, the sensitivity analysis potentially provides a more reliable representation of the stress response. At this point, however, it is difficult to assess which components (sample size, reliability of the constructs, etc.) weigh more heavily. However, we think that the effect of *sweetener* on the stress response, especially in women, should be investigated further before drawing final conclusions, although we would not want to omit the significant finding from the main analysis. To be able to draw meaningful conclusions from follow-up studies, it would be recommended to plan sample size a priori based on our and other effect size estimations to ensure sufficient power while testing the hypothesized effects. In contrast to that, we planned our sample size based on feasibility assessments prior to the conductance of the study, which could be a point of criticism. Yet, our sample size was still comparable to published studies in this context to date (von Dawans et al., 2020).

It is also noteworthy that the water group had significantly higher cortisol baseline levels, but comparable levels at stressor start. On the one hand, this could be due to the fact that different cortisol assays were applied in the first and second wave of the research project. Yet, we are confident that the conversion of raw values into cortisol factor scores (Miller et al., 2013b) has adequately addressed this issue. On the other hand, the higher cortisol baseline in the *water* group could – at least in parts – also be related to seasonal variations that might have affected cortisol concentrations (Persson et al., 2008). We are however not aware of studies showing an effect of seasonality on cortisol stress reactivity. We believe it is more plausible that the fact that the water group was tested predominantly at 0800 h could play a role here. Although we tried to account for the baseline differences by focusing on the time during and after the stressor and controlling for the effects of session start or cortisol baseline statistically, the heightened baseline might still have dampened overall reactivity in the water group (Kudielka et al., 2004), which could have critical effects on the interpretation of some of our results: As such, it is possible that the dampened response after *water* load in comparison to *sugar* or *sweetener* did not occur because sugar or sweetener load increased cortisol reactivity, but because the water group's initial high values prevented a comparable response from the start. If that was the case, all conclusions that included the water group as a comparison would be distorted and possible effects exaggerated artificially. Consequently, we need to interpret the reported effects with caution. To avoid such potential disruptive factors in future studies, we would therefore highly recommend asking participants to get up at least 2 h prior to the start of the experimental session, or recording awakening time if sessions take place in the morning. In addition to that, other potentially modulating variables like sleep and dietary habits were not assessed in the current study and should be assessed in the future. Further, the erroneous dissolvement of 25 g of sugar or sweetener in 200 or 400 ml of water might have resulted in an unintended variation of sweetness intensity. Unfortunately, we did not ask participants to rate the sweetness of the drinks, yet they estimated how much sugar their sweet drink contained in comparison to the same amount of Coke®. As this rating did not differ between groups consuming different drink volumes and content, we indirectly inferred that participants rated the drinks as comparably sweet, independent of the volume. Finally, it is possible, that a saturation effect and the lack of a direct comparison to another drink has diminished the effect of drink volume. In the light of the comparability of results across studies, it is further a limitation that we used a modified version of the TSST-G. We have used this version successfully in other studies (Meier et al., 2021; Popovic et al., 2020). The changes from the original protocol became necessary to adjust the original procedure for space and availability of the testing rooms. We cannot tell whether the modifications influenced our results. A meta-analysis comparing protocol variations of the TSST showcases that some variations, e.g., a negative instead of neutral panel, significantly affected cortisol reactivity, and thus, stricter adherence to standardized protocols might be warranted to guarantee comparability and transferability of results (Goodman et al., 2017). Lastly, the data assessment for this project was conducted over several years and possible effects of storage times on saliva samples and batch effects have been reported. As recommended, we analyzed the samples in batches to reduce storage times (longest storage duration did not exceed 6 months) (Strahler et al., 2017), yet it is possible that these differences introduced variability. In the light of these limitations, our results need to be interpreted with caution.

Apart from this, our study is one of the first to investigate mechanisms behind the restoring effect of glucose on the cortisol stress response after a fasting period of at least 8 h. The increase in topic-related publications in the last year shows that the modulating effects of caloric and non-caloric sweeteners on the endocrine system receives increased scientific interest. So far, a handful of published studies that specifically investigated the effects of sugar and sweeteners on the cortisol stress response after fasting (Gonzalez-Bono et al., 2002; Kirschbaum et al., 1997; von Dawans et al., 2020; Zänkert et al., 2020) vary considerably in the applied methodology. As such, the differences in results could be caused by sex-specific effects, the selection and amount of sugar or sweetener used, the duration and daytime of fasting, or the lag between drink consumption and stressor onset. Exemplary, the time of the day during which the food restriction took place could be a modulating factor (Jensen et al., 2013), because the metabolic rate

depends on the circadian rhythm of the studied species (nocturnal vs. diurnal) (Maughan et al., 2010). Thus, an overnight fast in the same species could have different effects compared to a fast that took place during the day (Jensen et al., 2013). Overall, the mechanistic basis of sweetener effects is still poorly understood at this point, which strongly merits follow-up studies.

In conclusion, our results emphasize the link between the endocrine and metabolic system (McEwen and Akil, 2020). On the one hand, we confirmed a boosting effect of glucose on the cortisol stress reactivity in the fasted state. Since this was not related to blood glucose levels, the underlying mechanisms of this effect are still unclear. On the other hand, given that we found at least some evidence for effects of non-caloric sweeteners, it raises the question whether sweet taste alone can act as endocrine modulator (Rother et al., 2018). While the effects need to be tested more rigorously in future studies, this knowledge is highly relevant in the field of endocrine stress research, as it might help to understand nutritive modulators of the physiological stress response and how they might contribute to the progression of metabolic and stress-related disorders.

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CRediT authorship contribution statement

Maria Meier: Formal analysis, Investigation, Data curation, Writing – original draft, Visualization, Project administration, Conceptualization, Methodology. **Ulrike U. Bentele:** Investigation, Writing – review & editing, Project administration, Conceptualization, Methodology. **Annika B.E. Benz:** Writing – review & editing. **Bernadette Denk:** Writing – review & editing. **Stephanie Dimitroff:** Writing – review & editing. **Jens C. Pruessner:** Formal analysis, Resources, Writing – original draft, Supervision, Funding acquisition, Conceptualization, Methodology. **Eva Unternaehrer:** Formal analysis, Writing – original draft, Conceptualization, Methodology.

Competing interest statement

The authors declare to have no conflict of interest.

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Preregistration

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