1. Study Protocol

Human Subjects Protection Review approval no.: APK.002.107.2024

Note: This document is an English translation of the protocol approved by Bioethics Committee of the Medical University of Bialystok

Date: 01.02.2026

Official Title: Breathwork and stress: investigating the mechanisms of action and effectiveness of breathing interventions in modulating the psychophysiological response to acute stress.

The following blinded clinical trial design focuses on evaluating the impact of a breathing method utilizing Breathwork (breathing exercises) and Mindfulness components on physiological and psychological stress levels in response to an acute stress test. The efficacy of coherent breathing will be compared with both a breathing sham (15 bpm) and a control condition in the form of spontaneous, undirected breathing. This design will allow for a more precise determination of whether the decisive therapeutic factor is a specific form of breathing or rather a general effect related to attention, rhythm, and participant expectations. The preliminary research hypothesis assumes that, compared to the sham intervention, performing the breathing method will significantly lower both physiological and psychological stress parameters.

Background:

Excessive and negative stress affects a significant portion of modern society. Long-term exposure to psychological stress leads to the development of allostatic load, which is associated with the disruption of the proper functioning of the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA) axis, as well as significant emotional and cognitive dysregulation. Consequently, stress is a key risk factor for the development of both mental and physical illnesses, particularly cardiovascular diseases [1–5]. Chronic stress is associated with disturbances in sleep, cardiovascular function, metabolism, and immunity. In epidemiological studies, chronic stress has been linked to a significantly higher risk of depression, anxiety disorders, and cardiovascular diseases. Reduced heart rate variability (HRV), as a marker of allostatic load, serves as an independent prognostic factor for all-cause mortality [4–10].

One method that has gained popularity in recent years as a stress reduction tool is breathwork—the conscious regulation of the rhythm and depth of breathing [2,11,12]. Breathing techniques, often based on yoga or mindfulness traditions, are increasingly appearing in clinical interventions, mobile applications, and wellness practices. A growing

body of research confirms their effectiveness in improving psychological well-being, reducing symptoms of anxiety and depression, and strengthening parasympathetic nervous system functioning. Despite the wide variety of techniques, the common denominator of their action appears to be a positive impact on the ANS and the synchronization of physiological rhythms, making breathwork a promising, non-pharmacological strategy for mental health prevention [2,13-17].

Coherent breathing (breathing at a frequency of approximately 5.5 breaths/min) is currently recognized as one of the most effective breathing methods for modulating autonomic nervous system (ANS) activity and improving heart rate variability (HRV). Through the synchronization of cardiorespiratory rhythms and baroreflex activation, coherent breathing leads to increased parasympathetic activity and promotes a state of physiological calm (RSA), as confirmed by numerous imaging and psychophysiological studies [2,18–23].

However, recent large-scale blinded randomized controlled trials indicate that differences in long-term effects between various breathing techniques may be smaller than previously assumed. Fincham et al. demonstrated a lack of significant differences between coherent breathing (5.5 bpm) and placebo breathing at a natural rate (12 bpm). Conversely, a systematic review by Bentley et al. suggests that it is not the tempo, but rather the structuring and conscious control of breath itself that may constitute the primary therapeutic factor. Additionally, research shows that different breathing rates (both slow and fast) lead to similar improvements in mental health over the long term [19,21,24].

In light of these findings, it becomes crucial to empirically investigate which of these two potential mechanisms—specific physiological properties or a general psychological effect—plays a decisive role in the effectiveness of breathing techniques used in stressful situations.

Study Objectives:

The objective of this study is an in-depth analysis of the mechanisms underlying the effectiveness of breathing techniques in reducing responses to acute stress. Specifically, the study aims to determine whether the observed effects of breathing interventions result from a specific and key physiological parameter in breathwork—the optimal breathing rate (5.5 breaths/min in coherent breathing), which, according to current research, maximizes baroreflex activation and cardiorespiratory synchronization—or whether a psychological component related to the act of performing a structured, relaxing activity plays the key role. The preliminary research hypothesis assumes that, compared to the sham intervention, performing the breathing method will significantly lower both physiological and psychological stress parameters.

A significant aspect of this research experiment is ensuring uniform perceptual conditions across all interventions. To avoid potential suggestions regarding the superiority of one stress-management method over another—which could lead to so-called performance bias—it is crucial to present each technique appropriately to the study participants. In the

information materials, both strategies will be presented as equivalent and widely used stress regulation methods that can yield beneficial effects. Such an approach will satisfy the requirement for cognitive blinding, thereby increasing the internal validity of the study [25,26].

Inclusion Criteria:

The study will ultimately include up to 120 healthy, professionally active individuals or university students aged 18–60 (60 females and 60 males).

Exclusion Criteria:

Severe chronic diseases, including metabolic (diabetes) and psychiatric disorders; history of myocardial infarction or stroke; history of cardiac surgery; regular use of medications such as anxiolytics or beta-blockers (excluding hormonal contraception); pregnancy; participation in other scientific experiments; previous extensive experience with breathing techniques or regular independent breathing/meditation practice; professional athletes; Raynaud's disease.

Method of Physiological and Psychological Stress Induction:

A vital component of the experiment is a valid, effective, and, most importantly, fully safe method of inducing stress in study participants. Psychophysiological responses to distress can be reliably induced under laboratory conditions using stress induction protocols. To this end, participants will be subjected to the **Maastricht Acute Stress Test (MAST)** protocol, which is designed as a simple, rapid, and non-invasive procedure aimed at activating the human stress response system. MAST is a reliable method for inducing robust autonomic, glucocorticoid, and subjective stress responses. The MAST protocol combines physical stress induction, unpredictability, uncontrollability, and the social-evaluative nature characteristic of other stress induction protocols [27,28].

The main components of the MAST protocol include:

- **Introductory Presentation:** A brief 5-minute presentation familiarizing participants with the conditions and procedure of the test.
- **Video Recording:** A dummy camera will be used; in reality, participants will never be recorded. This element is intended to evoke an additional social-evaluative effect reflecting realistic conditions, such as public speaking.
- **Main Phase (10 minutes):** Alternating exposure to cold (hand immersion in cold water) and the instruction to perform mental arithmetic aloud (subtracting two-digit numbers from four-digit numbers). Both stress stimuli will have interval durations not exceeding 90 seconds, of which participants will be notified during the introductory presentation.

Study Groups and Interventions:

Through block randomization stratified by sex, participants will be assigned to three equal groups: control, sham, and experimental. The only difference between the groups is the breathing rate. A detailed description of the experimental protocol is provided below.

Stress Management Methods Used in the Experiment (Research Interventions):

- **Coherent Breathing (~6 breaths per minute, 5.5s inhale / 5.5s exhale):** An active intervention with documented physiological benefits utilizing slow, conscious diaphragmatic breathing. Participants will practice diaphragmatic breathing for 10 minutes at a resonance frequency of ~0.1 Hz, guided by auditory cues, while focusing on the sensations accompanying the ventilation process.
- **Sham Breathing (15 breaths per minute, 2s inhale / 2s exhale):** A structural placebo, devoid of deep physiological impact but maintaining the form of a conscious intervention. The breathing rate in the sham intervention corresponds to the spontaneous breathing rate. This will also be performed for 10 minutes.
- **Spontaneous Breathing (No instructions):** A control condition reflecting the everyday way of responding to stress. Participants in the control group will be asked to close their eyes and attempt to relax, without any instructions regarding breath modulation.

This study design allows for determining whether the specificity of the intervention (e.g., rate and depth of breath) indeed determines the strength of the physiological stress response, or whether this effect can be attributed to more general psychological mechanisms such as a sense of control, expectations, and focus of attention.

General Study Design:

Each participant will undergo a remote one day preparation phase and an in-person experimental phase on the third day:

1. **Preparation Phase (Remote):** Participants will receive the following: a basic demographic questionnaire, the **DASS-21** (21-item Depression Anxiety and Stress Scale [29–32]), and information materials regarding the breathing method used in the experimental phase. Participants will be required to complete both questionnaires once, review the information materials, and perform a 10-minute breathing protocol not used in the actual study (breathing at a rate of 3s inhale / 3s exhale).
2. **Experimental Phase (In-person):** Participants will be seen at the research center (**Department of Physiology, Medical University of Bialystok**), where they will undergo a research protocol sequence involving two research interventions. To avoid natural **cortisol fluctuations**, appointments will be scheduled between 11:00 AM and 7:00 PM. Participants will be informed in advance of the required 24-hour abstinence from alcohol or other psychoactive substances, as well as a minimum 3-hour break

from caffeine (also known by other names such as theine, guaranine, or mateine), physical exercise, nicotine intake, or consuming anything other than water.

Physiological and Psychological Assessments:

The following measurements will be conducted during the experimental phase:

- **Salivary cortisol concentrations:** Analyzed using an **ELISA** (Enzyme-Linked Immunosorbent Assay) kit. Samples will be collected using **Salivette® Cortisol** tubes with synthetic swabs.
- **RR intervals (HRV):** Measured using a **POLAR H10** heart rate monitor paired with the **Elite HRV** mobile application [33,34] as well as KubiosHRV mobile application.
- **Blood pressure and heart rate:** Measured using an automated sphygmomanometer.
- **Current subjective stress levels:** Assessed using **Visual Analogue Mood Scales (VAMS)** [35].
- **State and Trait Anxiety:** Measured using the **State-Trait Anxiety Inventory (STAI)** [36–39].

Experimental Phase Protocol:

Regardless of the group assignment, the research protocol follows an identical structure, with the only variation being the specific stress-management method applied during the intervention. The sequence of the research protocol is as follows:

1. **Baseline (BASE):** Upon arrival, the participant will be led to a neutral room to complete the necessary documentation and prepare for the study (fitting the Polar H10 belt, rinsing the mouth with water). Baseline measurements of physiological and psychological stress parameters will then be conducted: completion of both parts of the STAI questionnaire; saliva sample collection; measurement of blood pressure, heart rate; subjective stress assessment on VAMS scales; and a 5-minute HRV measurement.
2. **First Research Intervention:** The participant will be asked to perform the 10-minute breathing/sham/control method according to the instructions provided during the preparation phase. The researcher will leave the room during this time.
3. **Transfer:** The participant will be moved to a second room specifically prepared for the **MAST protocol**. After the participant is seated, the researcher will ask them to sign a non-binding consent form for video and audio recording (participants will not actually be recorded).
4. **Pre-Stress (PRE):** The researcher will begin the MAST preparation phase, where the tasks will be explained via a graphical presentation. Following the presentation, "PRE" measurements will be taken: subjective stress on VAMS scales; saliva sample

collection; measurement of blood pressure, heart rate; and completion of the "State" portion of the STAI questionnaire, and a 5-minute HRV measurement.

5. **Acute Stress Phase (MAST):** The participant will undergo the main 10-minute portion of the MAST protocol. This phase involves alternating hand immersion in cold water (4.5-5.5°C) and counting backward aloud, starting from a random four-digit number. 10-minute HRV measurement will be done during the task.

Stimulus	Presentation	HI	MA	HI	MA	HI	MA	HI	MA	HI
Duration	5 min	90 s	45 s	60 s	60 s	60 s	90 s	45 s	60 s	60 s

6. **Post-Stress (POST):** Before the official end of the MAST protocol (the participant is only informed of a "short break" in testing), the researcher will perform "POST" measurements: VAMS scales, saliva collection, blood pressure, heart rate, the STAI "State" subscale, and a 5-minute HRV measurement.
7. **Debriefing:** Immediately after data collection, the researcher will inform the participant that there is no break, the MAST test has officially concluded, and that they were not actually recorded. The participant is then moved back to the first neutral room.
8. **Second Research Intervention:** The participant will again perform the 10-minute breathing/sham/control method. The researcher will leave the room and start 10-minute HRV recording.
9. **End of Intervention (END):** A 5-minute final measurement will be conducted, collecting all parameters again: VAMS scales, saliva collection, blood pressure, heart rate, and a 5-minute HRV measurement.
10. **Recovery:** After the "END" measurement, the participant will be asked to wait an additional 10 minutes in a seated position. This period accounts for the **delayed glucocorticoid response**, as salivary cortisol levels typically peak approximately 15 minutes after exposure to a stressor.
11. **Follow-up (FOLLOW):** The final stage involves a comprehensive reassessment of stress parameters: STAI "State" subscale; saliva collection; blood pressure, heart rate; VAMS scales; and a final 5-minute HRV measurement.

Fazy badania	Control Group	Sham Group	Experimental Group
1	Preparation + Baseline **measurement (BASE)**	Preparation + Baseline **measurement (BASE)**	Preparation + Baseline **measurement (BASE)**

2	Intervention I – Control (Spontaneous breathing) + DURING1 measurement	Intervention I – Sham breathing + DURING1 measurement	Intervention I – Coherent breathing + DURING1 measurement
3	Transfer to the second room	Transfer to the second room	Transfer to the second room
4	MAST presentation + **PRE measurement**	MAST presentation + **PRE measurement**	MAST presentation + **PRE measurement**
5	MAST Test – Exposure to stress stimulus + STRESS measurement	MAST Test – Exposure to stress stimulus + STRESS measurement	MAST Test – Exposure to stress stimulus + STRESS measurement
6	**POST measurement**	**POST measurement**	**POST measurement**
7	Conclusion of test and transfer to the first room	Conclusion of test and transfer to the first room	Conclusion of test and transfer to the first room
8	Intervention II – Control (Spontaneous breathing) + DURING2 measurement	Intervention II – Placebo breathing + DURING2 measurement	Intervention II – Coherent breathing + DURING2 measurement
9	**Final measurement (END)**	**Final measurement (END)**	**Final measurement (END)**
10	Recovery phase	Recovery phase	Recovery phase
11	**FOLLOW measurement**	**FOLLOW measurement**	**FOLLOW measurement**

The following parameters will be evaluated as part of the analysis:

1. STAI (State-Trait Anxiety Inventory) scores: Assessment of state and stress anxiety levels.
2. Cortisol Analysis: Salivary cortisol levels as a primary biomarker of the endocrine stress response.
3. **Subjective Stress (VAMS):** Intensity of perceived stress as measured on the Visual Analogue Mood Scale from 0 to 100 in terms of perceived:
    - Stress
    - Relaxation
    - Mental tension
    - Calmness

HRV (Heart Rate Variability) Parameters including:

- RMSSD (Root Mean Square of Successive Differences): A time-domain measure of HRV reflecting parasympathetic nervous system (PNS) activity.
- SDNN (Standard Deviation of NN intervals): An overall measure of heart rate variability.
- Kubios PNS Index: A composite indicator of parasympathetic nervous system activity.
- Kubios SNS Index: A composite indicator of sympathetic nervous system activity.
- LF Absolute Power: Low-frequency power, reflecting the balance between the sympathetic and parasympathetic systems.
- HF Absolute Power: High-frequency power, primarily associated with parasympathetic (vagal) activity.
- LF/HF Ratio**:** The ratio of low-frequency to high-frequency power, used as an index of autonomic balance.
- Mean RR: The average duration of RR intervals.
- Mean HR: Average heart rate.

Blood Pressure Parameters:

- **SBP (Systolic Blood Pressure):** The pressure in the arteries during heart contraction.
- **DBP (Diastolic Blood Pressure):** The pressure in the arteries during the heart's relaxation phase.

While a wide range of psychophysiological and biochemical markers is recorded according to the protocol, the final statistical analysis and primary publication may focus on a subset of the most relevant parameters to ensure clarity and avoid redundancy. All collected heart rate variability (HRV) parameters may undergo standard mathematical transformations (e.g., natural log transformation) where necessary to meet the assumptions of parametric statistical testing and ensure the validity of the results.

The primary objective of this analysis is to elucidate how chronic stress and heart rate variability influence psychophysiological outcomes. Furthermore, the study aims to determine the interdependencies between psychological test results, HRV parameters, cortisol levels, glucose levels, and subjective stress perception.

- Polish, validated version of the DASS-21 (21-item Depression Anxiety and Stress Scale) questionnaire. [29–32];

DASS21

Name: Date:

Please read each statement and circle a number 0, 1, 2 or 3 which indicates how much the statement applied to you **over the past week**. There are no right or wrong answers. Do not spend too much time on any statement.

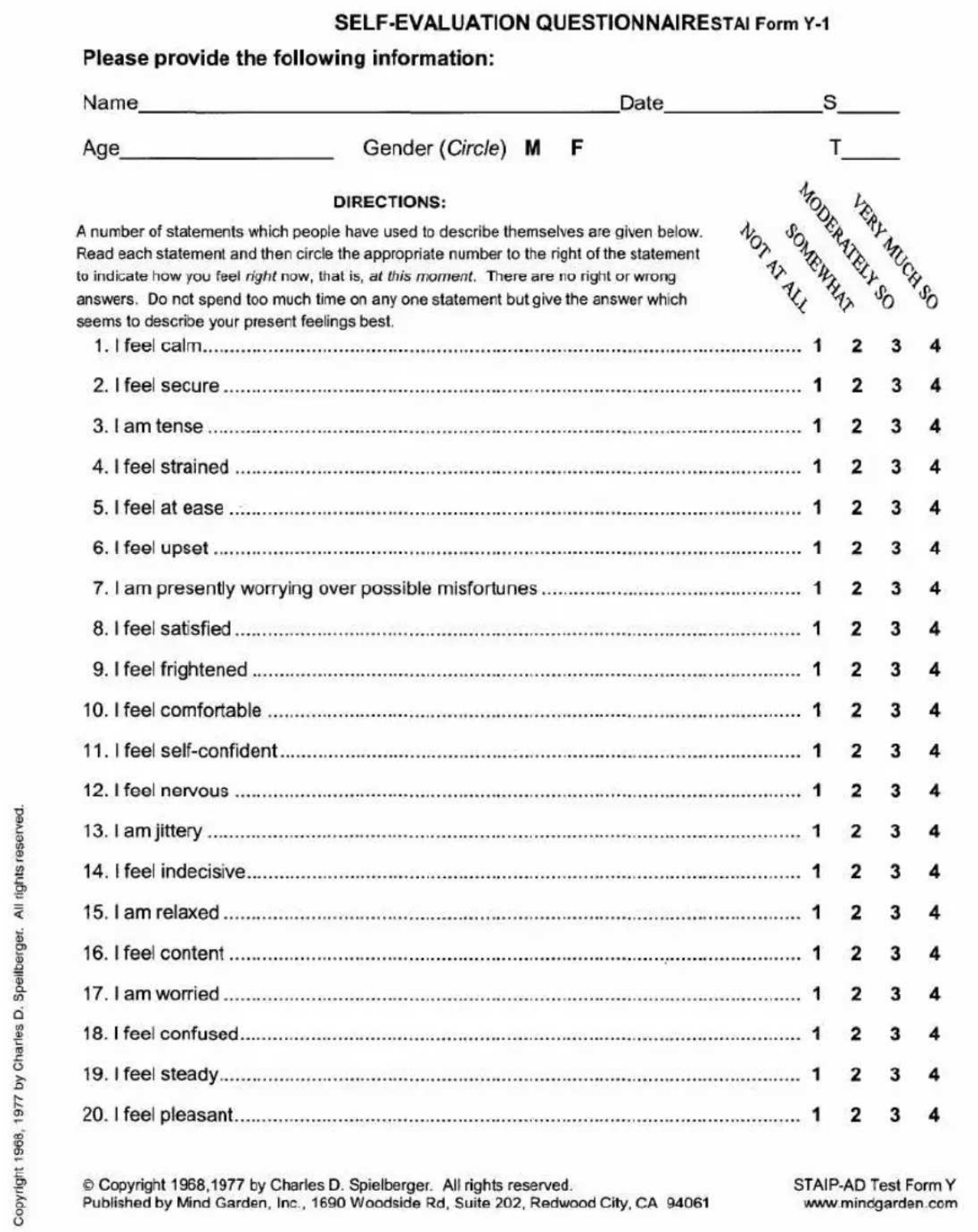
The rating scale is as follows:

0 Did not apply to me at all
1 Applied to me to some degree, or some of the time
2 Applied to me to a considerable degree or a good part of time
3 Applied to me very much or most of the time

1 (s)	I found it hard to wind down	0	1	2	3
2 (a)	I was aware of dryness of my mouth	0	1	2	3
3 (d)	I couldn't seem to experience any positive feeling at all	0	1	2	3
4 (a)	I experienced breathing difficulty (e.g. excessively rapid breathing, breathlessness in the absence of physical exertion)	0	1	2	3
5 (d)	I found it difficult to work up the initiative to do things	0	1	2	3
6 (s)	I tended to over-react to situations	0	1	2	3
7 (a)	I experienced trembling (e.g. in the hands)	0	1	2	3
8 (s)	I felt that I was using a lot of nervous energy	0	1	2	3
9 (a)	I was worried about situations in which I might panic and make a fool of myself	0	1	2	3
10 (d)	I felt that I had nothing to look forward to	0	1	2	3
11 (s)	I found myself getting agitated	0	1	2	3
12 (s)	I found it difficult to relax	0	1	2	3
13 (d)	I felt down-hearted and blue	0	1	2	3
14 (s)	I was intolerant of anything that kept me from getting on with what I was doing	0	1	2	3
15 (a)	I felt I was close to panic	0	1	2	3
16 (d)	I was unable to become enthusiastic about anything	0	1	2	3
17 (d)	I felt I wasn't worth much as a person	0	1	2	3
18 (s)	I felt that I was rather touchy	0	1	2	3
19 (a)	I was aware of the action of my heart in the absence of physical exertion (e.g. sense of heart rate increase, heart missing a beat)	0	1	2	3
20 (a)	I felt scared without any good reason	0	1	2	3
21 (d)	I felt that life was meaningless	0	1	2	3

Polish, validated version of the **STAI** (State-Trait Anxiety Inventory) questionnaire (**STAI-S** and **STAI-Y** subscales). [39]:

English versions:



SELF-EVALUATION QUESTIONNAIRE STAI Form Y-1

Please provide the following information:

Name_____ Date_____ S_____

Age_____ Gender (*Circle*) **M** **F** T_____

DIRECTIONS:

A number of statements which people have used to describe themselves are given below. Read each statement and then circle the appropriate number to the right of the statement to indicate how you feel *right* now, that is, *at this moment*. There are no right or wrong answers. Do not spend too much time on any one statement but give the answer which seems to describe your present feelings best.

	NOT AT ALL	SOMEWHAT	MODERATELY SO	VERY MUCH SO
1. I feel calm	1	2	3	4
2. I feel secure	1	2	3	4
3. I am tense	1	2	3	4
4. I feel strained	1	2	3	4
5. I feel at ease	1	2	3	4
6. I feel upset	1	2	3	4
7. I am presently worrying over possible misfortunes	1	2	3	4
8. I feel satisfied	1	2	3	4
9. I feel frightened	1	2	3	4
10. I feel comfortable	1	2	3	4
11. I feel self-confident	1	2	3	4
12. I feel nervous	1	2	3	4
13. I am jittery	1	2	3	4
14. I feel indecisive	1	2	3	4
15. I am relaxed	1	2	3	4
16. I feel content	1	2	3	4
17. I am worried	1	2	3	4
18. I feel confused	1	2	3	4
19. I feel steady	1	2	3	4
20. I feel pleasant	1	2	3	4

STAIP-AD Test Form Y
www.mindgarden.com

SELF-EVALUATION QUESTIONNAIRE

STAI Form Y-2

Name_____ Date_____

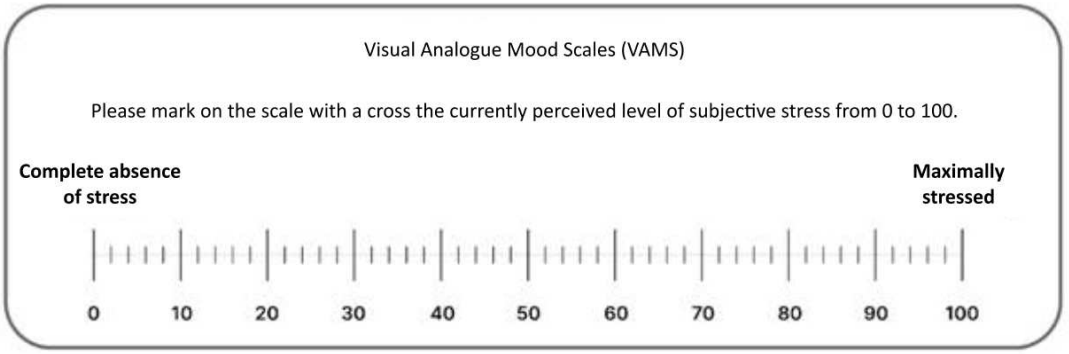
DIRECTIONS

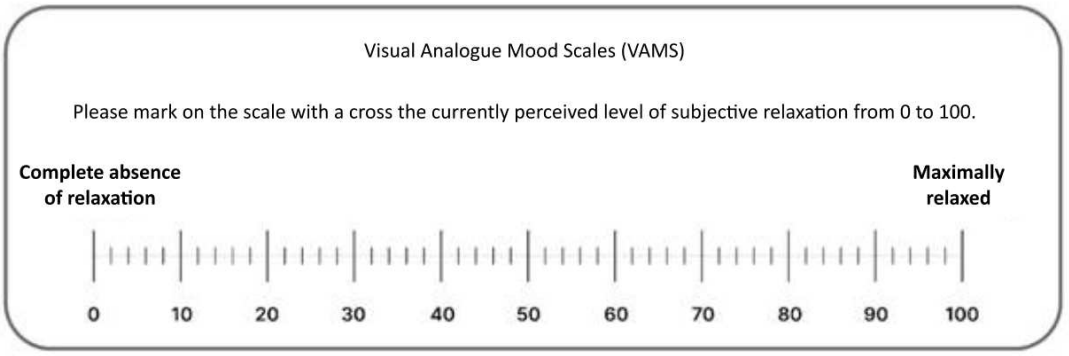
A number of statements which people have used to describe themselves are given below. Read each statement and then circle the appropriate number to the right of the statement to indicate how you *generally* feel. There are no right or wrong answers. Do not spend too much time on any one statement but give the answer which seems to describe how you generally feel.

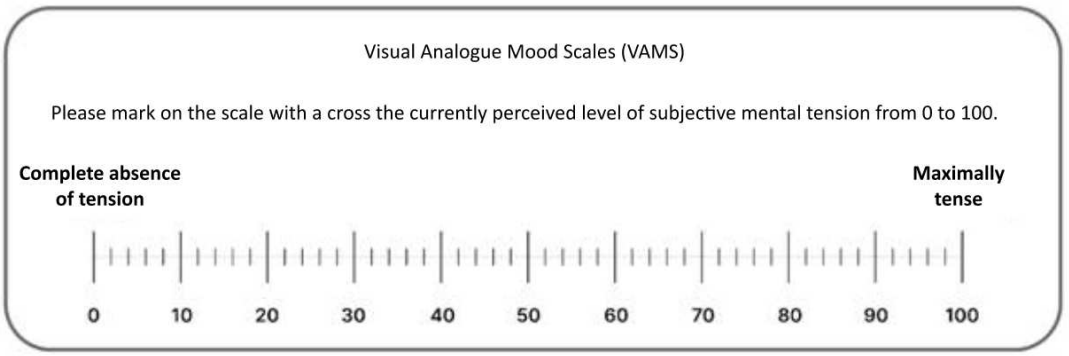
	ALMOST NEVER	SOMETIMES	OFTEN	ALMOST ALWAYS
21. I feel pleasant	1	2	3	4
22. I feel nervous and restless	1	2	3	4
23. I feel satisfied with myself	1	2	3	4
24. I wish I could be as happy as others seem to be	1	2	3	4
25. I feel like a failure	1	2	3	4
26. I feel rested	1	2	3	4
27. I am "calm, cool, and collected"	1	2	3	4
28. I feel that difficulties are piling up so that I cannot overcome them	1	2	3	4
29. I worry too much over something that really doesn't matter	1	2	3	4
30. I am happy	1	2	3	4
31. I have disturbing thoughts	1	2	3	4
32. I lack self-confidence	1	2	3	4
33. I feel secure	1	2	3	4
34. I make decisions easily	1	2	3	4
35. I feel inadequate	1	2	3	4
36. I am content	1	2	3	4
37. Some unimportant thought runs through my mind and bothers me	1	2	3	4
38. I take disappointments so keenly that I can't put them out of my mind	1	2	3	4
39. I am a steady person	1	2	3	4
40. I get in a state of tension or turmoil as I think over my recent concerns and interests	1	2	3	4

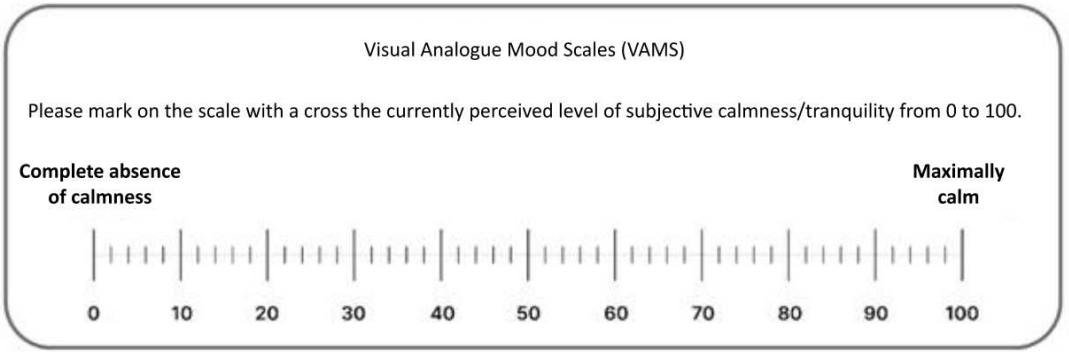
Published by Mind Garden, Inc., 1690 Woodside Rd, Suite 202, Redwood City, CA 94061

- VAMS (Visual Analogue Mood Scales) for assessing subjective mood (stress, relaxation, mental tension, calmness/tranquility).[35]:









References:

1. Stress Available online: https://www.who.int/news-room/questions-and-answers/item/stress (accessed on 1 January 2024).

2. Fincham, G.W.; Strauss, C.; Montero-Marin, J.; Cavanagh, K. Effect of Breathwork on Stress and Mental Health: A Meta-Analysis of Randomised-Controlled Trials. *Sci Rep* **2023**, *13*, 432, doi:10.1038/s41598-022-27247-y.

3. Hopper, S.I.; Murray, S.L.; Ferrara, L.R.; Singleton, J.K. Effectiveness of Diaphragmatic Breathing for Reducing Physiological and Psychological Stress in Adults: A Quantitative Systematic Review. *JBI Evidence Synthesis* **2019**, *17*, 1855, doi:10.11124/JBISRIR-2017-003848.

4. Liu, Y.-Z.; Wang, Y.-X.; Jiang, C.-L. Inflammation: The Common Pathway of Stress-Related Diseases. *Front Hum Neurosci* **2017**, *11*, 316, doi:10.3389/fnhum.2017.00316.

5. Salleh, Mohd.R. Life Event, Stress and Illness. *Malays J Med Sci* **2008**, *15*, 9–18.

6. Roberts, B.L.; Karatsoreos, I.N. Brain–Body Responses to Chronic Stress: A Brief Review. *Fac Rev* **2021**, *10*, 83, doi:10.12703/r/10-83.

7. Mariotti, A. The Effects of Chronic Stress on Health: New Insights into the Molecular Mechanisms of Brain–Body Communication. *Future Sci OA* **2015**, *1*, FSO23, doi:10.4155/fso.15.21.

8. Hussenoeder, F.S.; Conrad, I.; Pabst, A.; Luppa, M.; Stein, J.; Engel, C.; Zachariae, S.; Zeynalova, S.; Yahiaoui-Doktor, M.; Glaesmer, H.; et al. Different Areas of Chronic Stress and Their Associations with Depression. *Int J Environ Res Public Health* **2022**, *19*, 8773, doi:10.3390/ijerph19148773.

9. Dai, S.; Mo, Y.; Wang, Y.; Xiang, B.; Liao, Q.; Zhou, M.; Li, X.; Li, Y.; Xiong, W.; Li, G.; et al. Chronic Stress Promotes Cancer Development. *Front Oncol* **2020**, *10*, 1492, doi:10.3389/fonc.2020.01492.

10. Depressive Disorder (Depression) Available online: https://www.who.int/news-room/fact-sheets/detail/depression (accessed on 1 January 2024).

11. Breathwork - Quick Search Results | Oxford English Dictionary Available online: https://www.oed.com/search/dictionary/?scope=Entries&q=breathwork&tl=true (accessed on 2 January 2024).

12. Bahi, C.; Irrmischer, M.; Franken, K.; Fejer, G.; Schlenker, A.; Deijen, J.B.; Engelbregt, H. Effects of Conscious Connected Breathing on Cortical Brain Activity, Mood and State of Consciousness in Healthy Adults. *Curr Psychol* **2023**, doi:10.1007/s12144-023-05119-6.

13. Birdee, G.; Nelson, K.; Wallston, K.; Nian, H.; Diedrich, A.; Paranjape, S.; Abraham, R.; Gamboa, A. Slow Breathing for Reducing Stress: The Effect of Extending Exhale. *Complement Ther Med* **2023**, *73*, 102937, doi:10.1016/j.ctim.2023.102937.

14. Ma, X.; Yue, Z.-Q.; Gong, Z.-Q.; Zhang, H.; Duan, N.-Y.; Shi, Y.-T.; Wei, G.-X.; Li, Y.-F. The Effect of Diaphragmatic Breathing on Attention, Negative Affect and Stress in Healthy Adults. *Front Psychol* **2017**, *8*, 874, doi:10.3389/fpsyg.2017.00874.

15. Shaw-Metz, J.L. Coming up for Air: Breathwork Practice for Stress Management in the Healthcare Setting. *Journal of Interprofessional Education & Practice* **2023**, *30*, 100594, doi:10.1016/j.xjep.2022.100594.

16. Bentley, T.G.K.; D'Andrea-Penna, G.; Rakic, M.; Arce, N.; LaFaille, M.; Berman, R.; Cooley, K.; Sprimont, P. Breathing Practices for Stress and Anxiety Reduction: Conceptual Framework of Implementation Guidelines Based on a Systematic Review of the Published Literature. *Brain Sciences* **2023**, *13*, 1612, doi:10.3390/brainsci13121612.

17. Perciavalle, V.; Blandini, M.; Fecarotta, P.; Buscemi, A.; Di Corrado, D.; Bertolo, L.; Fichera, F.; Coco, M. The Role of Deep Breathing on Stress. *Neurol Sci* **2017**, *38*, 451–458, doi:10.1007/s10072-016-2790-8.

18. Laborde; Allen, M.S.; Borges, U.; Dosseville, F.; Hosang, T.J.; Iskra, M.; Mosley, E.; Salvotti, C.; Spolverato, L.; Zammit, N.; et al. Effects of Voluntary Slow Breathing on Heart Rate and Heart Rate Variability: A Systematic Review and a Meta-Analysis. *Neurosci Biobehav Rev* **2022**, *138*, 104711, doi:10.1016/j.neubiorev.2022.104711.

19. Sevoz-Couche, C.; Laborde, S. Heart Rate Variability and Slow-Paced Breathing:When Coherence Meets Resonance. *Neurosci Biobehav Rev* **2022**, *135*, 104576, doi:10.1016/j.neubiorev.2022.104576.

20. Fincham, G.W.; Strauss, C.; Montero-Marin, J.; Cavanagh, K. Effect of Breathwork on Stress and Mental Health: A Meta-Analysis of Randomised-Controlled Trials. *Sci Rep* **2023**, *13*, 432, doi:10.1038/s41598-022-27247-y.

21. Fincham, G.W.; Strauss, C.; Cavanagh, K. Effect of Coherent Breathing on Mental Health and Wellbeing: A Randomised Placebo-Controlled Trial. *Sci Rep* **2023**, *13*, 22141, doi:10.1038/s41598-023-49279-8.

22. Russo, M.A.; Santarelli, D.M.; O'Rourke, D. The Physiological Effects of Slow Breathing in the Healthy Human. *Breathe (Sheff)* **2017**, *13*, 298–309, doi:10.1183/20734735.009817.

23. Zaccaro, A.; Piarulli, A.; Laurino, M.; Garbella, E.; Menicucci, D.; Neri, B.; Gemignani, A. How Breath-Control Can Change Your Life: A Systematic Review on Psycho-Physiological Correlates of Slow Breathing. *Front Hum Neurosci* **2018**, *12*, 353, doi:10.3389/fnhum.2018.00353.

24. Bentley, T.G.K.; D'Andrea-Penna, G.; Rakic, M.; Arce, N.; LaFaille, M.; Berman, R.; Cooley, K.; Sprimont, P. Breathing Practices for Stress and Anxiety Reduction: Conceptual Framework of Implementation Guidelines Based on a Systematic Review of the Published Literature. *Brain Sciences* **2023**, *13*, 1612, doi:10.3390/brainsci13121612.

25. Karanicolas, P.J.; Farrokhyar, F.; Bhandari, M. Blinding: Who, What, When, Why, How? *Can J Surg* **2010**, *53*, 345–348.

26. Moustgaard, H.; Clayton, G.L.; Jones, H.E.; Boutron, I.; Jørgensen, L.; Laursen, D.R.T.; Olsen, M.F.; Paludan-Müller, A.; Ravaud, P.; Savović, J.; et al. Impact of Blinding on Estimated Treatment Effects in Randomised Clinical Trials: Meta-Epidemiological Study. *BMJ* **2020**, *368*, l6802, doi:10.1136/bmj.l6802.

27. Shilton, A.L.; Laycock, R.; Crewther, S.G. The Maastricht Acute Stress Test (MAST): Physiological and Subjective Responses in Anticipation, and Post-Stress. *Frontiers in Psychology* **2017**, *8*.

28. Smeets, T.; Cornelisse, S.; Quaedflieg, C.W.E.M.; Meyer, T.; Jelicic, M.; Merckelbach, H. Introducing the Maastricht Acute Stress Test (MAST): A Quick and Non-Invasive Approach to Elicit Robust Autonomic and Glucocorticoid Stress Responses. *Psychoneuroendocrinology* **2012**, *37*, 1998–2008, doi:10.1016/j.psyneuen.2012.04.012.

29. Makara-Studzińska, M.; Tyburski, E.; Załuski, M.; Adamczyk, K.; Mesterhazy, J.; Mesterhazy, A. Confirmatory Factor Analysis of Three Versions of the Depression Anxiety Stress Scale (DASS-42, DASS-21, and DASS-12) in Polish Adults. *Front Psychiatry* **2021**, *12*, 770532, doi:10.3389/fpsyt.2021.770532.

30. Tran, T.D.; Tran, T.; Fisher, J. Validation of the Depression Anxiety Stress Scales (DASS) 21 as a Screening Instrument for Depression and Anxiety in a Rural Community-Based Cohort of Northern Vietnamese Women. *BMC Psychiatry* **2013**, *13*, 24, doi:10.1186/1471-244X-13-24.

31. Henry, J.D.; Crawford, J.R. The Short-Form Version of the Depression Anxiety Stress Scales (DASS-21): Construct Validity and Normative Data in a Large Non-Clinical Sample. *Br J Clin Psychol* **2005**, *44*, 227–239, doi:10.1348/014466505X29657.

32. Osman, A.; Wong, J.L.; Bagge, C.L.; Freedenthal, S.; Gutierrez, P.M.; Lozano, G. The Depression Anxiety Stress Scales-21 (DASS-21): Further Examination of Dimensions, Scale Reliability, and Correlates. *J Clin Psychol* **2012**, *68*, 1322–1338, doi:10.1002/jclp.21908.

33. Gambassi, B.B.; Neves, V.R.; Brito, E.Z.A.; da Silva Fernandes, D.S.; Sá, C.A.; da Rocha Nogueira, R.M.; de Jesus Furtado Almeida, F.; de Araújo Cavalcanti, P.A.; Gomes Gonçalves e Silva, D.C.; Neto, D.S.; et al. A Validation Study of a Smartphone Application for Heart Rate Variability Assessment in Asymptomatic Adults. *Am J Cardiovasc Dis* **2020**, *10*, 219–229.

34. Vondrasek, J.D.; Riemann, B.L.; Grosicki, G.J.; Flatt, A.A. Validity and Efficacy of the Elite HRV Smartphone Application during Slow-Paced Breathing. *Sensors (Basel)* **2023**, *23*, 9496, doi:10.3390/s23239496.

35. Lesage, F.-X.; Berjot, S.; Deschamps, F. Clinical Stress Assessment Using a Visual Analogue Scale. *Occup Med (Lond)* **2012**, *62*, 600–605, doi:10.1093/occmed/kqs140.

36. State-Trait Anxiety Inventory for Adults Available online: https://psycnet.apa.org/doiLanding?doi=10.1037%2Ft06496-000 (accessed on 1 February 2024).

37. Thomas, C.L.; Cassady, J.C. Validation of the State Version of the State-Trait Anxiety Inventory in a University Sample. *SAGE Open* **2021**, *11*, 21582440211031900, doi:10.1177/21582440211031900.

38. Spielberger, C.; Gorsuch, R.; Lushene, R.; Vagg, P.; Jacobs, G. *Manual for the State-Trait Anxiety Inventory (Form Y1 – Y2)*; 1983; Vol. IV;

39. Wrześniowski, K.; Matusik, D.; Sosnowski, T. Inwentarz Stanu i Cechy Lęku STAI: Polska Adaptacja STAI: Podręcznik. *Pracownia Testów Psychologicznych Polskiego Towarzystwa Psychologicznego* **2002**.

2. Informed Consent Form

INFORMED CONSENT FORM FOR STUDY PARTICIPATION (TRANSLATED)

Title: "Breathwork and stress: investigating the mechanisms of action and effectiveness of breathing interventions in modulating the psychophysiological response to acute stress."

Full Name: …….....

I hereby provide my consent to participate in a study aimed at evaluating the effectiveness of stress reduction methods utilizing breathing exercises. The objectives, conditions of participation, potential risks and benefits, as well as all procedures and stages of the research protocol, have been explained to me. I also confirm that I have been informed of the opportunity to ask questions regarding this medical experiment. I understand that I may withdraw my consent at any stage of the study without providing a reason and without facing any adverse consequences or claims. I declare my consent for the processing of my clinical data by the individual conducting the study, provided that my personal data is protected and all information obtained during the study is treated as confidential.

Białystok, date: ……

Participant's Signature .....

Researcher's Signature .....

3. Statistical Analysis Plan

Data Preparation and Normality Testing: The normality of the data distribution for all physiological and psychological variables will be assessed using the **Shapiro-Wilk test**. For any parameters demonstrating a non-normal distribution, a **natural log transformation (ln)** will be applied prior to parametric testing to meet the necessary assumptions for Analysis of Variance (ANOVA).

Data Integrity and Quality Control: To ensure high data quality and minimize loss due to technical issues, HRV data is recorded simultaneously using two mobile applications: **Elite HRV** and **Kubios HRV**, both connected to a **Polar H10** monitor. This redundancy is implemented because pilot testing revealed occasional freezing of the Kubios HRV app; in such instances, identical data segments will be recovered from the Elite HRV backup.

HRV Exclusion Criteria: For the 5-minute static HRV measurement windows, a minimum of **4 minutes of valid RR interval data** is required. This 1-minute buffer accounts for potential movement artifacts (e.g., chest strap displacement) or software synchronization errors. Participants with recordings shorter than 4 minutes in any primary measurement window will be excluded from the final HRV analysis to ensure the physiological validity of the results.

Statistical analysis:

1. **Primary Statistical Model (Discrete Points):** A **two-way mixed-design Analysis of Variance (ANOVA)** will be utilized to analyze changes across the five primary 5-minute measurement points: **BASE, PRE, POST, END, and FOLLOW**. The model will include **Intervention Group** (Coherent, Sham, Control) as the between-subjects factor and **Time** as the within-subjects factor.

2. **Secondary Statistical Analysis (Process Windows):** To evaluate the immediate psychophysiological dynamics, separate statistical analyses will be conducted for the three 10-minute continuous monitoring windows: **First Intervention (DURING1)**, **Acute Stress Phase (STRESS)**, and **Second Intervention (DURING2)**. These analyses will specifically:

- Verify the efficacy of the **MAST stressor** by comparing SNS Index and Heart Rate across groups during the STRESS window.
- Assess the **"buffering" effect** of coherent breathing by analyzing RMSSD and PNS Index during the DURING1 and STRESS windows compared to sham and control groups.
- Confirm **protocol adherence** using the **Kubios RESP algorithm** to compare respiratory rates between the 6 bpm and 15 bpm groups during both intervention windows.

Post-hoc Analysis: To explore significant main effects or Group x Time interactions, **Tukey's HSD post-hoc test** will be employed. This will allow for multiple pairwise

comparisons between groups at specific time points and within groups across the study phases to identify where significant differences occur. The level of statistical significance for all tests is set at **$p < 0.05$**.

Biochemical Analysis (Technical details): Salivary cortisol concentrations are determined using **ELISA kits (Abcam)**. The absorbance of the samples is measured using a **BayoTec microplate reader**. All samples will be analyzed in accordance with the manufacturer's instructions.