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Investigating the Interaction of Sympathetic and Parasympathetic Nervous System Under Stress and Relaxation Using Guided Imagery

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The autonomic nervous system and the neuroendocrine system enable the body to switch between states of “fight/flight/freeze” and of “rest/digest” when coping with stressors or during recovery. The “rest/digest” or "relaxation" response, is crucial for regeneration processes, physiological homeostasis, and sustainment of physiological and psychological health. Here we asked whether a chronically stressed state is associated with an absence of an autonomic physiological relaxation response after acute stress. To do this, we investigated the effects of a relaxation intervention in acutely stressed individuals on neuroendocrine and autonomic markers trying to illustrate the interaction of sympathetic and parasympathetic nervous system. Healthy participants (N = 71) completed the socially evaluated cold pressor test before receiving a relaxation induction consisting of diaphragmatic breathing and guided imagery. Heart rate, heart rate variability (continuous electrocardiogram), salivary cortisol and salivary alpha amylase (saliva samples) were assessed as biological stress and relaxation markers. Mixed ANOVAs revealed a significant effect of the socially evaluated cold pressor test on cortisol levels and subjective stress. Additionally, a significant effect of the relaxation intervention on heart rate variability and heart rate was revealed (all $p < .001$). No significant differences in the ratio of the reactivity of the autonomic branches under stress and relaxation were found. The study confirms a successful induction of a neuroendocrine stress response via the socially evaluated cold pressor test and a successful induction of autonomic relaxation using the relaxation induction. Methodological limitations and indications for future studies are discussed.

Keywords: sympathetic nervous system, parasympathetic nervous system, guided imagery, diaphragmatic breathing, HPA, HRV

The autonomic nervous system (ANS) and the neuroendocrine system (NES) enable our bodies to switch between states of “fight/flight/freeze” and of “rest/digest” when coping with stressors, and during recovery. The ANS divides into the enteric system, the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS; Callara et al., 2021; Chadderdon et al., 2020; LeBouef et al., 2021; Pinel et al., 2018). The SNS, or “fight/flight”-system, is important for the quick, ergotropic autonomic stress response (Callara et al., 2020, July 15; Chadderdon et al., 2020; LeBouef et al., 2021; Pinel et al., 2018) whereas the PNS, or “rest/digest”-system, is important for the trophotropic relaxation response (Ali & Nater, 2020; Callara et al., 2020, July 15; O'Connor et al., 2021; Pinel et al., 2018). The NES encompasses multiple system with the endocrine system, made up of exocrine and endocrine glands, being responsible for regulation and communication of hormones throughout the body (Pinel et al., 2018; Trasko, 2018). It compromises two important axis, with the hypothalamus-pituitary-adrenal axis (HPA) being especially important for the delayed neuroendocrine stress response (Andrews et al., 2013; Toni, 2004).

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Stress can be defined as an unspecific reaction of the organism to endo- and exogenous challenges or threats of the bodily homeostasis (Agorastos et al., 2019), for example social expectations (Hopper et al., 2019). For the experimental investigation of stress, the *cold pressor test* (CPT; Schwabe et al., 2008), which requires participants to immerse their dominant hand in cold water up to the wrist for 3 min (McRae et al., 2006; Smeets et al., 2012), is among the most used methods for stress induction. To ensure a reliable HPA axis activation , the CPT was expanded to a socially evaluated CPT by adding an experimenter watching and videotaping the participant Schwabe and Schächinger (Dickerson & Kemeny, 2004; 2018).

Once stress is induced, the SNS, responsible for the immediate "fight/flight/freeze" response eliciting rapid physiological alterations within seconds (Chadderdon et al., 2020; Glier et al., 2022; O'Connor et al., 2021; Pinel et al., 2018), is activated facilitating the release of catecholamines from the adrenal glands and the locus coeruleus. This then triggers the release of epinephrine and norepinephrine from the adrenal glands (Chadderdon et al., 2020; Dib et al., 2020; Glier et al., 2022; O'Connor et al., 2021), which leads to a release of salivary alpha amylase (sAA), an enzyme to break down starch (Ali & Nater, 2020; Glier et al., 2022) from salivary glands promoting, for example, an acceleration of HR (Chadderdon et al., 2020; Dib et al., 2020; O'Connor et al., 2021; Wascher, 2021).

For the measurement of autonomic stress, HR and sAA have emerged as a reliable and highly sensitive markers indicative of SNS dominance and stress-related changes (Ali & Nater, 2020; Hensten & Jacobsen, 2019; O'Connor et al., 2021; Skoluda et al., 2015; Wascher, 2021). Different studies confirmed increased activity and predominance of the SNS in response to stressors, for example the SECPT, indicated by increased HR (Ritvanen et al., 2006; Schwabe et al., 2008), significant sAA increases (Ehlert et al., 2006) or overall higher sAA levels (Becker et al., 2019; Buttlar et al., 2022) as well as increased subjective stress levels (Schwabe et al., 2008; Schwabe & Schächinger, 2018). However, several researchers hypothesise that the release of sAA might reflect an interaction or combination of stress-dependent PNS and SNS activity (Ali & Nater, 2020; Ehlert et al., 2006; Nagy et al., 2015), as biologically the SNS stimulating protein release while the PNS facilitates salivary flow rate (Bosch et al., 2011; Strahler et al., 2017).

Slightly delayed, compared to the SNS activation, the neuroendocrine stress response is triggered within minutes mainly via the HPA axis (Glier et al., 2022; von Dawans & Heinrichs, 2018). Once the paraventricular nucleus in the hypothalamus releases the corticotropin-releasing hormone (O'Connor et al., 2021; von Dawans & Heinrichs, 2018), the release of the adrenocorticoid hormone in the pituitary gland is triggered, leading to the secretion of glucocorticoids, like cortisol, from the adrenal cortex (O'Connor et al., 2021; van Bodegom et al., 2017; von Dawans & Heinrichs, 2018). Cortisol is, for example, responsible for the mobilisation of energy and the heightening of alertness, irritability, and attention during stress (Averill et al., 2018; Bruce et al., 2013). It has become a "gold standard" marker for measuring HPA axis activity and the body's neuroendocrine stress response, mostly due to its quick and uncomplicated measurement (Ali & Nater, 2020; Andrews et al., 2013; Skoluda et al., 2015). Numerous studies confirmed a reliable measurement of the neuroendocrine stress response assessing cortisol in response to a laboratory stressor (e.g. CPT, SECPT; Becker et al., 2019; Giles et al., 2014; Schwabe & Schächinger, 2018).

In healthy individuals, the described stress response is followed by the *relaxation* response, which can be defined as state of undivided attention, free from nervous and physiological tension with PNS dominance and absence of anxiety, physiological arousal, or stress (Cumbie, 1989; Luberto et al., 2020; Titlebaum, 1988). One prominent technique to increase PNS activity and induce physiological relaxation is diaphragmatic breathing (DB), which requires individuals to breathe deeply into the abdomen activating the diaphragm in a given breathing pace (Hopper et al., 2019). Studies have shown that DB effectively induces relaxation and reduces physiological and psychological stress by reducing the sympathetic reaction (Bergland, 2019; Hamasaki, 2020; Hopper et al., 2019). Another common mind-body relaxation intervention is guided imagery (GI; Dib et al., 2020), where individuals are guided through imagining and experiencing something with all senses. Positive imagery has been proven to reduce stress and promote relaxation (Bashir & Goswami, 2020; deLeyer-Tiarks et al., 2020; Dib et al., 2020).

Physiologically, relaxation following stress cessation is characterized by PNS dominance promoting the "rest/digest" response (Chadderdon et al., 2020; Luberto et al., 2020; Shaffer & Ginsberg, 2017). As the PNS dominance blocks the physiological processes induced by stress, the relaxation response

facilitates, for example, lower HR and decreased respiration rate (Chadderdon et al., 2020; Luberto et al., 2020).

For the measurement of the autonomic relaxation response, HR and HRV have emerged as reliable, easy to measure, physiological markers (Malik et al., 1996; Tian et al., 2018). Studies report decreased HR (Nakao, 2019; Varvogli & Darviri, 2011) and increased HRV (Hamasaki, 2020; Luberto et al., 2020; Toussaint et al., 2021) in response to an intervention subjectively perceived as relaxing (e.g. GI, meditation, music listening; Dib et al., 2020). As already outlined, sAA might be another important physiological marker for measuring the autonomic relaxation response (Ali & Nater, 2020). **S**tudies too reported significantly decreased sAA levels in response to a relaxation intervention (e.g., listening to Tibetan music, autogenic training;Ali & Nater, 2020; Cotoia et al., 2018; Heckenberg et al., 2018).

So far, we looked at SNS and PNS under stress and relaxation in isolation while in our daily lives SNS and PNS show inter-individually different coordinate activity to maintain the regulatory balance of autonomic function (Pham et al., 2021; Weissman & Mendes, 2021). However, the exact interaction patterns are not yet fully understood and remain controversial. Some describe the interaction as antagonistic (Callara et al., 2021; Shaffer & Ginsberg, 2017; Smeets et al., 2012; Weissman & Mendes, 2021), others as a more complicated interaction (Callara et al., 2021; Ulrich-Lai & Herman, 2009). According to the autonomic space model (ASM), the coordinate activity happens in a bivariate autonomic space ranging from sympathetic to parasympathetic dominance along orthogonal axes resulting in different so-called autonomic modes being either (a) coupled reciprocal mode, (b) coactive mode, or (c) uncoupled mode (Berntson et al., 1991; Berntson et al., 1993; Weissman & Mendes, 2021).

Based on this theoretical background, we planned on investigating the effects of a relaxation intervention in healthy, acutely stressed individuals to illustrate the interaction of the two branches of the ANS. We combined a stress induction (SECPT) with a subsequent relaxation induction (GI and DB) measuring stress and relaxation on autonomic (HR, HRV, sAA) and neuroendocrine (cortisol) level. To our knowledge, this is one of the first studies providing such a complete framework.

Hypothesis: We expected the SECPT to induce a significant stress response compared to a control group (CG) as measured by a significant increase in (1a) salivary cortisol, (1b) sAA and (1c) in the subjective stress response (affect grid [AG]). We expected the relaxation intervention (GI, DB) to induce significantly more relaxation compared to a CG as measured by (2a) a significant increase in HRV (Root Mean Square of Successive Differences [RMSSD]), (2b) a significant decrease in HR, (2c) a significant change in sAA and (2d) a significant increase in the subjective relaxation response (AG). Finally, we hypothesised that (3a) under stress, the sympathetic branch of the ANS (sAA) and the parasympathetic branch (RMSSD) of the ANS would work reciprocally whereas (3b) under relaxation, autonomic (sAA) and parasympathetic nervous system (RMSSD) would coact.

Methods and Material

The experimental study was conducted at the Neuropsychology unit of the Department of Psychology at the University of Konstanz. The data collection took place from May 2022 to August 2022 at the Centre for Psychiatry at Reichenau (ZfP) in houses 15 and 22. The study was approved by the Ethics Committee of the University of Konstanz, according to the ethical principles of the Declaration of Helsinki (IRB statement 12/2017). The study was preregistered to the Open Science Framework (OSF; osf.io/m32c4). To determine the sample size, an a priori power analysis was conducted using G*Power 3 (Faul et al., 2007). Results indicated a required sample size of $N = 72$ to detect a group (four, between-subjects factor) by time (seven, within-subjects factor) interaction effect of small to moderate size ($f = .175$) with a power of 95% ($\alpha = .05$, $r = .5$ between repeated measures). Due to spontaneous cancellations of laboratory appointments by participants as well as time restraints data of $N = 71$ participants was collected.

Participants

In total, $N = 71$ individuals aged 18 to 34 ($M_{age} = 23.35$ years, $SD_{age} = 3.27$) participated in the study. Participants were of male ($n = 34$) and female ($n = 37$) sex. Further demographic and psychological characteristics of the sample are summarized in Appendix Table S1.

Participants were continuously recruited from 1st of May 2022 to 11th of August 2022. Flyers were distributed in Konstanz, over social media (Whats-App),

over the platform SONA of the University of Konstanz as well as via e-mail. To ensure study eligibility according to exclusion criteria, participants filled out a 15-minutes prescreening questionnaire on Qualtrics (Qualtrics, 2005). Individuals who were (a) not of male or female sex, (b) younger than 18 years or older than 35 years, (c) under- or overweight (BMI <17.5 or > 30), (d) currently pregnant, (e) working in night shift, (f) consuming more than 5 cigarettes per day or drugs during the last two weeks, (g) diagnosed with psychiatric illness, (h) moderately to severely depressed (Beck's Depression Inventory II [BDI-II] sum score > 19; Beck et al., 1996; Kühner et al., 2007) or (i) belonging to a Covid-19 risk group were excluded. Further exclusion criteria were (a) somatic illnesses influencing the cardiovascular system, the HPA-system, the metabolic system, or other endocrine systems as well as (b) consumption of pharmaceuticals influencing the described systems. These exclusion criteria were chosen to avoid confounding influences on the biological measurements (Heim et al., 2008; Koch et al., 2019; Laborde et al., 2017; Strahler et al., 2017).

In sum, n = 267 individuals completed the prescreening with n = 147 (55.1 %) being eligible for participation in the actual study. Of the invited individuals N = 71 (48.3 %) took part in the study. Participants gave their written consent to participate and received 20€ or 2 *Versuchspersonenstunden* as reimbursement. Participants were allowed to quit the testing at any time without consequences or specification of reasons. An individual code ensured the extensive pseudonymisation of the collected data.

Procedures

Using a between-subjects 2x2 design for this quantitative study, participants firstly were exposed to a stress- or control condition for the induction of acute stress followed by a relaxation- or control intervention. The assignment of participants to groups was done quasi-randomised. Due to reasons of practicability, it was alternated between EG and CG of the SECPT per day of laboratory appointments. For the relaxation intervention, it was tried to alternate between EG and CG within the sex groups, which was not always possible due to unexcused no-shows and spontaneous cancellations of the appointment by the participants. Generally, it was aimed at an equal distribution of individuals per group per sex. The detailed assignment process is depicted in Appendix Figure S1. The laboratory appointments were conducted in two time slots from 3 p.m. to 5 p.m. and 5.15 p.m. to 7.15 p.m. to control for the diurnal cycle of cortisol and to ensure comparability of the biological measurements between participants (Bruce et al., 2013; Laborde et al., 2017). All laboratory assessments were conducted by the same experimenter and took approximately 105 to 120 min. For their laboratory appointment participants were asked to be fasting (no drinks, except water, no food and no smoking 2 hr prior to the appointment, no alcohol 18 hr prior) and to abstain from exercising 24 hr prior to the appointment in order to eliminate possible confounding variables of the saliva sampling as well as the HRV measurement (Biondi & Picardi, 1999; Kirschbaum et al., 1993; Laborde et al., 2017; Strahler et al., 2017). Three participants reported to have eaten and drunk something within 2 hr before their laboratory appointment and one participant did not abstain from exercising. To control for possible confounding effects, these variables were included in the preliminary analysis.

First, participants were informed about the hygiene protocol and the collection of biological data (continuous ECG using MindWare Mobile; saliva samples using salivettes), generated their individual code, filled out the informed consent and filled in questionnaires on an iPad during the acclimatisation period of approximately 25 min. Second, a baseline period of 5 min followed. Participants were instructed to place their feet on the ground, bring their legs to a 90-degree angle, place their hands facing upwards on their thighs and remain steady for 5 min (Laborde et al., 2017). Participants were asked to keep their eyes open and focus on one point without looking around. Then, participants again filled in questionnaires. Third, during the first experimental manipulation participants completed the socially evaluated stress test (SECPT), or a control condition followed again by questionnaires during the recovery 1 period. Finally, participants received a relaxation intervention, consisting of diaphragmatic breathing (DB) and guided imagery (GI), or a control intervention and again filled in questionnaires before being thanked, debriefed and reimbursed. A schematic flow of a laboratory appointment is depicted in Figure 1.

Stress Induction

For the stress induction, the SECPT (Schwabe et al., 2008) was used as it induces a psychological as well as a physiological stress response and thereby ensures the activation of the HPA axis and the release

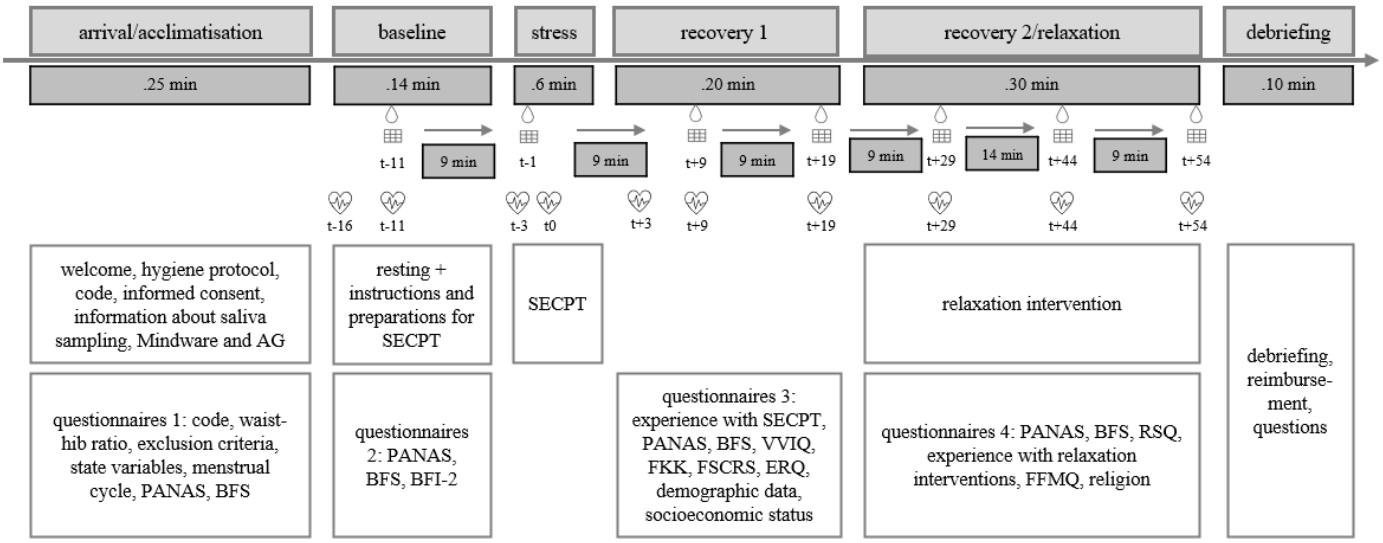

Figure 1. Schematic flow of the laboratory appointment of approximately 105 minutes. Drop symbol = saliva sample; table symbol = AG; heart symbol = MWM Marker; AG = Affect grid; SECPT = socially evaluated cold pressor test. Definitions of the abbreviations of the questionnaires can be found in the Appendix.

of cortisol in addition to eliciting an autonomic and subjective stress response (Buttlar et al., 2022; Dickerson & Kemeny, 2004; Giles et al., 2014; Schwabe et al., 2008; Schwabe & Schächinger, 2018; Skoluda et al., 2015). This study followed the guidelines and the described procedure by Schwabe and Schächinger (2008; 2018), as they stated the importance of the adherence to the protocol for successful stress induction. As the study was conducted by one experimenter, the experimenter interacted in a curt and neutral way with the participants in the EG of the SECPT from the beginning of the study until the termination of the SECPT. After reading the instructions, participants had to immerse their opened hand including the wrist into the water on a signal of the experimenter. In this study, the water container was filled with approximately 3 L of water that either had a temperature of 0 to 4 °C in the experimental group (EG) or a temperature of 35 to 37 °C in the CG. The duration of the task was measured with a stopwatch to make sure that each participant remained in the water for 3 min in total. If participants removed their hand too early, they were asked to put it back immediately until 3 min were reached; n = 7 participants removed their hand too early and were asked to put it back. In our study n = 16 participants had participated in the SECPT before. On average the participation dated back 5.2 months [1 month; 24 months]. To control for habituation effects, prior participation in the SECPT was included in the statistical analysis of group differences. Once the SECPT was terminated the experimenter interacted in a less reserved manner with the participants in the EG of the SECPT (Schwabe & Schächinger, 2018).

Relaxation Induction

For the 14-minute relaxation intervention two established methods for relaxation induction, DB and GI, were combined to ensure the induction of physiological as well as psychological relaxation in the experimental group. The effectiveness, individual's satisfaction, and potential impact on autonomic function as well as absence of reported side effects for both methods have been described previously (Trasko, 2018; Varvogli & Darviri, 2011; Wood & Patricolo, 2013). Studies confirmed a significant downregulation of the SNS, reduced physiological and psychological responses to stress, and induced relaxation by a significant increase in PNS activity via an increase in vagal activity in response to DB (Bergland, 2019; Gerritsen & Band, 2018; Hamasaki, 2020; Hopper et al., 2019; Luberto et al., 2020; Tian et al., 2018; Toussaint et al., 2021; Zaccaro et al., 2018). Regarding GI too, studies have shown that GI induces relaxation, supports coping with stress, effectively reduces

harmful effects of stress, disease symptoms, negative thoughts, levels of anxiety and depression; and supports coping with stress (Bashir & Goswami, 2020; Carter, 2006; Cumbie, 1989; deLeyer-Tiarks et al., 2020; Dib et al., 2020; Giacobbi et al., 2017; Nakao, 2019; Trasko, 2018; Varvogli & Darviri, 2011). The CG was a resting condition.

In this study, the relaxation intervention began with DB for 3 min, enabling the individual to focus, release tension and facilitate relaxation, then suggesting images of a pleasant, positive, calm and relaxing place for 11 min(Hart, 2008; Lindquist et al., 2014; Luberto et al., 2020; Skeens, 2017), as this type of GI has been reported to be the most effective relaxation technique (Jerath et al., 2020). Prior to the induction, participants received written instructions. They were asked to remain in a comfortable seated position, close their eyes, place one hand on their chest and the other hand on their stomach, breathe in for 4 sec through the nose and breathe out for 6 sec through their slightly pursed lips, which equals six breaths per min. The exact instructions for the relaxation intervention can be found on the OSF. Participants in the CG were simply asked to remain seated and relax while reading provided magazines, as in the study by Skoluda and colleagues (2015). To reduce additional stress and difficulties of choosing between multiple options, only three different magazines were provided (*"GEO – Die Welt mit anderen Augen sehen"* [05-2022], *"hygge – Vom Glück, das Leben mit anderen zu teilen"* [Nr. 30], *"bike"* [05-2022]). Previous experience with relaxation interventions as well as a prior infection with Covid-19 or long covid symptoms were assessed and included in the statistical analyses, as different studies have shown more pronounced and significant effects of relaxation interventions in experienced individuals (Morton et al., 2020; Skeens, 2017), and as long covid symptoms, for example difficulties in breathing, might have influenced the effectiveness of DB.

Physiological Measures

For the non-invasive and quick measurement of the development of the neuroendocrine and the autonomic stress response over time, saliva samples (S1 – S7) were collected at seven timepoints in intervals of 10 min (t-11, -1, +9, +19, +29, +44, +54) assessing salivary cortisol and sAA, as they can be easily combined in one assessment of saliva (Ali & Nater, 2020). The saliva samples were collected using salivettes (Sarstedt, Nümbrecht, Deutschland). Participants kept a cotton roll in their mouth for 1 min while exerting chewing movements before putting the salivette back into the plastic container. The $N = 497$ saliva samples were stored at -20 °C and defrosted to room temperature the day before the analysis. Analyses were conducted at the Biochemical laboratory of the University of Konstanz. Each saliva sample was centrifugalised at 2500 revolutions per minute (rpm) for 10 min. For cortisol, commercially available competitive ELISA Assays were executed following the manufacturer instructions (Cortisol Saliva ELISA, RE-52611, IBL International GmbH, Hamburg, Germany). The inter- and intra-assay variance coefficients ranged between 7.3 and 9.3% according to the manufacturer. All saliva samples were analysed in duplicate, and the resulting mean values were indicated in nmol/l. Then, commercially available alpha-amylase saliva assays were executed following the manufacturer instructions (alpha-Amylase Saliva Assay, RE80111, IBL International GmbH, Hamburg, Germany). The inter-assay variance ranged between 3.7 and 2.3% and the intra-assay variance coefficients ranged between 6.2 and 6.9% according to the manufacturer. Due to a refrigeration interruption, the used saliva assays might have been suboptimal. To test for possible distortions, a correlation between sAA concentrations analysed with a potentially suboptimal plate and sAA concentrations analysed with an intact plate was calculated. The resulting correlation coefficient ($r_p = 0.59$, $p < .00$) was assumed to indicate sufficient reliability of the potentially suboptimal plates used for the subsequent analyses. All saliva samples were analysed in duplicate, and the resulting mean values were indicated in U/ml.

For the continuous assessment of HR over the course of the experiment, the MWM (MindWare Technologies LTD, 2021) was used measuring a continuous ECG. Over the course of the experiment, 10 markers (t-16, t-11, t-3, t0, t+3, t+9, t+19, t+29, t+44, t+54) were placed in time intervals of 3 to 15 min for the computation of HRV. HRV can be measured via time- and frequency-domain markers with RMSSD, reflecting the root mean square of successive differences based on the differences between successive interbeat intervals or on the interbeat intervals, being one of the most used HRV time-domain markers of predominantly parasympathetic tone (Malik et al., 1996; Schumacher et al., 2013; Thayer, 2009). In this study we decided to use RMSSD as a time domain HRV marker of the PNS (1) as several researchers confirmed RMSSD to robustly reflect vagal cardiac influence (Pham et al., 2021; Thayer, 2009), (2) as

RMSSD is assumed to be less affected by respiratory changes than, for example high frequency power, (Shaffer & Ginsberg, 2017) and (3) as it can be classified as valid relaxation marker (Shaffer & Ginsberg, 2017; Thomas et al., 2019). Based on the recorded ECG, RMSSD was analysed using the software HRV Analysis 3.2.3 implemented by the MindWare System (MindWare Technologies LTD, 2021). RMSSD was evaluated for the baseline segment (MWM1-MWM2; 5 min), the stress segment (MWM4-MWM5; 3 min) and the relaxation segment (MWM8-MWM9; 15 min) following the instructions of the manufacturer.

Psychological Measures

The collection of psychological data was conducted via questionnaires, which were answered on an Apple iPad in Qualtrics XM (Qualtrics, 2005). Multiple possible confounding variables that may have affected biological markers, the handling of stress and the ability to relax, for example the ability to visualise or the regulation of emotions, were assessed. Only the instruments statistically evaluated in detail are described hereinafter.

Affect Grid

The subjective-emotional affective state of the participants was assessed using paper-pencil AGs (AG1 - AG7; Russell et al., 1989) at seven timepoints concurrently to saliva sampling. The scale consists of one item that assesses the affective state of the participant on two bipolar dimensions being valence (ger. *Wohlbefinden*) and arousal (ger. *Erregung*). The AG is composed of 9x9 boxes with valence being displayed horizontally from 1 *"unwell"* (ger. *unwohl*; sinistral) to 9 *"well"* (ger. *wohl*; dexter) and arousal being displayed vertically from 1 *"sleepy"* (ger. *schläfrig*; bottom) to 9 *"aroused"* (ger. *erregt*; top), as shown in Appendix Figure **S**2a. Participants select one box to indicate their momentary affective state. Besides the single scores for arousal and valence a composite stress score ranging from 1 to 81 can be derived (see Appedix Figure 2b; Meier et al., 2020). Russell and colleagues (1989) reported sufficient reliability and convergent as well as discriminant validity of the AG. They recommend their use for a quick and repeated assessment of the momentary affective state.

Relaxation State Questionnaire

To assess the momentary and short-lasting relaxation effects after the relaxation intervention and the immediate effectiveness of the intervention the Relaxation State Questionnaire (RSQ) was used, which was reported to be suitable as an instrument to assess the relaxation state (Steghaus & Poth, 2021). The questionnaire consists of 10 statements, which allows for a non-time-consuming assessment. The statements must be rated on a 5-point Likert scale ranging from 1 *"not at all"* (ger. *"trifft überhapt nicht zu"*) to 5 *"absolutely"* (germ. *"trifft voll und ganz zu"*) by the participant. The statements can be assigned to four factors being *muscle tension*, *sleepiness*, *cardiovascular activity*, and *general relaxation*. The individual scores for each statement are summed up resulting in sum scores for each factor. As studies reported, it remains an open question, whether the RSQ is sensitive in the detection of effects of relaxation interventions on subjective relaxation, which is the reason why the RSQ was only used in addition to the AG (Steghaus & Poth, 2021). Steghaus and Poth (2021) confirmed that, based on their study, the RSQ has a 4-factorial structure and is a highly reliable and valid instrument. As reliability analysis, Cronbach's alpha was calculated, which suggested internal consistency was defective, with $\alpha = .57$ (Kline, 1999).

Statistical Analyses

The statistical analysis was done in R statistical software version 4.2.0 (R Core Team, 2022) and RStudio for Windows version 4.2.0 (R Core Team, 2022) mainly using the packages afex (Singmann et al., 2022), carData (Fox et al., 2020), DescTools (Signorell, 2022), ez (Lawrence, 2016), ggplot2 (Wickham, 2016), Hmisc (Harrell, 2022) and tidyverse (Wickham et al., 2019). For calculating statistical power G*Power 3 was used (Faul et al., 2007).

Data Preprocessing and Cleaning

Prior to the analysis the repeated measures of cortisol, HR, RMSSD and sAA were preprocessed as follows. (1) To allow for statistical analysis, missing values were replaced (cortisol: 0 data points, HR: 1 data point, RMSSD: 1 data point, sAA: 0 data points). For cortisol and sAA, if only one of the duplicate analyses had resulted in a valid concentration, a single instead of a mean value was used (cortisol: 10 data points, sAA: 0 data points). Single missing values (a) at first, last or peak assessment timepoint were replaced by

the group mean values of the correspondent timepoint (HR: 1 data point), (b) missing values at all other timepoints as well as (c) sAA concentrations outlying the maximum or minimum detectable concentration of the plate were interpolated. For cortisol and sAA, if more than three values within one participant had been missing, for example due to too little amount of provided saliva, the participant was excluded from analyses (cortisol: 0 participants, sAA: 1 participant). For RMSSD, if more than 10% of heart beats per segment had to be edited in the HRV data editing in more than 3 segments per participant, the participant was excluded from the analysis as the data did then not allow for valid statistics ($HRV_{baseline}$: 1 participant; $HRV_{relaxation}$: 1 participant; Morgan, 2017). Thus, statistical analyses were based on $N = 71$ (cortisol, HR) or $N = 70$ (sAA and HRV) participants. (2) To reduce the impact of external variables prior to the laboratory appointment and to avoid systematic error, measurement timepoint 1 of AG, cortisol and sAA was excluded from the statistical analyses and figures. (3) To reduce the impact of statistical outliers, defined as values exceeding the mean of the group the individual belonged to by more than 3SDs, these values were winsorized across groups to $M \pm 3SD$. (4) To use parametric tests, requirements were tested, and skewed data were transformed. To test for normality, q-q-plots and the function shapiro.test () (Shapiro & Wilk, 1965) in R was used. To test for homoscedasticity, the function levene.test () (Levene, 1960) in R was used. To correct for skewness, natural log transformations of cortisol levels and RMSSD values were applied. Statistical analyses with HR, sAA and subjective stress levels relied on untransformed values. Figures reflect untransformed values. (5) To test for sphericity, the Mauchly Test (Mauchly, 1940) was used. If the data lacked sphericity, it was transformed using the Greenhouse-Geisser correction (G-G; Greenhouse & Geisser, 1959).

Preliminary, confirmatory, and exploratory analyses were based on a two tailed significance level of $\alpha = .05$. Significant effects were followed up by Bonferroni-corrected post-hoc t-tests. Effect sizes were indicated as η^2 as small ($\eta^2 = .01$), medium ($\eta^2 = .06$) or large ($\eta^2 = .14$) effect (Cohen, 1988; Field et al., 2012). For the analyses, parametric methods (Pearson-correlation, χ^2-test, mixed ANOVAs) were used. If the statistical requirements had not been met, non-parametric methods (Spearman correlation, Kruskal-Walli's test, Fisher's Exact Test for Count Data) were used. The mixed ANOVA was even calculated if the statistical requirements had not been met after transforming the values relying on the robustness of the ANOVA (Field et al., 2012).

For the Preliminary Analysis possible confounding variables affecting HRV, cortisol and sAA were compared between groups using Analyses of Variance (ANOVA), Kruskal-Wallis Tests or Chi-Square Tests. The following variables were included as possible confounding variables: ability to visualise [VVIQ], alcohol consumption, BMI, depressive symptomology [BDI], drink consumption, emotion regulation [ERQ], experience with SECPT or relaxation techniques, female cycle phase, food consumption, infection with covid-19, mindfulness [FFMQ], personality [BFI-2], physical activity, religiousness, self-criticism and self-reassurance [FSCSR], sex, sleep duration, smoking, time of laboratory assessment, and WHR. For time of laboratory assessment and female cycle phase two additional factors (laboratory appointment at 3 p.m. [1] or 5.15 p.m. [2]; luteal, follicular, oral contraceptives) were generated. Variables significantly differing between groups were included in the subsequent confirmatory analyses as confounding variables controlling for possible main- and interaction-effects.

For the confirmatory analyses of hypotheses 3a) and b) a ratio between sAA and RMSSD was calculated assumed to reflect the interplay of the reactivity of the sympathetic branch (sAA) and the parasympathetic branch (RMSSD) of the autonomic nervous system under stress and relaxation. Areas under the curve with respect to increase (AUCi; Pruessner et al., 2003) were calculated as first step using z-standardised RMSSD- and sAA-values for the stress and relaxation period. To ensure each sAA/RMSSD data point could be assigned to a corresponding sAA/RMSSD value, the participants excluded for RMSSD data, as described before, were excluded for sAA too and vice versa. The different time intervals between measurement timepoints for RMSSD (3min SECPT divided into three 1-minute-intervals; 15min relaxation divided into fifteen 1-minute intervals) and measurement timepoints for sAA (5-minute-interval for stress; 15-minute-interval for relaxation) were considered in the calculation. For hypothesis 3a) $\frac{(sAA - RMSSD)}{RMSSD}$ was used as dependent variable while for hypothesis 3b) $\frac{sAA}{RMSSD}$ was used as dependent variable. In line with the autonomic space model (Berntson et al., 1994; Berntson et al., 1991; Berntson et al., 1993) and the Polyvagal theory (Porges, 1995, 2001, 2007) SNS activity was expected to increase, corresponding to activity of the young vagus and a release of the vagal brake (Porges, 2007) reflected as a positive AUCi,

and PNS activity was expected to decrease, reflected as a negative AUCi, corresponding to mode (a) of the autonomic space model (Berntson et al., 1991), resulting in a larger negative ratio for hypothesis 3a. For hypothesis 3b, SNS activity was expected to decrease while there still is residual activity, reflected as a negative AUCi, and PNS activity was expected to increase, corresponding to activity of the young vagus and an active vagal brake (Porges, 2007) reflected as a positive AUCi, corresponding to mode (b) of the autonomic space model (Berntson et al., 1991), resulting in a smaller negative ratio close to zero.

Exploratory Analysis

As exploratory analyses, the autonomic stress response to the SECPT as measured by HR was analysed by calculating a mixed 2 (groups) x 15 (measurement timepoints)-ANOVA. This marker was not included in the main hypotheses as the cold stress in the SECPT might have caused vasoconstriction, elevating blood pressure, activating baroreceptors and thereby decreasing HR to bring blood pressure back to "normal" (Pham et al., 2021; Schwabe et al., 2008). This is contrary to what one would expect in response to stress, potentially making HR not a suitable physiological marker for the autonomic stress response using the SECPT.

Second, the subjective relaxation response as indicated by the self-reported RSQ score was explored, as alternative to using the AG, by computing a one-way ANOVA with the RSQ score as dependent variable and the relaxation groups (EG, CG) as independent variables.

Third, analyses 2a) to 2d) were reran excluding individuals from the sample who indicated to have followed the relaxation induction with 3 of 5 points on MC3 to test whether the subjective perception of following the instructions influenced the relaxation response.

Fourth, the AUCi of sAA and RMSSD under stress and relaxation per stress and relaxation group were correlated to further explore possible interaction patterns of SNS and PNS under stress and relaxation following on Berntson and colleagues (Berntson et al., 1991), who translated the autonomic modes into correlation patterns.

Results

$N = 71$ participants participated in the study with $n = 35$ (49.30%) receiving a stress intervention and $n = 37$ (52.11%) receiving an additional relaxation intervention. $N = 18$ (25.35%; $n_{female} = 9$, $n_{male} = 9$) participants were in the stress EG/relaxation EG, $n = 17$ (23.94%; $n_{female} = 9$, $n_{male} = 8$) in the stress EG/relaxation CG, $n = 19$ (26.77%; $n_{female} = 10$, $n_{male} = 9$) in the stress CG/relaxation EG and $n = 17$ (23.94%; $n_{female} = 9$, $n_{male} = 8$) received neither a stress intervention nor a relaxation intervention.

Preliminary Analyses

There were no significant differences between groups regarding the possible confounding variables ability to visualise (VVIQ), alcohol consumption, BMI, depressive symptomology (BDI), drink consumption, emotion regulation (ERQ), experience with the SECPT or relaxation techniques, female cycle phase, food consumption, infection with covid-19, mindfulness (FFMQ), physical activity, religiousness, self-criticism and self-reassurance (FSCSR), several facets of personality (BFI-2), sex, sleep duration, smoking, time of laboratory appointment or WHR.

There were significant differences between groups regarding the personality facets anxiousness, $F(3, 67) = 2.95$, $p = .039$, $\eta 2 = .12$, and tidiness, $F(3, 67) = 4.64$, $p = .005$, $\eta 2 = .17$, as measured with the BFI-2. Bonferroni tests for anxiousness revealed significant differences between individuals in the stress CG/relaxation CG ($M_{anxious} = 12.53$) and the stress EG/relaxation EG group ($M_{anxious} = 9.73$), $p = .035$. Bonferroni tests for tidiness revealed significant differences between individuals in the stress CG/relaxation CG ($M_{tidy} = 15.88$) and the stress CG/relaxation EG group ($M_{tidy} = 12.42$), $p = .013$. Additionally, there were significant differences between individuals in the stress CG/relaxation EG ($M_{tidy} = 15.88$) and the stress EG/relaxation CG ($M_{tidy} = 15.71$), $p = .021$, as well as the stress EG/relaxation EG group ($M_{tidy} = 15.39$), $p = .043$. Therefore, the variables anxiousness and tidiness were included as covariates in the confirmatory analyses.

Results of the confirmatory analyses will be reported excluding covariates, if sequential inclusion did not show any significant effects. A summary of all results of the confirmatory and exploratory analyses can be found in Appendix Table S2; hereinafter, only vital results regarding the hypotheses will be reported.

Hypothesis 1a

The mixed 2 (groups) x 6 (measurement timepoints)-ANOVA to test whether the SECPT induced a significant stress response compared to a CG as measured by a significant increase in salivary cortisol revealed a significant Stress Group x Time interaction, G-G (ε = .43), $F(5, 345) = 10.46$, $p < .001$, η2 = .03, as depicted in Figure 2. The actual statistical power of the mixed ANOVA was 1-ß = .85.

Hypothesis 1b

The mixed 2 (groups) x 6 (measurement timepoints)-ANOVA to test whether the SECPT induced a significant stress response compared to a CG as measured by a significant increase in sAA revealed a non-significant Stress Group x Time interaction, G-G (ε = .85), $F(5, 340) = 1.00$, $p = .412$. The actual statistical power for the mixed ANOVA was 1-ß = .08.

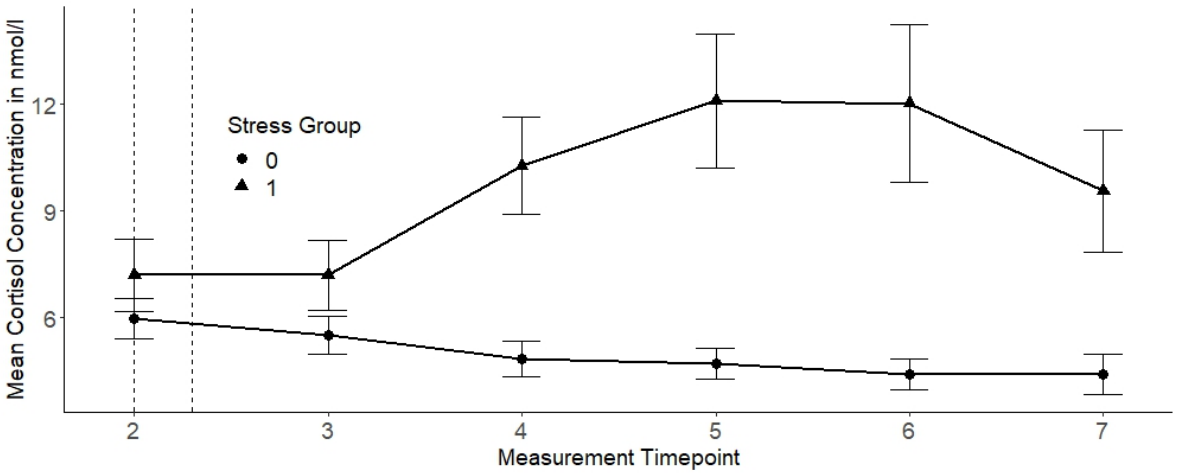

Figure 2. $N = 71$. Mean cortisol levels in nmol/l per stress group per measurement timepoint. Timepoint 1 is left out to eliminate possible confounding effects prior to the laboratory appointment. The dashed line depicts the SECPT between measurement timepoint 2 and 3. Error bars represent standard errors. Stress group 0 = SECPT CG; stress group 1 = SECPT EG.

Hypothesis 1c

The mixed 2 (groups) x 6 (measurement timepoints)-ANOVA to test whether the SECPT induced a significant stress response compared to a CG as measured by a significant increase in the subjective stress response revealed a significant Stress Group x Time interaction, G-G (ε = .80), $F(5, 345) = 7.45$, $p < .001$, η2 = .06, as depicted in Figure 3. The actual statistical power of the mixed ANOVA was 1-ß = 1.00.

Hypothesis 2a

The mixed 2 (groups) x 15 (measurement timepoints)-ANOVA to test whether the relaxation intervention as a combination of GI and DB induced significantly more relaxation compared to a CG as measured by a significant increase in HRV (RMSSD) revealed a significant Relaxation Group x Time interaction, G-G (ε = .61), $F(14, 952) = 4.60$, $p < .001$, η2 = .01, depicted in Figure 4. The actual statistical power of the mixed ANOVA was 1-ß = .61.

Hypothesis 2b

The mixed 2 (groups) x 15 (measurement timepoints)-ANOVA to test whether the relaxation intervention as a combination of GI and DB induced significantly more relaxation compared to a CG as measured by a significant decrease in HR revealed a significant Relaxation Group x Time interaction, G-G (ε = .46), $F(14, 966) = 6.65$, $p < .001$, η2 = .01, as shown in Figure 5. The actual statistical power of the mixed ANOVA was 1-ß = .13.

Hypothesis 2d

The mixed 2 (groups) x 6 (measurement timepoints)-ANOVA to test whether the relaxation intervention as a combination of GI and DB induced significantly more relaxation compared to a CG as

measured by a significant increase in the subjective relaxation response revealed a non-significant Relaxation Group x Time interaction, G-G (ε = .72), $F(5, 345) = 1.55$, $p = .196$. The actual statistical power of the mixed ANOVA was 1-ß = .48.

Hypothesis 2c

The mixed 2 (groups) x 6 (measurement timepoints)-ANOVA to test whether the relaxation intervention as a combination of GI and DB induced significantly more relaxation compared to a CG as measured by a significant change in sAA revealed a non- significant Relaxation Group x Time interaction, G-G (ε = .86), $F(5, 340) = 0.52$, $p = .74$. The actual statistical power of the mixed ANOVA was 1-ß = .06.

Hypothesis 3a

The one-way ANOVA to test whether under stress the sympathetic branch of the autonomic system (sAA) and the parasympathetic branch of the autonomic system (HRV: RMSSD) work reciprocally revealed a non-significant main effect of stress group, $F(1, 68) = 0.65$, $p = .424$. The actual power of the one-way ANOVA was 1-ß = .05. A comparison of sAA and RMSSD values under stress is shown in Appendix Figure **S**3.

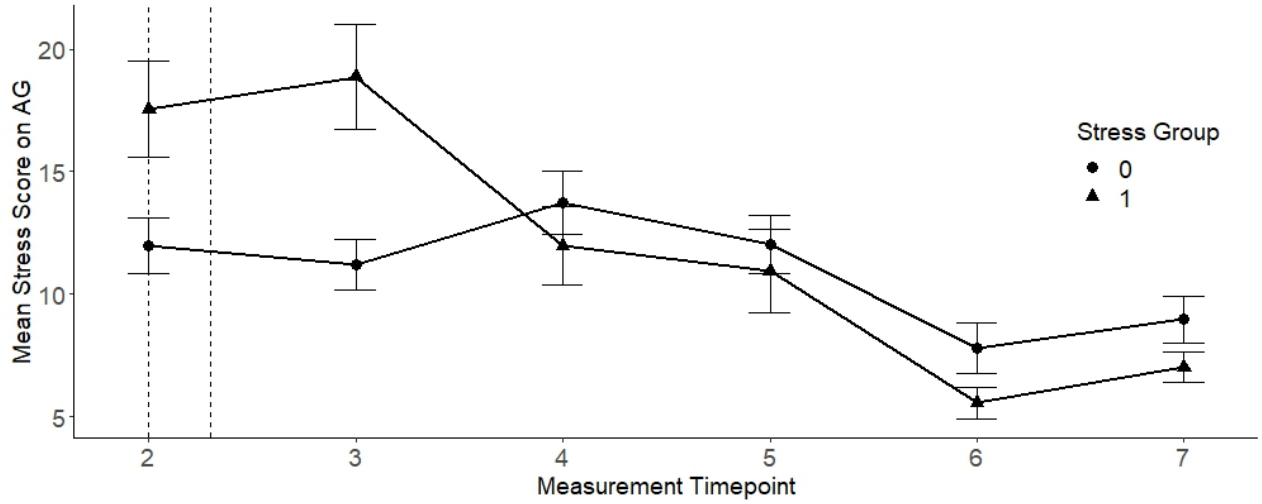

Figure 3. $N = 71$. Mean stress scores on the AG per stress group per measurement timepoint. Values can range between 1 and 81. Timepoint 1 is left out to eliminate possible confounding effects prior to the laboratory appointment. The dashed line depicts the SECPT between measurement timepoint 2 and 3. Error bars represent standard errors. Stress group 0 = SECPT CG; stress group 1 = SECPT EG; AG = Affect Grid.

Hypothesis 3b

The one-way ANOVA to test whether under relaxation the autonomic nervous system (sAA) and PNS

(HRV: RMSSD) coact revealed a non-significant main effect of relaxation group, $F(1, 67) = 0.39$, $p = .54$. The actual power of the one-way ANOVA was 1-ß = .05. A comparison of sAA and RMSSD values under stress is shown in Appendix Figure **S**4.

Exploratory Analyses

First, the mixed 2 (groups) x 15 (measurement timepoints)-ANOVA to test whether the SECPT induced a significant stress response compared to a CG as measured by a significant increase in HR revealed a non-significant Stress Group x Time interaction, G-G (ε = .75), $F(2, 138) = 2.67$, $p = .089$. Second, the one-way ANOVA to exploratively test whether the relaxation intervention as a combination of GI and DB induced significantly more relaxation compared to a CG as measured by the RSQ revealed a non-significant main effect of relaxation group, $F(1,69) = 0.18$, $p = .670$. Third, the repeated analyses of hypotheses 2a) to 2d) excluding participants from the sample who indicated to have followed the relaxation instructions with 3 points (MC3) did not change the results of the analyses as reported above.

Fourth, the correlation between the $AUCi_{sAA}$ and the $AUCi_{RMSSD}$ under stress revealed a nonsignificant positive correlation in the EG stress ($r_s = .06$, $p = .729$) as well as a nonsignificant negative correlation in the CG stress ($r_s = -.09$, $p = .616$). The correlation

between the $AUCi_{sAA}$ and the $AUCi_{RMSSD}$ under relaxation revealed a nonsignificant negative correlation in the EG relaxation (r_s = -.15, p = .363) as well as a nonsignificant negative correlation in the CG relaxation (r_s = -.03, p = .880).

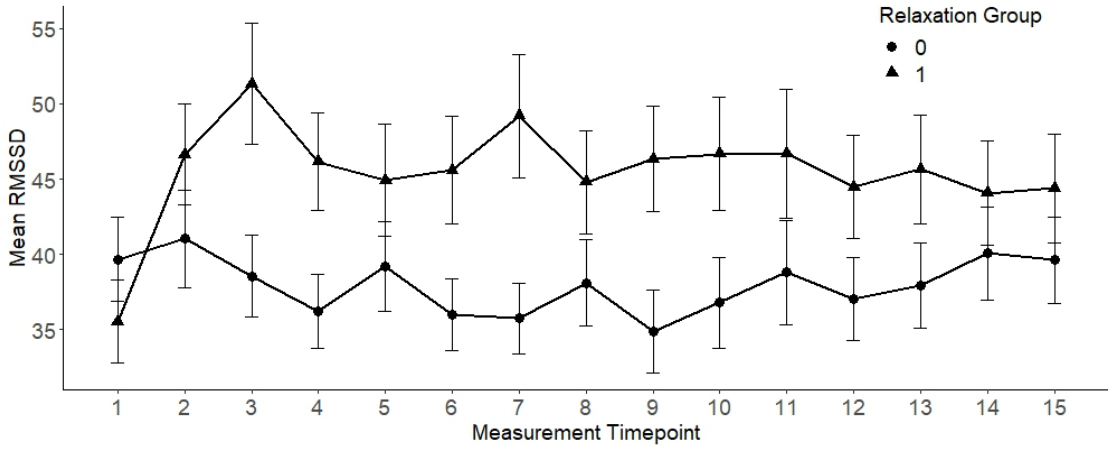

Figure 4. N = 70. Mean RMSSD (HRV) per relaxation group per measurement timepoint. Measurement timepoints on x-axis represent the relaxation period of 15 minutes. The intervention started at timepoint 1 and ended at timepoint 15. Error Bars represent standard errors. Relaxation group 0 = CG; relaxation group 1 = EG, RMSSD = root mean square of successive differences.

Discussion

The goal of this study was to investigate the interaction of sympathetic- (SNS) and parasympathetic nervous system under relaxation following acute stress as well as the effects of a stress induction (socially evaluated cold pressor test [SECPT]; Schwabe et al., 2008; Schwabe & Schächinger, 2018) and subsequent relaxation induction (diaphragmatic breathing [DB] and guided imagery [GI]) on neuroendocrine and autonomic stress and relaxation response. As expected, confirmatory analyses (1) did show that the neuroendocrine and autonomic stress and relaxation response can be reliably measured on autonomic (heart rate [HR], heart rate variability [HRV]) and neuroendocrine (cortisol) level using the described stress- and relaxation induction methods controlling for several confounding variables. However, the confirmatory analyses neither confirmed (2) the assumed reciprocal interplay of SNS and PNS under stress nor the coactive interplay of SNS and PNS under relaxation.

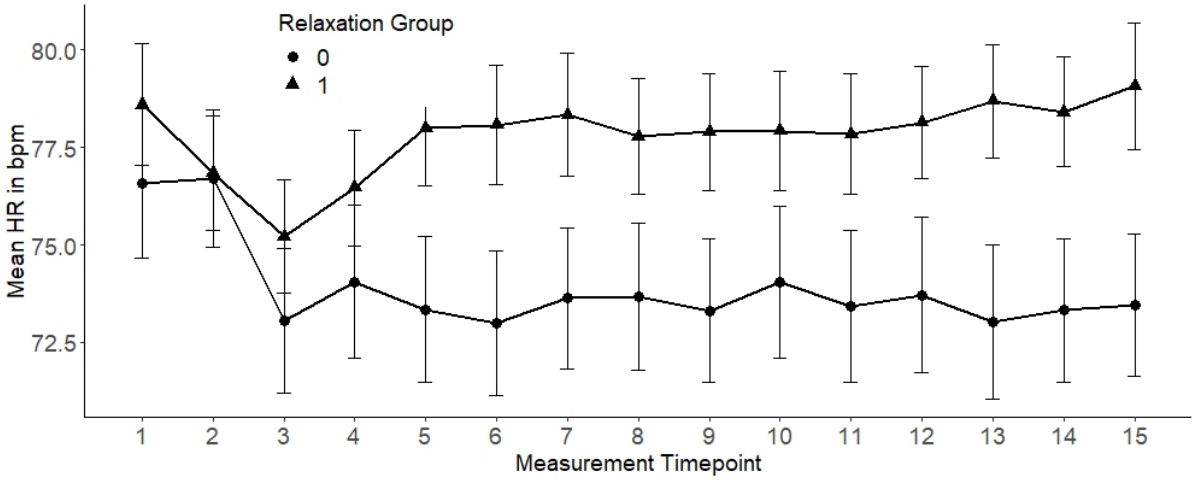

Figure 5. N = 71. Mean HR in bpm per relaxation group per measurement timepoint. Measurement timepoints on x-axis represent the relaxation period of 15 minutes. The intervention started at timepoint 1 and ended at timepoint 15. Error bars represent standard errors. Relaxation group 0 = CG; relaxation group 1 = EG, bpm = beats per minute, HR = heart rate.

First (H1a), the study did reveal a significant effect of the SECPT on the cortisol stress reaction depending on the stress group with an effect size indicating a little to medium effect. On a descriptive level, individuals completing the SECPT overall expressed higher cortisol levels than individuals in the control group [CG]. Individuals in the experimental group [EG] expressed significantly increased cortisol levels to the stressor 24 to 38 min after the end of the SECPT whereas individuals in the CG showed a slight decrease of cortisol levels over time. This is in line with current literature as several researchers have firstly confirmed a slightly delayed cortisol peak response at approximately 20 to 40 min after the termination of a stressor (Glier et al., 2022; Li-Tempel et al., 2016; von Dawans & Heinrichs, 2018) and secondly confirmed significant increases in cortisol levels in response to the SECPT (Li-Tempel et al., 2016; Rubio et al., 2015; Schwabe et al., 2008; Schwabe & Schächinger, 2018).

Second (H1b), the study did not reveal a significant effect of the stress induction on sAA over time. On a descriptive level, individuals in the stress group exhibited higher sAA levels over all measurement timepoints compared to the CG. Individuals in the EG showed the peak sAA levels approximately 6 to 7 min after the SECPT, whereas in the CG, levels dropped from the beginning of the SECPT to 6 to 7 min after the stress test. But as these observations are not based on statistically significant results they should not be interpreted. The non-significant interaction is unexpected and partially contradicts current literature as some researchers report increased salivary alpha-amylase (sAA) levels in response to a standardised laboratory stressor (Ehlert et al., 2006; Hensten & Jacobsen, 2019; Weigensberg et al., 2022) while others reported a blunted or even no sAA response (Becker & Rohleder, 2020; Giles et al., 2014). There are several possible explanations for the unexpected non-significant interaction. First, the SECPT is a passive coping task, which might have led to diminished sympathetic control compared to active coping tasks like the Trier Social Stress Test (TSST; Schwabe et al., 2008; Weissman & Mendes, 2021). Second, since sAA seems to be sensitive to environmental changes and several health factors (e.g. burnout, tinnitus, oral health and hygiene, napping, sleep quality), unknown influences might have confounded the effect of the SECPT on sAA levels (Ali & Nater, 2020; Skoluda et al., 2015; Strahler et al., 2017). Third, studies reported no effect of stress induction on sAA levels in women who experienced early-life adversities (Mielock et al., 2017; Hensten & Jacobsen, 2019), which we did not assess.

Third (H1c), the study did reveal a significant effect of the SECPT on the subjective-emotional stress response over time dependent on stress group with an effect size indicating a large effect. On a descriptive level, individuals in the SECPT EG reported a higher subjective-emotional stress score on the AG at the beginning of the SECPT as well as 10 min later. The significant interaction of the subjective-emotional stress response and measurement timepoints is in line with current literature. Several studies reported strikingly increased subjective stress levels in response to the SECPT (Schwabe & Schächinger, 2018) as well as subjective ratings of increased stressfulness, unpleasantness, and painfulness (Schwabe et al., 2008), increased arousal, anxiousness, activity, and tension (Li-Tempel et al., 2016).

Fourth (H2a), the study did reveal a significant effect of the relaxation intervention on HRV over time dependent on relaxation group with an effect size indicating a small effect. On a descriptive level, aside from measurement timepoint 1, participants in the relaxation EG overall showed higher HRV (RMSSD) with peaks 3 and 7 min after the beginning of the intervention, while individuals in the CG showed a relatively constant HRV. This is in line with existing literature. Numerous studies reported overall significantly higher or significantly increased HRV in response to DB in healthy individuals (Gerritsen & Band, 2018; Hunt et al., in print; Laborde et al., 2022; Van Diest et al., 2014; You et al., 2021; Zaccaro et al., 2018), with a rhythm of six breaths per minute, as in this study, seeming to elicit the largest increases in HRV (Steffen et al., 2022).

Fifth (H2b), the study did reveal a significant effect of the relaxation intervention on HR over time dependent on relaxation group with an effect size indicating a small effect. However, on a descriptive level, participants in the relaxation EG overall showed higher HR with a decrease during the first 3 min of the intervention and a subsequent increase whereas the CG exhibited lower HR with a peak at measurement point 2, a sharp decrease at timepoint 3 and a relatively constant HR after. This finding is unexpected and contradicts existing research. Several authors reported decreased HR in response to GI (Carter, 2006; deLeyer-Tiarks et al., 2020; Hunt et al., in print; Laborde et al., 2022) as well as DB (Gerritsen & Band, 2018; Hunt et al., in print; Perciavalle et al., 2017; Varvogli & Darviri, 2011). Gerritsen and Band (2018) explained that via the vagus nerve conveying

efferent fibres of the PNS to organs of the abdominal and thoracic cavities, a respiratory biofeedback of slowing and deepening of breath and deceleration of HR is sent, facilitating the "rest/digest" mode even further (Bergland, 2019; Gerritsen & Band, 2018; Hamasaki, 2020; Meier et al., 2020; Pinel et al., 2018). Magnon and colleagues (2021) added that conversely to inhalation, exhalation facilitates the restorage of vagal outflow resulting in a slowing-down of the heart rate (McCraty & Shaffer, 2015).

Sixth (H2c), the study did not reveal a significant effect of the relaxation intervention on sAA over time dependant on relaxation group. On a descriptive level, individuals in the relaxation EG exhibited higher sAA levels at the beginning (measurement timepoint 5) of the relaxation intervention although at the end (measurement timepoint 6) the EG as well as the CG showed comparable sAA levels. Additionally, both groups exhibited a slight decrease of sAA levels. But as these observations are not based on statistically significant results they should not be interpreted. The number of studies assessing sAA under relaxation is scarce. Available studies too did not report a significant change of sAA in response to a relaxation induction, for example GI (Weigensberg et al., 2022). Weigensberg and colleagues (2022) conducted a GI group intervention over 4 weeks and reported no statistically significant amylase changes, demonstrating that even regular practise of GI might not elicit an autonomic relaxation response as measured by sAA. As an explanation, Weigensberg and colleagues (2022) proposed that sAA might change only in emotional states of high arousal but not following lower arousal as in relaxation. Potentially, sAA quickly returned to baseline after the stress induction, which might be the reason why the relaxation induction did not induce further reduction of sAA levels.

Seventh (H2d), the study did not reveal a significant effect of the relaxation intervention on the subjective-emotional relaxation response over time dependent on relaxation group. On a descriptive level, participants in both groups of the relaxation intervention reported a similar subjective-emotional feeling of stress, which was lower than in response to the stress intervention. Participants in both groups showed a decrease in self-reported stress over the course of the intervention with participants in the EG showing a steeper decline. But as these observations are not based on statistically significant results they should not be interpreted. As already described, the subjective-emotional relaxation response as indicated with the Relaxation State Questionnaire (RSQ) was analysed additionally, which as well did not reveal a significant effect of the relaxation intervention on subjective-emotional relaxation. The non-significant interaction of stress levels and measurement timepoints under relaxation was unexpected and contradicts to current literature, as numerous studies reported participants to feel significantly more relaxed in response to DB (Hunt et al., in print; Toussaint et al., 2021; Van Diest et al., 2014). However, some researchers found no association between subjective markers of relaxation and physiological markers of relaxation (Dib et al., 2020). Toussaint and colleagues (2021) suggested that unexperienced individuals might have difficulties to understand how to obtain maximum relaxation benefits from DB leading to a subjectively unrelaxing experience. Additionally, unexperienced individuals might have had difficulties to engage in GI and DB for 15 min, which could have either elicited boredom or even unwellness diminishing the expected subjective-emotional relaxation effect. However, experience with relaxation techniques did not differ significantly between groups in this study. At the same time, participants already experienced in using GI and DB for relaxation might have felt disturbed by the constant instructions of the experimenter as they would have been able to engage in DB and GI without it. This again might have diminished the subjective-emotional relaxation effect. Additionally, factors like preferred coping style (Lindquist et al., 2014), relationship with the imagery instructor, outcome expectancy or prior experience and practice (Hart, 2008) might affect the outcome of an imagery intervention. Moreover, it was not assessed whether an individual liked to relax in silence while reading a magazine or engaging in an actual relaxation technique. Future studies should consider whether participants experience the chosen relaxation induction as a way of relaxation.

The study did not reveal a significant effect of the stress induction on the ratio between SNS and PNS activity over time dependant on group (H3a). Additionally, the exploratory correlations between SNS and PNS did not reveal a significant relation pattern. Descriptively, individuals in the EG of the stress induction exhibited on average increasing sAA levels as well as increasing root mean square of successive differences (RMSSD) values, as indicative of increasing HRV. Individuals in the CG of the stress induction exhibited decreasing sAA levels and increasing RMSSD values, as indicative of increasing HRV. Since these observations are of descriptive nature and not based on statistically significant results they

should not be interpreted. Literature on studies investigating the interaction of SNS and PNS under stress is scarce, which makes it difficult to integrate the results of this study into a context and reflects the need for further investigations on the exact mechanisms of SNS and PNS interaction. Available studies reported reciprocal coupling of PNS and SNS under stress (Berntson et al., 1994; Chadderdon et al., 2020; Glier et al., 2022; Kim et al., 2018; von Dawans & Heinrichs, 2018; Weissman & Mendes, 2021). There are several possible explanations for the nonsignificant result of the analyses. First, as Berntson and colleagues (1994) pointed out, there are profound individual differences in the autonomic mode in response to stress. In this study, merging all individuals of the stress EG into one analysis might have masked possible effects of stress on the interaction of SNS and PNS due to large inter-individual variability in autonomic mode. Kim and colleagues corroborated the assumption by Berntson and colleagues (1994) reporting participants to exhibit either a reciprocal patter of autonomic response, or sympathetic activation, or vagal withdrawal emphasizing inter-individual differences in the autonomic response to stress. As studies queried the sensitivity of sAA to stress (Becker & Rohleder, 2020; Giles et al., 2014) it might have been questionable to integrate sAA as a SNS marker into the ratio of SNS and PNS. Considering the debate on whether sAA is a "pure" SNS marker or whether it is an autonomic marker influenced by PNS and SNS activity (Ali & Nater, 2020; Bosch et al., 2011; Ehlert et al., 2006; Nagy et al., 2015; Thomas et al., 2019) future studies should compare a distinct SNS marker, for example PEP (Krohova et al., 2017), to RMSSD.

The study did not reveal a significant effect of the relaxation induction on the ratio between SNS and PNS activity over time dependant on group (H3b). Additionally, the exploratory correlations between SNS and PNS did not reveal a significant relation pattern. Descriptively, individuals in the EG of the relaxation induction exhibited, on average, decreasing sAA levels while RMSSD values, as indicative of HRV, increased. Individuals in the CG of the relaxation induction exhibited decreasing sAA levels as well as decreasing RMSSD values, as indicative of HRV. Since these observations are of descriptive nature and not based on statistically significant results they should not be interpreted. As for stress, literature on studies investigating the interaction of SNS and PNS under relaxation is scarce, as most of the times studies assess the interaction of SNS and PNS only during recovery after stress instead of in response to a dedicated relaxation intervention. Thus, it is difficult to integrate the results of this study into a context, which, at the same time, reflects the need for further investigations on the exact mechanisms of SNS and PNS interaction under relaxation. There are few available studies reporting increased activation of both SNS and PNS during the recovery period after a stressor suggesting a coactivation pattern (Mezzacappa et al., 2001; Tian et al., 2018; Weissman & Mendes, 2021). There are several possible explanations for the nonsignificant result of the analyses. First, as the relaxation intervention did not induce a significant change in sAA per relaxation group over it is highly unlikely that there are significant differences in the interaction pattern of SNS and PNS per group under relaxation, which leads back to the already made statement to use another SNS marker (e.g., PEP) in future studies. Second, as for stress, there appear to be different response types referring to the activity patterns of SNS and PNS in response to relaxation (Glier et al., 2022), which has to be considered in the statistical analyses in future studies. As for H3b, considering the ongoing debate about sAA being an autonomic or a pure SNS marker, the ratio of SNS and PNS regarding relaxation might have been distorted influencing the result of the analysis. Again, as for H3a, it might have been critical to compare a salivary marker of ANS to a non-salivary marker of ANS and future studies should focus on two autonomic markers both originating, for example, from HR. Finally, as a possible explanation that also holds true for H3b, it is highly likely that there are multiple possible, complex, nonlinear interaction patterns between PNS and SNS, regulated and influenced by different mechanisms difficult to summarise as one distinct interaction pattern in response to stress and relaxation (Callara et al., 2021).

As already mentioned, there are some methodological limitations, which must be considered when interpreting the results of this study. First, as the study was conducted in the summer months high room temperatures might have caused feelings of uncomfortableness confounding measures of subjective wellbeing. Second, we did neither control for a specific posture nor for whether participants stuck to DB. Therefore, we cannot certainly conclude that the combination of DB and GI induced autonomic relaxation rather than one technique in isolation and as even slight changes of posture and position can significantly change vagal modulation (Meier et al., 2020; Yokogawa et al., 2018) the reported effects of the relaxation induction must be interpreted cautiously and

future studies should consider these variables. Third, the essays used for the laboratory analyses of sAA were suboptimal and might have lacked sensitivity and numerous sAA concentrations ranged outside of the essay's detectable range and were therefore interpolated linearly. The assumption of a linear development of sAA levels might have led to artificial values that did not represent the real sAA concentrations of the participant. Fourth, to adhere to the 10-minute intervals between saliva assessments, the saliva sample to detect a sAA stress response was collected 6 min after the termination of the SECPT, which might be the reason we did not see a significant difference in the acute autonomic stress response between EG and CG. The same holds true for the measurement of the autonomic relaxation response via sAA. Fifth, as it cannot be made sure that the difference of sAA and RMSSD, used as enumerator in H3a, represented "pure" SNS activation, the whole ratio of SNS and PNS might be distorted. Additionally, using the area under the curve with respect to increase (AUCi) limits the possibilities of potential interpretation of results, as, for example, reduced but residual SNS activity cannot be translated into an AUCi value, which is either negative, indicative of a decrease, or positive, indicative of an increase. However, as this study yielded only non-significant results, the mathematical concerns regarding interpretation might only be interesting to future studies. In future studies, alternative statistical approaches must be developed to ensure interpretability.

Despite these limitations and the restricted generalisability to young males and females, the study has numerous benefits for psychological research and future studies. First, a new and comprehensive study design for the investigation of stress and relaxation responses on autonomic, neuroendocrine, and subjective level as well as a new relaxation protocol for the induction of psychological and physiological relaxation were presented. Second, numerous factors potentially influencing sAA responses were identified, which can serve as methodological starting point for future studies. Third, expanding on the debate on sAA, guture studies could compare PEP and sAA, as markers of the SNS, in relation to RMSSD, as marker of the PNS, potentially offering interesting insights into the nature of sAA as SNS, PNS or general ANS marker. To summarise, this study marks a starting point in the investigation of autonomic and neuroendocrine relaxation processes offering a "status quo" in healthy individuals. Future studies could offer insight into predispositions for the development and progression of psychological and physiological illnesses by revisiting the topic in affected individuals as well as individuals at risk.

Appendix

Supplementary information is available at the end of this article.

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Appendix

Appendix Table S1. Overview of Demographic Data of the Study Sample.

Demographic Characteristic	Total Sample	Stress EG ($n_{stressEG}$ = 35)		Stress CG ($n_{stressCG}$ = 36)	
		Relax EG	Relax CG	Relax EG	Relax CG
N	71	*n* = 18 (25.35%)	*n* = 17 (23.94%)	*n* = 19 (26.76%)	*n* = 17 (23.94%)
Age (in years)	23.35 (3.27)	22.67 (2.03)	23.29 (3.89)	24.00 (3.90)	23.41 (3.02)
Sex	n_{female} = 37; n_{male} = 34	n_{female} = 9; n_{male} = 9	n_{female} = 9; n_{male} = 8	n_{female} = 10; n_{male} = 9	n_{female} = 9; n_{male} = 8
Citizenship					
German	*n* = 62	*n* = 17	*n* = 13	*n* = 16	*n* = 16
Hungary	*n* = 1			*n* = 1	
Albania	*n* = 1		*n* = 1		
Austria	*n* = 2	*n* = 1		*n* = 1	
Madagascar	*n* = 1		*n* = 1		
France	*n* = 1		*n* = 1		
Russian Federation	*n* = 1		*n* = 1		
Italy	*n* = 1				*n* = 1
Ukraine	*n* = 1			*n* = 1	
Ethnicity					
European	*n* = 67	*n* = 17	*n* = 15	*n* = 18	*n* = 17
African	*n* = 1		*n* = 1		
Asian	*n* = 1	*n* = 1			
Highest Level of Education					
Secondary School	*n* = 2	*n* = 1			*n* = 1
Abitur	*n* = 42	*n* = 13	*n* = 11	*n* = 8	*n* = 10
Vocational Training	*n* = 1				*n* = 1
Bachelor	*n* = 21	*n* = 3	*n* = 5	*n* = 8	*n* = 5
Master/Diploma/State Examination	*n* = 5	*n* = 1	*n* = 1	*n* = 3	
Employment Status/Occupation (Psychology students: 17.46%)					
Full Time Student	*n* = 63	*n* = 16	*n* = 15	*n* = 16	*n* = 16
Minijob	*n* = 34	*n* = 10	*n* = 8	*n* = 10	*n* = 6
Part Time Occupation	*n* = 8		*n* = 3	*n* = 1	*n* = 4
Full Time Occupation	*n* = 6	*n* = 2	*n* = 1	*n* = 2	*n* = 1

Demographic Characteristic	Total Sample	Stress EG ($n_{stressEG}$ = 35)		Stress CG ($n_{stressCG}$ = 36)	
		Relax EG	Relax CG	Relax EG	Relax CG
Marital Status					
Single	*n* = 70	*n* = 18	*n* = 17	*n* = 18	*n* = 17
No Information	*n* = 1			*n* = 1	
BMI in kg/m^2	22.48 (2.88)	22.20 (3.54)	22.47 (2.63)	23.42 (2.82)	21.75 (2.35)
Smoking	*n* = 7	*n* = 1	*n* = 3	*n* = 2	*n* = 1
Cannabis Consumption	*n* = 8	*n* = 1	*n* = 3	*n* = 2	*n* = 2
Cycle Phase					
Luteal	*n* = 15	*n* = 4	*n* = 2	*n* = 6	*n* = 3
Follicular	*n* = 10	*n* = 3	*n* = 2	*n = 4*	*n* = 1
Oral Contraceptives	*n* = 12	*n* = 2	*n* = 5		*n* = 5
BDI-Score	4.14 (4.09)	3.89 (3.91)	3.82 (2.81)	3.53 (3.22)	5.41 (5.92)
Chronic Diseases					
Cl. D. Physiological	*n* = 6	*n* = 3	*n* = 1	*n* = 2	
Cl. D. Psychological					
Cl. nD. Physiological	*n* = 1			*n* = 1	
Cl. nD. Psychological	*n* = 1				*n* = 1

Note. Summary of demographic data of the study sample, *N* = 71, per combination of stress and relaxation group. Values are shown as mean and standard deviation (*M* [*SD*]) or absolute frequencies for each demographic characteristic; EG = experimental group; CG = control group; Relax = relaxation; BMI = Body Mass Index; BDI = Beck's Depression Inventory; Cl. D. = clinically diagnosed; Cl. nD. = not clinically diagnosed.

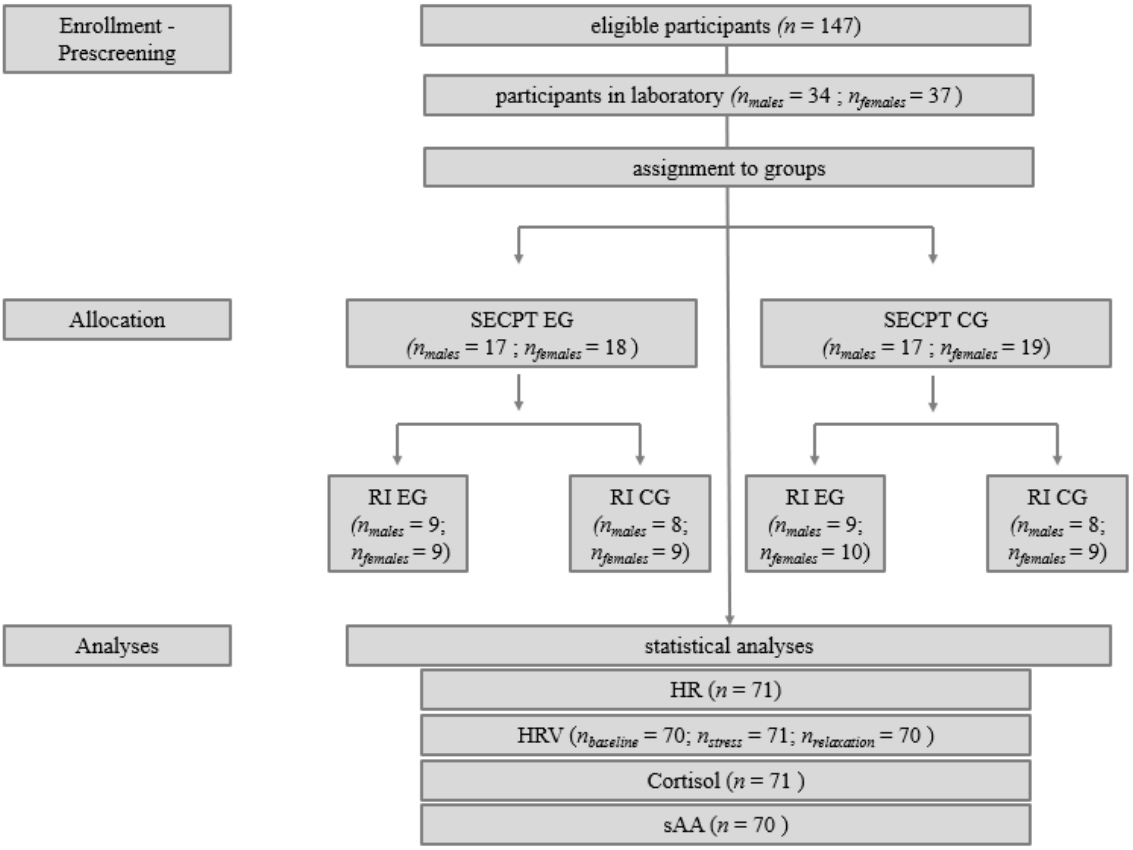
Appendix Table S2. Overview of the Results of the Statistical Analyses

Test, Effects	Statistics	*p*	η^2
Confirmatory Analyses			
Hypothesis 1a – *Cortisol Stress Response*			
Mixed ANOVA (MT 2-7)			
Measurement Timepoint	G-G (ε = .430), $F(5, 345) = 3.66$	< .05*	.009
Stress Group	$F(1, 69) = 7.29$	< .01**	.081
Time x Stress Group	G-G (ε = .430), $F(5, 345) = 10.46$	< .001***	.025
Hypothesis 1b – *Salivary Alpha Amylase*			
Mixed ANOVA (MT 2-7)			
Measurement Timepoint	G-G (ε = .851), $F(5, 340) = 5.11$	< .001***	.021
Stress Group	$F(1, 68) = 1.49$	.227	
Time x Stress Group	G-G (ε = .851), $F(5, 340) = 1.00$	.412	
Hypothesis 1c – *Stress Score Affect Grid*			
Mixed ANOVA (MT 2-7)			
Measurement Timepoint	G-G (ε = .800), $F(5, 345) = 18.47$	< .001***	.136
Stress Group	$F(1, 69) = 0.70$	.406	
Time x Stress Group	G-G (ε = .800), $F(5, 345) = 7.45$	< .001***	.060
Anxiousness	$F(1, 67) = 19.42$	< .001***	
Hypothesis 2a – *Heart Rate Variability*			
Mixed ANOVA (MT 2-7)			
Measurement Timepoint	G-G (ε = .612), $F(14, 952) = 2.70$	< .01**	.007
Relaxation Group	$F(1, 68) = 2.25$	.138	
Time x Relaxation Group	G-G (ε = .612), $F(14, 952) = 4.60$	< .001***	.013
Hypothesis 2b – *Heart Rate*			
Mixed ANOVA (MT 2-7)			
Measurement Timepoint	G-G (ε = .461), $F(14, 966) = 5.92$	< .001***	.006
Relaxation Group	$F(1, 69) = 2.87$	.095	
Time x Relaxation Group	G-G (ε = .461), $F(14, 966) = 6.65$	< .001***	.006
Hypothesis 2c – *Salivary Alpha Amylase*			

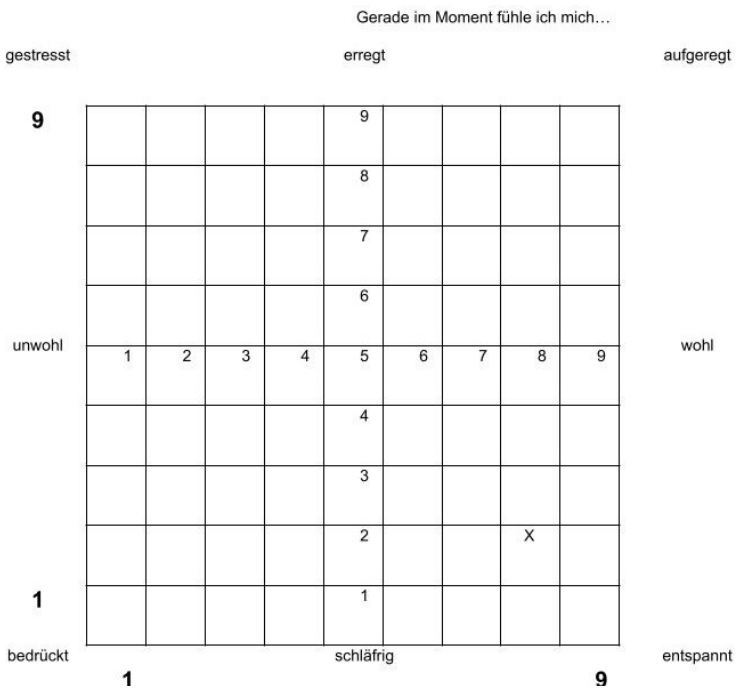
Test, Effects	Statistics	*p*	η^2
Mixed ANOVA (MT 2-7)			
Measurement Timepoint	G-G (ε = .855), *F*(5, 340) = 5.08	< .001***	.020
Relaxation Group	*F*(1, 68) = 0.53	.469	
Time x Relaxation Group	G-G (ε = .855), *F*(5, 340) = 0.52	.768	
Hypothesis 2d – *Stress Score Affect Grid*			
Mixed ANOVA (MT 2-7)			
Measurement Timepoint	G-G (ε = .715), *F*(5, 345) = 17.05	< .001***	.130
Relaxation Group	*F*(1, 69) = 0.45	.503	
Time x Relaxation Group	G-G (ε = .715), *F*(5, 345) = 1.55	.175	
Time x Anxiousness	G-G (ε = .776), *F*(5, 330) = 2.45	< .05*	
Hypothesis 3a – *Interaction SNS and PNS under Stress*			
One-Way ANOVA (sAA: MT 2-3; RMSSD stress: MT 1-3)			
Stress Group	*F*(1, 68) = 0.65	.424	
Hypothesis 3b – *Interaction SNS and PNS under Relaxation*			
One-Way ANOVA (sAA: MT 5-6; RMSSD relaxation: MT 1-15)			
Relaxation Group	*F*(1, 67) = 0.38	.537	
Exploratory Analyses			
Relaxation RSQ			
One-Way ANOVA			
Relaxation Group	*F*(1, 69) = 0.18	.670	
Stress Response Heart Rate			
Mixed ANOVA (MT 2-7)			
Measurement Timepoint	G-G (ε = .754), *F*(2, 138) = 3.88	< .05*	.003
Stress Group	*F*(1, 69) = 1.32	.254	
Time x Stress Group	G-G (ε = .754),	.089	

Note. Summary of all results of the confirmatory and exploratory statistical analyses of the effects of stress and relaxation group on cortisol, HR, RMSSD and sAA as well as the interaction of SNS (sAA) and PNS (RMSSD). The dependent variable is italicised. ANOVA = Analysis of Variance; G-G = Greenhouse-Geisser; MT = measurement timepoint; RSQ = Relaxation State Questionnaire.

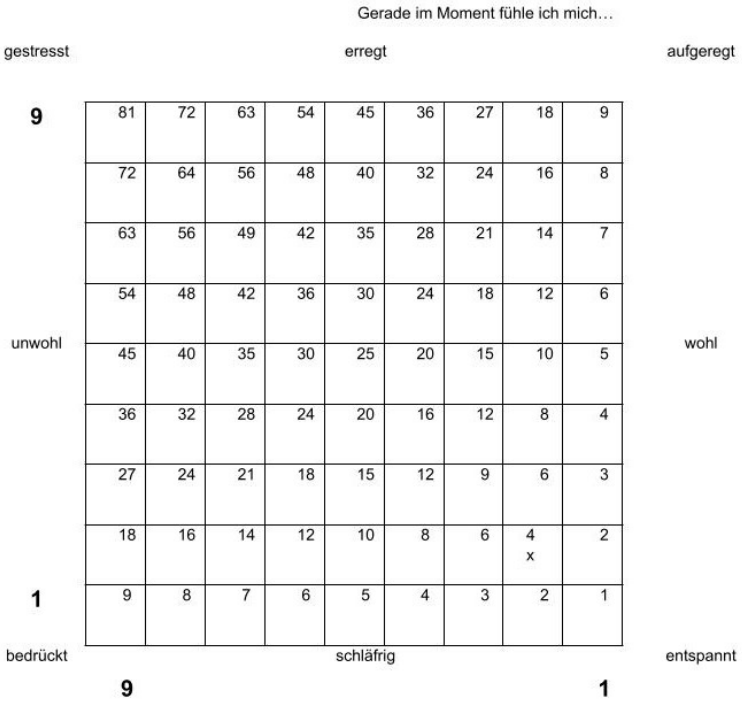
* $p < .05$ ** $p < .01$ *** $p < .001$.



Appendix Figure S1. Depiction of assignment and analyses processes. Determination of sample sizes per statistical analyses is explained in the statistical analyses part of this assignment. SECPT = socially evaluated cold pressor test. EG = experimental group; CG = control group; RI = relaxation intervention; HR = heart rate; HRV = heart rate variability.; sAA = salivary alpha amylase.

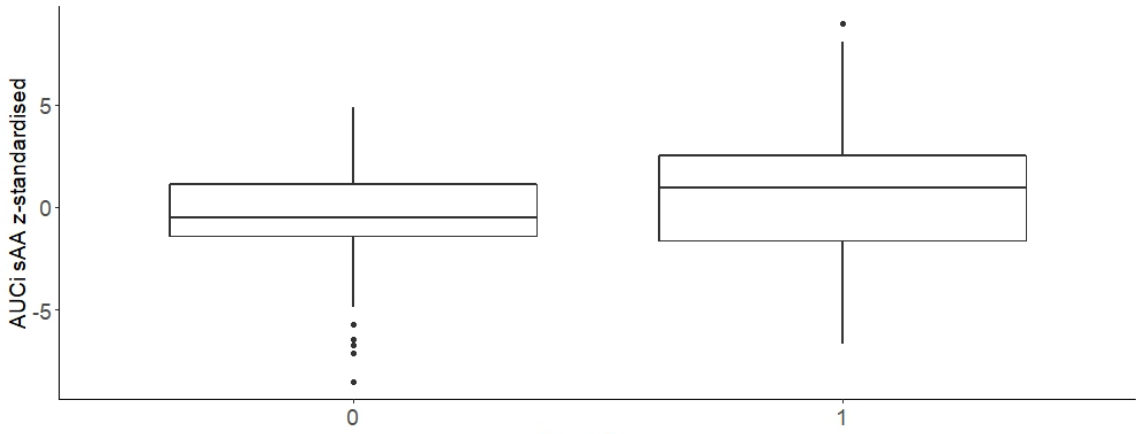


Appendix Figure S2a. AG for assessment of subjective-emotional well-being. Horizontally, valence is assessed from 1 to 9. Vertically, arousal is assessed again from 1 to 9. Higher scores indicate higher valence/arousal. Participants select one box to indicate their momentary affective state receiving point values for the two dimensions per assessment timepoint with the AG. AG = Affect Grid.

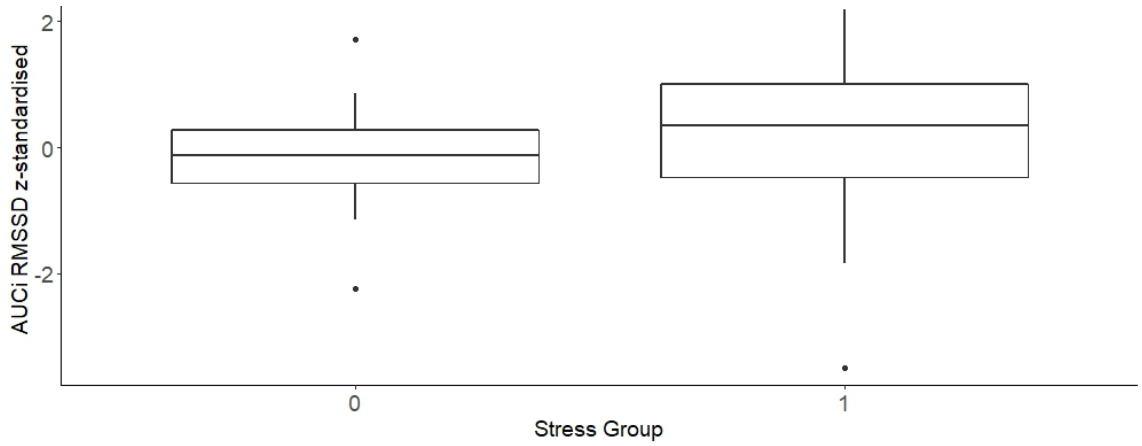


Appendix Figure S2b. AG for the computation of a subjective-emotional stress sum-score based on the self-reported values for the two dimensions arousal (vertical) and valence (horizontal), as shown in Appendix E. For the calculation, the scoring of valence is reversed. The stress sum-score is calculated by multiplying the arousal- by the valence score. The subjective-emotional stress sum-score can range between 1 and 81. AG = Affect Grid.

a)

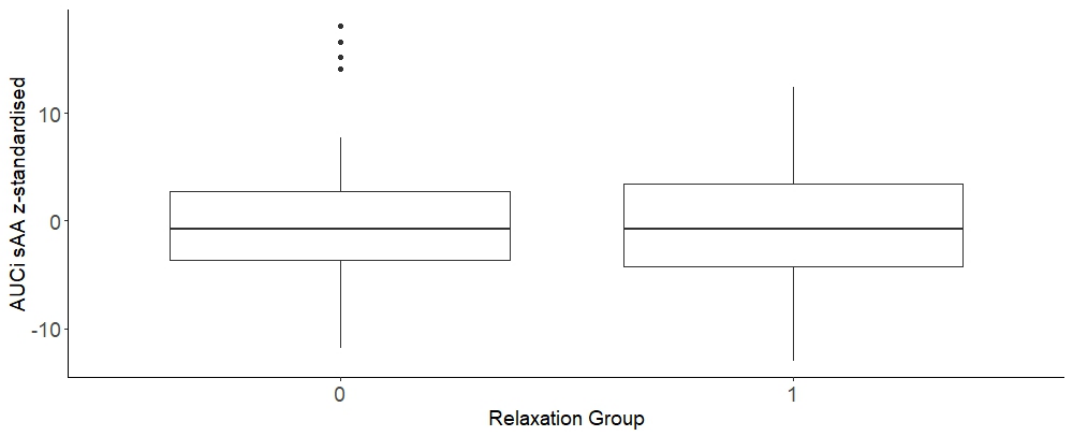


b)

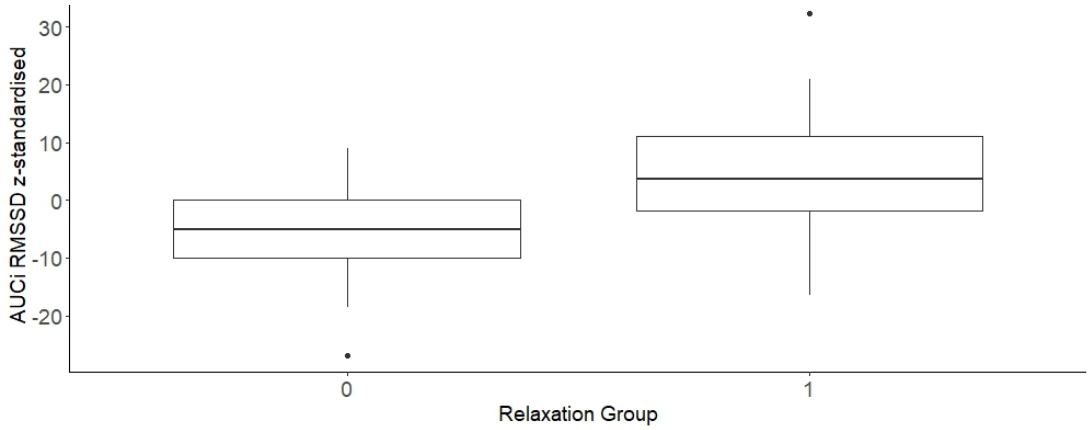


Appendix Figure S3. $N = 69$. a1) Boxplot of AUCi z-standardised sAA values per stress group during the stress induction. Measurement timepoint 2 and 3 of saliva sampling were included in the calculation and the plot. a2) Boxplot of AUCi z-standardised RMSSD values per stress group during the stress induction. For the calculation of RMSSD as well as the plot, the 3min-interval between MWM marker 4 and 5 was included. AUCi = area under the curve with respect to increase; sAA = salivary alpha amylase; RMSSD = root mean square of successive differences; stress group 0 = CG; stress group 1 = EG.

a)



b)



Appendix Figure S4. N = 70. a1) Boxplot of AUCi z-standardised sAA values per relaxation group during the relaxation induction. Measurement timepoint 5 and 6 of saliva sampling were included in the calculation and the plot. a2) Boxplot of AUCi z-standardised RMSSD values per relaxation group during the relaxation induction. For the calculation of RMSSD as well as the plot, the 15min-interval between MWM marker 8 and 9 was included. AUCi = area under the curve with respect to increase; sAA = salivary alpha amylase; RMSSD = root mean square of successive differences; relaxation group 0 = CG; relaxation group 1 = EG.