



NAVAL MEDICAL RESEARCH UNIT DAYTON

CO₂ Effects on Memory when Combined with 28 Hours of Continuous Wakefulness

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STATEMENTS

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Source of Support

This work was supported by Defense Health Program and funded by work unit number H1815.

Human Research/Institutional Review Board (IRB) statement

The study protocol was approved by the Naval Medical Research Unit Dayton Institutional Review Board in compliance with all applicable federal regulations governing the protection of human subjects (NAMRUD.2019.0008).

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ACKNOWLEDGEMENTS

We would like to thank all the people involved in this study, particularly all the airmen who volunteered to participate. They gave many hours of their time to contribute to the data in an effort to help military and civilian communities struggling with the consequences of long schedules and chemical exposure that may occur during operations. Without their participation, this study would not have been possible. We also express gratitude to the physicians who were on call for any medical issues that occurred: CDR Matthew R. Doubrava, MD, III, MD; and LtCol Dara D. Regn, MD. Many thanks go to Dr. Barbara Shykoff for teaching the team to work with gases safely; Dr. Dan Warkander for programming our physiological monitoring equipment and teaching us how to read the graphs; LCDR Matthew Shipman for his input into the study design and application; Mr. Jonathan Sagan for helping with the literature review and setting up the equipment; Mr. Ryan Everidge for assistance with data entry. Finally, we would like to thank the Defense Health Agency for funding this study.

1.0 INTRODUCTION

Physiological episodes (PEs) remain a top safety priority for U.S. Navy (USN), U.S. Marine Corp (USMC) and U.S. Air Force (USAF) aviation, but the root causes of PEs have remained elusive. Over the last several years, there have been repeated and increasing instances of PEs in tactical aviation (TACAIR) throughout the USN, USMC and USAF TACAIR fleets. PEs, inducing hypoxia or hypoxia-like symptoms, continue to be reported despite numerous efforts to mitigate or eliminate them. One potential root cause that has been given significant attention is the possibility of contaminants present in the aviator's breathing air delivered by the on-board oxygen-generated system (OBOGS). Studies, including one conducted by NASA, have shown the presence of hundreds of contaminants present in OBOGS-generated air (Cragg *et al.*, 2017). None of the contaminants identified, however, are understood to be present at levels of concern. In other words, no identified contaminant, taken individually, was determined to be present at or above levels where adverse responses would be anticipated.

Exposure to increased levels of carbon dioxide (CO₂), a typical component of inhaled air and exhaled breath and a potential contaminant, may occur as a result of human error and/or system malfunction. Exposure may occur during oxygen mask removal (e.g., due to mask discomfort, eating breaks, or insufficient oxygen flow through the mask). During these instances, pilots breathe ambient cockpit air. System malfunctions (i.e., OBOGS failure) interrupt essential O₂ supply and results in inhalation of increased CO₂.

Regardless of the reason for increased exposure to CO₂, it can be a danger for pilots if they unknowingly are exposed to elevated levels of CO₂ over an extended period. Exposure to elevated levels of CO₂ in breathing air leads to hypercapnia, the build-up of CO₂ in the bloodstream. Studies have suggested that CO₂ has a significant impact on neuronal excitability as well as nervous system-regulation of physiology (Dulla *et al.*, 2005), and this can have a pronounced effect on multiple physiological systems. Elevated levels of CO₂ will cause changes to the acid-base chemistry in the blood and result in additional CO₂ being stored in the tissues rather than being exhaled (Andreasson & Rees, 2005; Rees & Andreasson, 2005). When concentrations of CO₂ in inspired gas increase, there is a reflexive metabolic increase in ventilation rate (Ellingsen, Sydnes, Hauge, Zwart, Liestol, & Nicolaysen, 1987) and depth (Vandor, Sherman, & Luciano, 1985). Consequently, additional excretion of CO₂ in the pilot's breath combined with ambient CO₂ may fill dead space in the mask/cockpit. At certain altitudes, the ability to measure the amount of CO₂ filling the dead space during a system malfunction is impossible. Even small amounts, however, can affect a pilot who may be unable to recognize signs of CO₂ due to the colorless and odorless composition of the gas. Respiration begins to increase at exposure levels of 1% and above (Scott, Kraemer, & Keller, 2009). The increase in laborious breathing will begin to physically fatigue the pilot and can possibly result in decrements in the performance of operationally relevant tasks (Slonim & Hamilton, 1987).

The human body is often able to compensate for changing environments according to physiological needs, but physical and cognitive limits must be considered when evaluating a warfighter's ability to achieve optimum performance. In military flight operations, pilots are at risk of experiencing adverse effects from elevated CO₂ exposure that may exceed normal

exposures. While CO₂ is inhaled in ambient air (~0.04%), elevated levels can be hazardous to human health (Zhang, Wargocki, Lian, & Thyregod, 2016). Studies have focused primarily on physiological effects and offer inconsistent information regarding consequences on higher order cognitive processes, including memory, task performance, decision making, and attention. For example, a study investigating inhalation of 0, 4.5, 5.5, 6.5, and 7.5% CO₂ found a significantly slower performance on reasoning tasks at the higher concentrations, but no impairment to long-term memory (Sayers, Smith, Holland, & Keatinge, 1987). Another study investigating cognitive effects of excessive CO₂ in the blood stream (hypercapnia) of divers found significant differences in some cognitive domains, but short-term memory remained unaffected after inhalation of 1.5% and 3% of surface equivalent value (SEV) CO₂ (Selkirk, Briggs, & Shykoff, 2010).

Other studies have revealed that acclimation can occur at low levels, and exposure to 3% CO₂ presents no cognitive threat to healthy individuals (Scott et al., 2009). The severity of effects depends on the concentration of CO₂, the amount of available O₂ for compensation, duration of exposure, and volume of occupied space (Ellingsen *et al.*, 1987; Slonim & Hamilton, 1987). An evaluation of these variables is important for understanding CO₂'s potential impact on cognition and overall mission effectiveness. If other stressors that affect performance, such as inadequate sleep, additionally compromise the pilot, there may be additive effects that alter pilot performance even further. What is not known is how physical factors, such as fatigue, impact susceptibility to the effects of increased CO₂ exposure.

A sufficient amount of sleep has been recognized as one of the most important factors to help ensure the success of a mission or field operation (Lindsay & Dyche, 2012) and is as important as maintaining adequate food and transportation logistics (Angus, Pigeau & Heslegrave, 1992). The amount of sleep that the average adult obtains each night has decreased (Banks & Dinges, 2007), and the detrimental effects of sleep loss, both in the civilian workplace (Dawson & McCulloch, 2005; Krueger, 1989; Lerman, Flower, Gerson, & Hursh, 2012) and military operations (Giam, 1997; Lindsay & Dyche, 2012; Miller, Matsangas, & Shattuck, 2007), are alarming. Fatigue due to sleep loss has been named as a contributing factor to 12% of the Air Force's Class A mishaps as well as 4% of the Army's Class A-C mishaps (Caldwell & Caldwell, 2005).

The neurobehavioral effects of total sleep deprivation are clear and pronounced. Impairment of various functions occurs quickly, including an increase in involuntary microsleeps, attentional instability, and judgement errors with a simultaneous decrease in response speed, response accuracy, learning, task-shifting ability, and situational awareness (Balkin, Rupp, Picchioni, & Wesensten, 2008; Banks, & Dinges, 2011; Lim & Dinges, 2010; Killgore, 2010). The pace and temporal organization of operations often force warfighters to perform in sleep deprived conditions, including long flight assignments. Long labor periods, high workload environments, circadian disruptions, and deficient recovery time between flights or missions ensures that fatigue will continue to be a problem for military personnel (Caldwell, Mallis, Caldwell, Paul, Miller, & Neri, 2009; Neville, Bisson, French, Boll, & Storm, 1994; Samel, Wegmann, & Vijvoda, 1995). Along with the threat of fatigue, warfighters are at risk for inflight mishaps that expose pilots to additional bodily stressors, including impeded ventilation as a result of changes in atmospheric pressure.

The individual impact of increased CO₂ exposure and sleep deprivation on cognitive impairment is still under investigation and have relevance for health and safety in military aviation. Assessments of the combined cognitive effects of inspired increased CO₂ and sleep-deprivation have not been conducted and may be of particular interest in the effort to identify potential root causes of PEs. This study, which examined the cognitive effects of exposure to 3% CO₂ in combination with sleep deprivation, was conducted to assess the combined effect of these two physiologic stressors on cognitive performance among aviators.

Hypotheses tested:

1. Individuals exposed to increased CO₂ after 28 hours of continuous wakefulness will perform worse on the memory task than those exposed to increased CO₂ while rested.
2. Individuals exposed to increased CO₂ after 28 hours of continuous wakefulness will perform worse on the memory task than those not exposed to increased CO₂ (ambient levels of CO₂) but continuously awake for 28 hours.

2.0 METHODS AND PROCEDURES

The protocol was a repeated measures study in which individuals were tested during 4 separate conditions: 1) participants were well-rested and tested during the day on the memory task (the baseline condition); 2) participants were well-rested and tested on the memory task during exposure to 3% CO₂ for 25 minutes (10 min pre-exposure, 15 minutes during the task); 3) participants were continuously awake for 28 hours and tested on the memory task; and 4) participants were continuously awake for 28 hours and tested on the memory task during exposure to 3% CO₂ for 25 minutes (10 min pre-exposure, 15 minutes during the task). The baseline condition was always the first condition; the other three conditions were counterbalanced to offset any order effects that may occur. Each condition was separated by a minimum of 3 days with most separated by 7 days. While the sleep deprivation and rested conditions could not be blinded, the CO₂ exposure was blinded to the participant only.

A total of six participants were enrolled into the study, however the final analyses only consist of four subjects who completed at least three conditions. The protocol was approved by the Naval Medical Research Unit Dayton Institutional Review Board (NAMRU-D 2019.0008).

2.1 Pre-study questionnaires

2.1.1 Questionnaires. Personality and circadian-type questionnaires were administered prior to data collection in order to determine whether participants fell into extreme morning or evening types and to quantify certain personality factors (e.g., neuroticism, extraversion) that may impact an individual's response to sleep deprivation (Killgore, Richards, Killgore, Kamimori, & Balkin, 2007; Taillard, Philip, Coste, Sagaspe, & Bioulac, 2003).

2.1.1.1 The Horne and Östberg Morningness/Eveningness Questionnaire (MEQ) (Horne & Östberg, 1976) was used to evaluate each participant's circadian type. The MEQ's main purpose is to measure whether a person's circadian rhythm (biological clock) produces peak alertness in the morning, in the evening, or in between. This questionnaire has 19 questions that asks the time

a person prefers to perform specific activities. Each question's point was added to determine the morningness-eveningness score, ranging from 16-86. Scores of 41 and below indicated "evening types." Scores of 59 and above indicate "morning types." Scores between 42 and 58 indicated "intermediate types." Participants answered this questionnaire on a printed copy. This questionnaire was administered on the consent day and scored by a research staff member.

2.1.1.2 The Revised NEO Personality Inventory (NEO-PI-R™) was used for the assessment of personality (Costa & McCrae, 1992). The inventory consists of 240 items answered on a 5-point scale, ranging from "strongly disagree" to "strongly agree." The five domains measured are: Neuroticism, Extraversion, Openness to Experience, Agreeableness, and Conscientiousness. Each domain is further subdivided into six facets that measure specific features of the primary personality factor. Participants answered this questionnaire on a printed copy. A research staff member scored the completed questionnaire from the template provided by Psychological Assessment Resources, Inc.

2.1.1.3 The Epworth Sleepiness Scale (ESS) was used to assess a participants' sleep propensity during normal daily activities (Johns, 1991). Participants were asked to rate their likelihood of dozing off or falling asleep on a 4-point scale (0-3) while engaged in 8 different life activities. Scores above 10 indicate excessive daytime sleepiness. The participant answered this questionnaire from a printed copy. A research staff member scored the completed questionnaire. This questionnaire was administered on the consent day.

2.2 Sleep and physiological monitoring

2.2.1 Activity/sleep monitor. Participants' activity was measured using an Actiwatch 2™ (Philips Respironics, Murrysville, PA). The Actiwatch 2™ (wrist activity monitor) is a water-resistant, wrist-worn device that measures frequency and intensity of wearer movement using a precision motion sensitive piezoelectric assembly. A 1-minute data capture epoch was used to collect movement data. The movement results were plotted using accompanying software to track participants' sleep patterns throughout the study. Participants were instructed to wear the wrist activity monitor 3 days prior to each condition of the study. The data were downloaded and reviewed for compliance with the sleep requirement on the first day of the in-house portion of each condition. Study participants who did not sleep at least 7 hours per night were either rescheduled until the sleep requirements were met or dismissed from the study.

Sleep/wake data were collected to ensure participants attained adequate sleep prior to their test days. In addition to using the wrist activity monitor, participants also completed a sleep diary for 3 days prior to each of the in-lab phases of the study. This diary included bedtime and rise time for each of the days in which the participant was required to be in bed a minimum of 7.5 hours. The diary data were used to corroborate the data obtained from the wrist activity monitor. If the wrist monitor failed during data collection, sleep estimation was obtained from the sleep diary.

2.2.2 Vital signs. Research participants wore a Zephyr BioHarness™ with a data module (Medtronic, Boulder, CO) to measure their heart rate and breathing rate. These were fitted to the participants prior to the start of each study session. The BioHarness™ was equipped with contacts (patches of a copper metallic-looking fabric) that were moistened with tap water to

make appropriate electrical contact with the participant's skin. (For privacy purposes, participants were dismissed to the restroom or a small procedure room in order to place the BioHarness™ against their skin.) Data modules were installed into the BioHarness™, turned on, and data transmission/reception were checked immediately prior to the beginning of the run. The data modules were turned off until immediately prior to the start of the test exposures and left in the "off" mode in case of delays in order to minimize the size of the data files and to minimize battery usage.

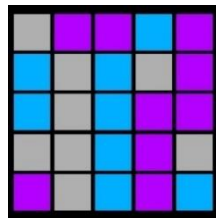
Each participant was also outfitted with a Nonin™ Pulse Oximetry Sensor (Nonin Medical, Plymouth, MN) to measure arterial oxygen blood saturation (SpO₂) at the cranium level. The forehead area above each participant's left eye was initially cleaned with a small alcohol wipe and allowed to dry. A Nonin™ 8000H Pulse Oximetry Sensor holder was affixed to the participant's forehead above the left eye using an adhesive ring and was secured with two additional strips of surgical tape. The Nonin™ 8000R Pulse Oximetry Sensor was then connected to the holder. The sensors were turned on, and data collection was confirmed immediately prior to the beginning of the session.

2.3 Performance tests, questionnaires, and physiological measurements

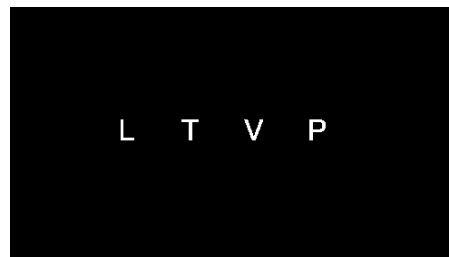
The following is a list of cognitive tasks, questionnaires, and physiological measurements included in the study.

2.3.1 Match to Sample Task (DMST) (NTI ATSTM) is designed to assess short-term spatial memory and pattern recognition skills by quickly and accurately choosing a test stimulus that is identical to a stimulus presented previously. In this task, the participant was shown a single 5 by 5 matrix that was centered on the screen. The matrix cells consisted of three colors (blue, grey, and purple). The number of cells of each color was randomly determined for each stimulus. After viewing the sample stimulus for up to 60 seconds to commit the stimulus to memory, the participant clicked the left or right mouse button to initiate a delay. During the delay, an interference task, which consisted of a series of four letters, appeared horizontally in the middle of the screen (Figure 1, middle). The participant responded with the right mouse button if the letters contained a vowel and with the left button if the letters did not contain a vowel. Following the participant's interference task response, the memory test began. This test consisted of two matrices of the same level as the previously displayed matrix, side by side on the screen (Figure 1). One of the matrices was identical to the previously-presented standard stimulus, while the other was different. The difference was always in the location of the cell(s), not the color of the matrix. Thus, if the lower right cell of the standard is blue, the different matrix might have the position of that cell and a grey cell elsewhere in the matrix swapped. In no case was the number of grey cells altered. The process of swapping rather than replacing ensured that the color ratios of the two stimuli remained the same. On half of the trials, the correct test stimulus was presented on the left side of the screen, and on half the trials, the correct test stimulus was presented on the right side of the screen (Figure 1, bottom). The position of the correct stimulus was randomized across all participants. The participant's task was to select the test stimulus that matched the standard by pressing the corresponding button (left or right) on the mouse. Following the participant's response, the screen was cleared and the standard stimulus for the next trial was presented. Data analyzed for this task included the response (either correct or

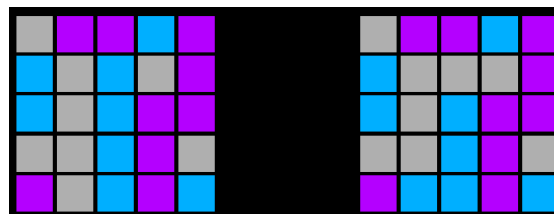
incorrect) and the reaction time to each response. This test took approximately 10 minutes to complete.



Sample Stimulus



Sample Interference Task



Sample Test Stimulus

Figure 1. The Match-to-Sample test.

2.3.2 Profile of Mood States (POMS) is a 65-item questionnaire used to measure affect on 6 scales: tension-anxiety, depression-dejection, anger-hostility, vigor-activity, fatigue inertia, and confusion-bewilderment (McNair, Lorr, & Droppleman, 1981). The questionnaire was digitally administered immediately after the memory task during both the consent/training and test days. Time to complete was less than 5 minutes.

2.3.3 Visual Analogue Scale (VAS) consists of eight 100 mm lines centered over the adjectives “alert/able to concentrate,” “anxious,” “energetic,” “feel confident,” “irritable,” jittery/nervous,” “sleepy,” and “talkative” (Penetar *et al.*, 1993). At the extremes of each line, “not at all” and “extremely,” were printed. Participants indicated the degree to which each adjective corresponded to their current state by clicking the mouse cursor along the rating scale. Scores (in mm) consisted of the distance of the participant’s mark from the left end of the line. The questionnaire was digitally administered immediately after the POMS during both the consent/training and test days. Time to complete was less than 5 minutes.

2.3.4 Side Effects Questionnaire included of a list of potential symptoms associated with long hours awake and/or increased CO₂ exposure (e.g., headache, tremor, anxiety, etc.). Participants indicated whether and to what degree they experienced a particular symptom. Responses were made on a 5-point scale, ranging from “not at all” to “extremely.” The questionnaire was digitally administered immediately after the VAS during both the consent/training and test days. Time to complete was under 5 minutes.

2.4 Participants

Participation was open to military men and women between the ages of 21 and 55. All participants were screened for inclusion/exclusion criteria prior to enrollment in the study. Exclusion criteria included the following: daily consumption of more than 300 mg of caffeine; living at an altitude above 5000 feet; consumption of more than 14 alcoholic drinks per week; diagnosis of any condition producing wheezing or dyspnea; a history of claustrophobia; loss of a significant amount of blood within 30 days of testing; high blood pressure; anemia; heart or circulatory disease; a history of significant psychiatric, neurological, or sleep-related problems; tobacco use within the last 6 months; insufficient sleep for the 3 days prior to the study days; and use of any medication (prescription or over the counter). Additionally, women who were pregnant or trying to become pregnant could not participate in the study. Following the informed consent, but prior to enrollment in the study, women participants completed a precautionary over-the-counter urine pregnancy test. Participants were compensated for their time and effort.

2.5 Duration of study

Participation in this study consisted of five visits to the laboratory. During the first visit, participants signed the informed consent agreement. Additionally, all participants were screened for inclusion/exclusion criteria prior to enrollment in the study. Upon enrollment in the study, participants completed training on the cognitive tasks and were scheduled for the in-house portions of the study.

Four in-house test days were scheduled for each participant. Before each test day, participants were required to obtain at least 7.5 hours in bed with at least 7 hours of sleep each night for the 3 nights immediately preceding the in-house portion of the study, with a rise time of 0600 in the morning, regardless of the condition of the testing day (rested, rested+CO₂, sleep deprived, and sleep deprived+CO₂). Participants were instructed to not nap between wake-up time and bedtime. Participants' wrist monitor data were checked for compliance with the sleep requirements prior to beginning the rested test days; compliance was checked the morning before their overnight sleep deprivation period to avoid unnecessary travel to the lab if they did not comply with sleep requirements. Two in-house sessions occurred during the day with participants arriving at the lab around 0800 with testing beginning at 0900. One of the sessions was rested only (the baseline condition), with the other being rested plus CO₂ exposure. Two additional in-house sessions occurred during the day but after the participants were awake for 28 hours. The participants arrived at the lab around 2200 the night before the test days and remained in the lab with staff members to ensure they stayed awake throughout the night. As in the rested conditions, testing began at 0900. One of the sessions was sleep deprived only (SD) with the other sleep deprived plus increased CO₂ exposure (SDGas).

Conditions were counterbalanced and separated by no less than 3 days, but no more than 21 days. At the end of each test condition, participants were dismissed and instructed on the requirements for the next test phase. They were not allowed to drive themselves home following the sleep-deprived conditions but could drive themselves home after the rested conditions.

2.6 Data Collection

All participants came to the NAMRU-D Fatigue Assessment and Countermeasures (FAC) lab for all phases of the study. On the first day, participants were consented and enrolled in the study if all exclusion and inclusion criteria were met. Following enrollment in the study, participants completed the questionnaires (Horne and Östberg MEQ, NEO-Pi-R™, and the ESS,), and 3 Match-to-Sample test training sessions (or as many as needed) to ensure stable performance was reached before test sessions. Participants were fitted with the mask to obtain correct fit and to assess trouble wearing it (e.g., claustrophobia, sensitivity). Prior to dismissal, participants were scheduled for their test days and given the wrist monitor before returning for their first in-house test day.

On the first test day (Day 1), participants arrived at the FAC lab at approximately 0800 to complete all intake procedures and for familiarity on the cognitive test. The actigraph was checked to be sure participants met the sleep requirements (at least 7.5 hours in bed with at least 7 hours of sleep per night for 3 nights immediately preceding the in-house portion of the study, and a rise time of 0600 the morning of the training day). If the sleep requirements were not met the first of the three nights, but were met the second two nights, the participant was given the option of obtaining the required sleep the next night. If the sleep requirements were met this next night, the participant was enrolled in the data collection portion of the study. If the wrist monitor malfunctioned during participants' activity away from the lab, sleep time was assessed from the sleep diary. Since the participants were not aware that the activity monitored malfunctioned, the accuracy of the sleep diary was assumed to be high since they knew their sleep/wake activity was monitored.

Days 2, 3, and 4 were set up in a similar fashion as Day 1. Generally, the four test conditions were the same except for exposure to CO₂ and time awake prior to testing. Although the rested and sleep deprivation conditions could not be blinded, the test gas was single-blinded to the participants. To maintain consistency, participants wore the same equipment during all conditions. Each was fit with a standard mask (Hans Rudolph, Shawnee, KS) and head harness and tested in the same room each time. The test gas (breathing air or breathing air with 3% CO₂) was drawn into the inspiratory port through large bore respiratory tubing, either from the room or from a reservoir at ambient pressure. The expiratory port was open to the room. Regardless of the test gas, the tubing was connected to a large respiratory-quality bag or other reservoir. The reservoir was refilled by bleeding gas into it from a cylinder of compressed test gas through an appropriate regulator. The reservoir served to ensure that no pressure other than the weight of the bag or equivalent was transmitted downstream.

In order to measure end tidal CO₂ (PETCO₂), a gas sample line was inserted into the mask near the front of the participant's lips to draw a continuous small sample of gas into a fast-response analyzer. This permitted the measurement of inhaled and exhaled gas with a minimum of mixing within the mask's dead space. The analyzer signal for CO₂ content of the gas was sampled at 100

Hz and was displayed in real time to permit a visual check of signal fidelity and of participant safety. Signals were accepted if the inspiratory gas was detected clearly, and if there was a clean transition from expired CO₂ to inspired, that is, a “shark fin” pattern on the capnogram. If this pattern was not detected, the sample line position was adjusted for a clearer reading. In post processing of acceptable gas tracings, end-tidal CO₂ (the CO₂ just prior to the inspiratory drop) was extracted on a breath-by-breath basis.

Inspiratory flow was measured using a respiratory pneumotachometer (Hans Rudolph, Shawnee, KS) in the inspiratory line. The flow meter was positioned several tube diameters away from both the valve and reservoir to avoid large disturbances to the flow. The flow signal provided measurements of tidal volume (V_T) and minute ventilation (V_e). Respiratory frequency could be obtained from either the flow or the gas concentration signal.

Participants completed the four experimental conditions of the study in separate phases, with the order being counterbalanced among participants. For all conditions, participants were monitored by at least one research staff member from the start of the test session to the time that the participant was approved to leave or escorted home, except for short periods needed for personal hygiene or use of restroom. Throughout each testing condition, sensors measured the delivered oxygen (O₂) and CO₂ levels. The participants’ heart rate (HR), respiratory rate (RR), and blood oxygen saturation (SpO₂) were continuously monitored to maintain safe limits. The study space was ventilated to minimize the possible accumulation of study gases.

Each of the four conditions is described below:

Daytime well-rested condition (Baseline): Participants arrived at the lab at 0800 after which the sleep amount/timing was checked and a urine pregnancy test for women was completed. Participants then donned their mask and monitoring equipment and acclimated to the equipment for approximately 5 minutes prior to the beginning of the test. Following acclimation, the participant wore the mask for 10 minutes while breathing bottled air in order to maintain consistency of procedures for all conditions. Following the 10-minute “exposure” period, testing on the memory task began and exposure to room air continued. Upon completion of the memory task, the participant answered the mood and side effects questionnaires. The entire testing sequence lasted approximately 15 minutes. A 5-minute break was given after the test session. Participants remained seated at the testing station, with the mask and monitoring equipment in place. After the 5-minute break, the second test session began. This test session was the same as the first test session with the exception of the 10-minute exposure period: the participant completed the memory task and questionnaires while continuing to breathe bottled air through the mask. Following a 5-minute break after the second test session, a third test session began and was the same as the second test session. After the last test session, the participant and researcher removed the mask and all sensors. The participant was debriefed and dismissed with instructions on when to return for the next test session. The schedule is shown in Table 1.

Table 1. Testing schedule for the daytime well-rested condition (Baseline)

Approximate Time	Event
0800	Arrive at Laboratory Check sleep diary and give pregnancy test as appropriate
0900 – 0915	Don mask (5-min acclimation); 10-min pre-test bottled air
0915 – 0930	Test Session 1 (room air only) (memory task; questionnaires)
0930– 0935	Break (5 minutes with mask on bottled air)
0935 – 0950	Test Session 2 (memory task; questionnaires; mask on bottled air)
0950 –0955	Break (5 minutes with mask on breathing bottled air)
0955 – 1010	Test Session 3 (memory task; questionnaires; mask on breathing bottled air)
1010	Remove gear; debrief/dismiss

Daytime well-rested exposure condition (3% CO₂): Participants arrived at the lab at 0800 after which the sleep amount/timing was checked and a urine pregnancy test for women was completed. Participants then donned their mask and monitoring equipment and acclimated to the equipment for 5 minutes prior to the beginning of the exposure period and testing. Following acclimation, the participant breathed bottled air containing 3% CO₂ for 10 minutes after which testing on the memory task began. Exposure to the bottled air containing 3% CO₂ continued. Upon completion of the memory task, the participant completed the mood and side effects questionnaires. The testing sequence lasted approximately 15 minutes. A 5-minute break was given after the test session; the participant remained seated at the testing station keeping the mask and monitoring equipment in place. After the break, the second test session began and was the same as the first test session, with the exception of the 10-minute exposure period, and the participant began the memory task and questionnaires. No additional CO₂ was administered during this test session, but the participant continued to wear the mask, breathing only bottled room air. Following a 5-minute break after the session, a third test session began and was the same as the second test session. After the last test, the participant and researcher removed the testing equipment. The participant was debriefed and dismissed with instructions on when to return for the next test session. The schedule is shown in Table 2.

Table 2. Testing schedule for the daytime well-rested exposure condition (3% CO₂)

Approximate Time	Event
0800	Arrive at Laboratory Check sleep diary and give urine pregnancy test as appropriate
0900 – 0915	Don mask (5-min acclimation); 10-min pre-test bottled air containing 3% CO ₂ exposure
0915 – 0930	Test Session 1 (continuing CO ₂ exposure) (memory task; questionnaires)
0930 – 0935	Break (5 minutes with mask on breathing bottled air)
0935 – 0950	Test Session 2 (memory task; questionnaires; mask on breathing bottled air)
0950 – 0955	Break (5 minutes with mask on breathing bottled air)
0955 – 1010	Test Session 3 (memory task; questionnaires; mask on breathing bottled air)
1010	Remove gear/debrief/dismiss

Overnight only condition (SD): Participants brought their wrist monitor to the lab between 0800 and 1200 on the day of overnight testing to confirm compliance with the sleep amount/timing. A urine pregnancy test was administered to women. If the sleep requirements were met, the participant was instructed to return to the lab at 2300. During the overnight period, participants remained awake in the lab accompanied at all times by research staff in order to ensure they remained awake. Activities during this time included reading, watching movies, playing video games, and/or quiet conversation. At approximately 0900, participants donned their mask and equipment and acclimated to the equipment for approximately 5 minutes prior to the beginning of the exposure period and testing. Following acclimation, the participant breathed bottled room air for 10 minutes after which testing on the memory task began. Upon completion of the task, the participant answered the mood and side effects questionnaires. The testing sequence lasted approximately 15 minutes. A 5-minute break was given after the test session, and the participant remained seated at the testing station, keeping the mask and monitoring equipment in place. Following the 5-minute break, the second test session began and was the same as the first test session, with the exception of the 10-minute exposure period. The participant continued to wear the mask and equipment, breathing only bottled air, during the memory test and questionnaires. After the second test session, a 5-minute break was given. The participant continued to wear the mask and monitoring equipment. Following the break, a third test session began and was the same as the second test session. After the last test, the participant and researcher removed all testing equipment. The participant was debriefed and dismissed with instructions on when to return for the next test session. The participant was not allowed to drive home and was instructed to not operate equipment until at least 8 hours of sleep were obtained. The schedule is shown in Table 3.

Table 3. Testing schedule for the overnight only condition (SD)

Approximate Time	Event
0800	Arrive at Laboratory Check sleep diary and give urine pregnancy test as appropriate
2300	Arrive at Laboratory to remain awake until testing begins
0900 – 0915	Don mask (5-min acclimation); 10-min pre-test bottled air
0915 – 0930	Test Session 1 (room air only) (memory task; questionnaires)
0930 – 0935	Break (5 minutes with mask on breathing bottled air)
0935 – 0950	Test Session 2 (memory task; questionnaires; mask on breathing bottled air)
0950 – 0955	Break (5 minutes with mask on breathing bottled air)
0955 – 1010	Test Session 3 (memory task; questionnaires; mask on breathing bottled air)
1010	Remove gear/debrief/dismiss

Overnight + exposure condition (SDCO₂): Participants brought their wrist monitor to the lab between 0800 and 1200 the day of overnight testing to confirm compliance to the sleep amount/timing as instructed. The urine pregnancy test was administered to women. If the sleep requirements were met, the participant was instructed to return to the lab at 2300. During the overnight period, participants remained awake in the lab accompanied at all times by research staff in order to ensure they remained awake. Activities during this time included reading, watching movies, playing video games, and/or quiet conversation. At approximately 0900, participants donned their mask and equipment and acclimated to the equipment for approximately 5 minutes prior to the beginning of the exposure period and testing. Following acclimation, the participant breathed bottled air containing 3% CO₂ for 10 minutes after which testing on the memory task began. Upon completion of the task, the participant answered the mood and side effects questionnaires. The testing sequence lasted approximately 15 minutes.

A 5-minute break was given before the next test session. During the 5-minute break, participants remained seated at the testing station, keeping the mask and monitoring equipment in place. Following the 5-minute break, the gas was switched from air containing 3% CO₂ to bottled air and the second test session began. This second test session was the same as the first test session, with the exception of the 10-minute exposure period. The participant continued to wear the mask and equipment, but breathing only bottled air, while performing the memory task and answering the questionnaires. After the second test session, a 5-minute break was given. Following the 5-minute break, a third test session was given and was the same as the second test session. After the last test session, the participant and researcher removed all testing equipment. The participant was debriefed and dismissed with instructions on when to return for the next test session. The participants were not allowed to drive home and were instructed to not operate equipment until at least 8 hours of sleep were obtained. The schedule is shown in Table 4.

Table 4. Testing schedule for the overnight + exposure condition (SDCO₂)

Approximate Time	Event
0800	Arrive at Laboratory Check sleep diary and give urine pregnancy test as appropriate
2300	Arrive at Laboratory to remain awake until testing begins
0900 – 0915	Don mask (5-min acclimation); 10-min pre-test bottled air containing 3% CO ₂ exposure
0915 – 0930	Test Session 1 (continuing CO ₂ exposure) (memory task; questionnaires)
0930 – 0935	Break (5 minutes with mask on but breathing bottled air)
0935 – 0950	Test Session 2 (memory task; questionnaires)
0950 – 0955	Break (5 minutes with mask on but breathing bottled air)
0955 – 1010	Test Session 3 (memory task; questionnaires; mask on breathing bottled air)
1010	Remove gear/debrief/dismiss

3.0 RESULTS

The SPSS statistical software package (Version 25; International Business Machines Corporation®, Armonk, NY) was used to analyze the Match to Sample task and questionnaire data. The analysis compared the performance on the memory task and subjective mood from the POMS and VAS to determine whether the stressor alone (sleep deprivation or CO₂ exposure) had a different effect on memory and mood than the stressors combined (sleep deprivation with CO₂ exposure). Analyses were conducted to directly test the stated hypotheses.

Hypotheses tested:

- 1) Individuals exposed to increased CO₂ while rested will perform worse on the memory task than those not exposed to increased CO₂.
- 2) Individuals exposed to increased CO₂ after 28 hours of continuous wakefulness will perform worse on the memory task than those not exposed to increased CO₂ (ambient levels of CO₂) but continuously awake for 28 hours.

A sample of 6 individuals enrolled in the protocol, with 3 successfully completing all data collection periods; 1 completed all but the sleep deprivation and gas exposure condition; 1 completed the baseline and sleep deprivation conditions; 1 was dismissed due to inadequate sleep prior to the data collection day. The participants who dropped out of the study discontinued participation due to COVID-19 restrictions or weather complications. All participants were active-duty military between the ages of 21 and 30 ($M = 25.83$, $SD = 3.89$; 4 male, 2 female). The demographic characteristics of the 6 volunteers and the 4 included in the data analysis are shown in Table 5. One participant did not complete the Horne-Östberg MEQ, so there are only 5 complete questionnaires and 3 for the completed data set.

Table 5. Demographics of study participants.

	N	Age	Horne-Östberg MEQ Score (N=5; 3)	NEO-PI-R Neuroticism Score	NEO-PI-R Extraversion Score	Epworth Sleepiness Score
Enrolled in study	6	25.83 (3.89)	57.40 (7.96)	56.50 (3.86)	119.83 (11.58)	4.50 (2.29)
Included in analyses	4	26.00 (3.54)	57.00 (9.42)	54.75 (2.28)	118.25 (11.54)	4.50 (2.60)

3.1 Data Analysis

The design of the study yielded four conditions (baseline [BL], gas exposure alone [Gas], sleep deprivation alone [SD], and sleep deprivation with gas exposure [SDGas]) to investigate the individual and combined effects of sleep deprivation and exposure to increased CO₂ on memory and mood. Difference scores were calculated for the data analysis; the scores from each condition were subtracted from the baseline scores (BL-condition), resulting in three scores for each condition (BL-Gas, BL-SD, and BL-SDGas). For each of these difference scores, a two-way repeated-measures analysis of variance (ANOVA) was conducted with condition (3) and session (3) as the repeated factors. Each analysis yielded three *F*-tests: a main effect for condition, a main effect for session, and a condition by session interaction. Mauchly's Test of Sphericity was used to test the assumption of sphericity. When the sphericity assumption was violated, the Huynh-Feldt correction and *F*-statistic are reported. Statistically significant interactions were followed with an analysis of simple effects and Fisher's Least Significant Difference (LSD) as appropriate. Statistically significant main effects were followed with LSD. Since one participant completed three of the four conditions (baseline, gas alone, and sleep deprivation alone), the data from the combined condition (gas with sleep deprivation) were estimated using the mean of the session from all participants, giving a total of 4 participants for the analysis. Given the small sample size and the exploratory nature of this study, an alpha level of $p \leq 0.1$ was used.

3.1.1 Match-to-Sample Task

The memory task data included metrics for number correct (N_{Corr}), number incorrect (N_{Incorr}), number correct reaction time (CorrRT), and number incorrect reaction time (InCorrRT). Scores from each condition were subtracted from the baseline condition, yielding a difference from baseline score (BL - Score).

N_{Corr}: The ANOVA did not reveal a statistically significant interaction between condition and session ($F(4, 12) = 1.16, p = .377, \eta_p^2 = .28$). The main effect for condition was not statistically significant as well ($F(2, 6) = 2.31, p = .180, \eta_p^2 = .44$).

The assumption of sphericity was violated for the session main effect ($\chi^2(2) = 4.77, p = .092, \varepsilon = .56$). The main effect for session was statistically significant ($F(1.12, 3.37) = 7.48, p = .062, \eta_p^2 = .71$). Analysis of simple effects indicated that the number correct during session 1 was significantly higher than sessions 2 and 3 ($p = .005$ and $.036$, respectively), with no difference

between sessions 2 and 3. The means (*SE*) for sessions 1 through 3 are 4.56 (.56), 2.83 (.78), and 2.23 (.22), respectively. The data are summarized in Figure 2.

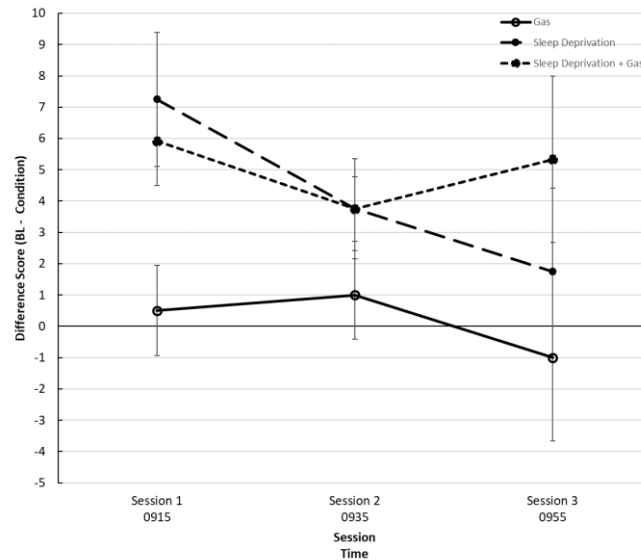


Figure 2. Match-to-Sample number correct; the graph represents the baseline difference scores for each condition at each session.

CorrRT: The ANOVA revealed there was not a statistically significant interaction between condition and session ($F(4, 12) = 1.06, p = .418, \eta_p^2 = .26$). The assumption of sphericity was violated for the condition main effect ($\chi^2(2) = 5.16, p = .076, \varepsilon = .55$). There was not a statistically significant main effect for condition ($F(1.10, 3.30) = 0.57, p = .517, \eta_p^2 = .160$) nor for the main effect of session ($F(2, 6) = 1.12, p = .388, \eta_p^2 = .27$). These results are illustrated in Figure 3.

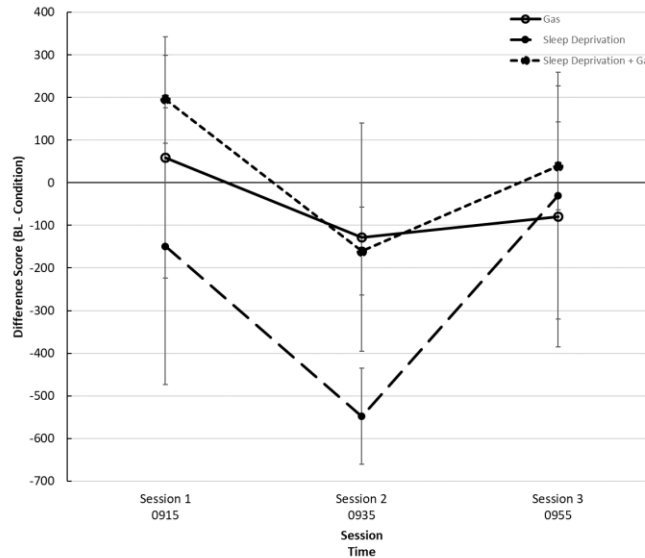


Figure 3. Match-to-Sample reaction time for number correct; the graph represents the baseline difference scores for each condition at each session.

NIncorr: The ANOVA did not show a statistically significant interaction between condition and session ($F(4, 12) = 1.16, p = .377, \eta_p^2 = .28$). There was not a statistically significant main effect for condition ($F(2, 6) = 2.31, p = .180, \eta_p^2 = .44$). The assumption of sphericity was violated for the session main effect ($\chi^2(2) = 4.76, p = .092, \varepsilon = .56$). There was a statistically significant main effect for session ($F(1.12, 3.37) = 7.48, p = .062, \eta_p^2 = .71$). Post hoc analyses indicated that the reaction time during session 1 was statistically worse than sessions 2 ($p = .006$) and 3 ($p = .036$), regardless of condition. These results are illustrated in Figure 4.

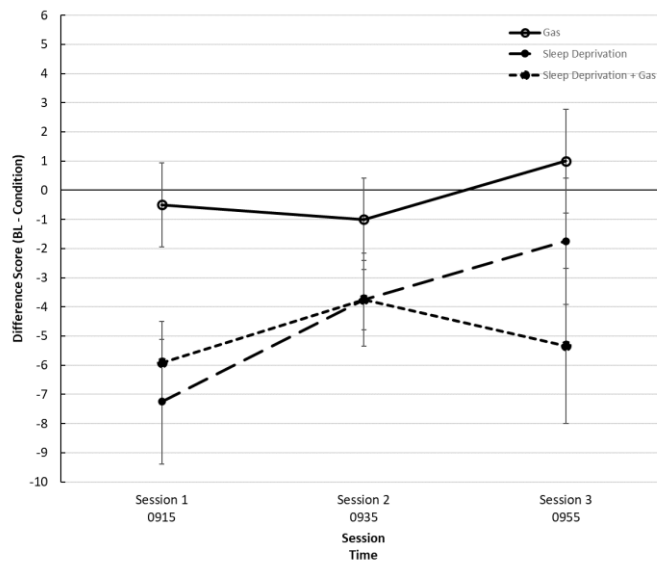


Figure 4. Match-to-Sample number incorrect; the graph represents the baseline difference scores for each condition at each session.

IncorrRT: The ANOVA revealed that there was not a statistically significant interaction between condition and session ($F(4, 12) = 0.45, p = .772, \eta_p^2 = .13$). There was not a statistically significant main effect for condition ($F(2, 6) = 1.28, p = .343, \eta_p^2 = .30$) or session ($F(2, 6) = 1.04, p = .409, \eta_p^2 = .26$). These results are illustrated in Figure 5.

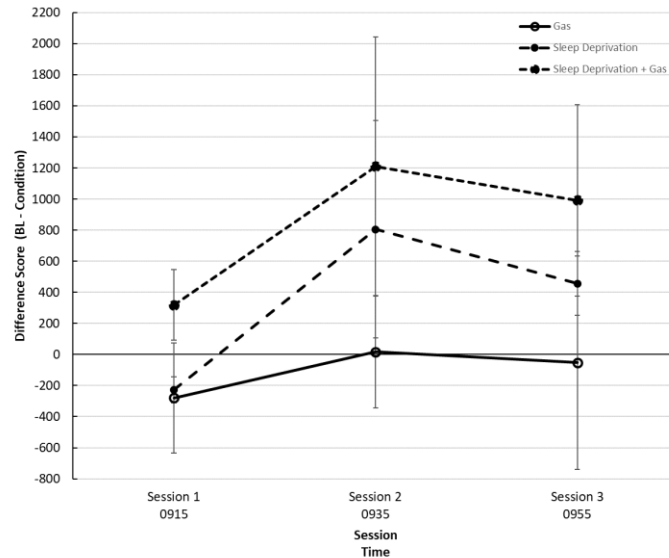


Figure 5. Match-to-Sample reaction time for number incorrect; the graph represents the baseline difference scores for each condition at each session.

3.1.2 Mood assessments

Mood was measured with the Profile of Mood States (POMS) and the Visual Analogue Scale (VAS). Scores from each condition were subtracted from the baseline condition, yielding a difference from baseline score (BL - Score).

3.1.2.1 Profile of Mood States (POMS): All six factors from the *POMS* – Tension, Depression, Anger, Vigor, Fatigue, Confusion, and the Total Mood Disturbance score – were calculated. However, due to technical errors, only two participants' data were collected, therefore, these data were not analyzed because of the small sample size.

3.1.2.2 Visual Analogue Scale (VAS): All eight adjectives from the *VAS* – alertness/able to concentrate, anxious, energetic, confident, irritable, jittery/nervous, sleepiness, and talkative – were analyzed, and the results from these analyses are presented below. Four participants are included in this analysis with the data from one participant's combined condition (gas with sleep deprivation) estimated using group means for each session.

Alertness/able to concentrate: The ANOVA did not show a statistically significant interaction between condition and session ($F(4, 12) = 1.56, p = .246, \eta_p^2 = .34$). There was a statistically significant main effect for condition ($F(2, 6) = 11.95, p = .008, \eta_p^2 = .80$). Analysis of simple

effects showed statistically significant differences between BL-Gas and BL-SD ($p = .036$) and between BL-Gas and BL-SDGas ($p = .033$). BL-SD and BL-SDGas were significantly worse than BL-Gas. The two sleep deprivation conditions did not differ from each other. The means (SE) for BL-Gas, BL-SD, and BL-SDGas are 0.17 (7.27), 48.33 (8.11), and 51.86 (7.38), respectively. There was not a statistically significant main effect for session ($F(2, 6) = 0.57$, $p = .596$, $\eta_p^2 = .16$). The means (SE) for sessions 1 through 3 are 31.14 (4.24), 36.56 (5.62), and 32.67 (3.39), respectively. These results are illustrated in Figure 6.

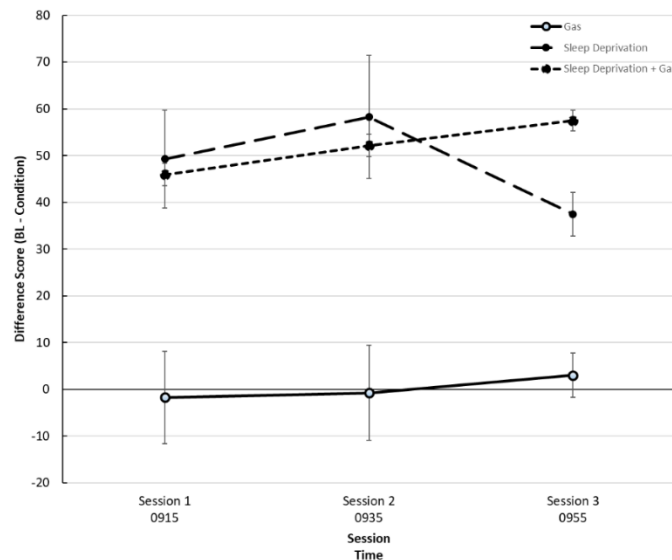


Figure 6. VAS Alertness/able to concentrate scores; the graph represents the baseline difference scores for each condition at each session.

Anxious: The ANOVA revealed a statistically significant interaction between condition and session ($F(4, 12) = 4.38$, $p = .021$, $\eta_p^2 = .59$). Post hoc analyses showed statistically significant differences at session 1 between the BL-Gas and BL-SD conditions ($p = .072$) and between the BL-Gas and BL-SDGas conditions ($p = .056$); scores were significantly lower for the BL-Gas condition than the other two conditions. At session 2, scores were significantly lower for the BL-Gas than the BL-SDGas condition ($p = .080$), but no differences between the BL-SD condition and the other conditions. No differences occurred between any of the conditions at session 3. Analyses comparing the sessions with each condition indicated that within the BL-Gas condition, scores at session 1 were significantly lower than at session 2 ($p = .099$). No differences between the sessions occurred within the other two conditions. This is illustrated in Figure 7.

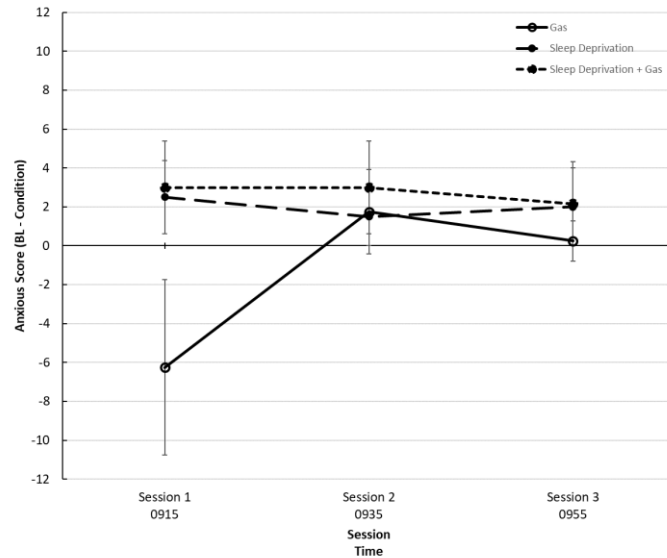


Figure 7. VAS Anxious scores; the graph represents the baseline difference scores for each condition at each session.

There was a statistically significant main effect for condition ($F(2, 6) = 16.34, p = .004, \eta_p^2 = .85$). Post hoc analyses revealed statistically significant differences between the BL-Gas and BL-SD conditions ($p = .035$) and between the BL-Gas and BL-SDGas conditions ($p = .015$). The BL-Gas condition scores were significantly lower than the other two conditions; this effect was due to the low scores at session 1. The means (*SEs*) for the BL-Gas, BL-SD, and BL-SDGas conditions are 1.42 (2.27), 2.00 (1.79), and 2.72 (2.30), respectively.

There was a statistically significant main effect for session ($F(2, 6) = 1.87, p = .234, \eta_p^2 = .38$). However, post hoc analyses did not reveal any statistically significant differences between any of the sessions. The means (*SEs*) for sessions 1 through 3 are -0.25 (2.77), 2.08 (2.00), and 1.47 (1.70), respectively.

Confidence: The ANOVA revealed that there was not a statistically significant interaction between condition and session ($F(4, 12) = 1.01, p = .439, \eta_p^2 = .25$). There was a statistically significant main effect for condition ($F(2, 6) = 7.00, p = .027, \eta_p^2 = .70$). Post hoc analyses showed statistically significant differences between the BL-Gas and BL-SDGas conditions ($p = .017$). The BL-Gas condition was significantly lower than the BL-SDGas condition. These means (*SEs*) for the BL-Gas, BL-SD, and BL-SDGas conditions are 3.67 (4.39), 21.03 (8.79), and 37.17 (2.26), respectively.

There was also a statistically significant main effect for session ($F(2, 6) = 4.87, p = .055, \eta_p^2 = .62$). Post hoc analyses revealed a statistically significant difference between sessions 1 and 3, with session 1 showing lower scores than session 3 ($p = .003$). The means (*SEs*) for sessions 1 through 3 are 15.50 (3.06), 19.69 (4.71), and 26.72 (2.90), respectively. These results also are illustrated in Figure 8.

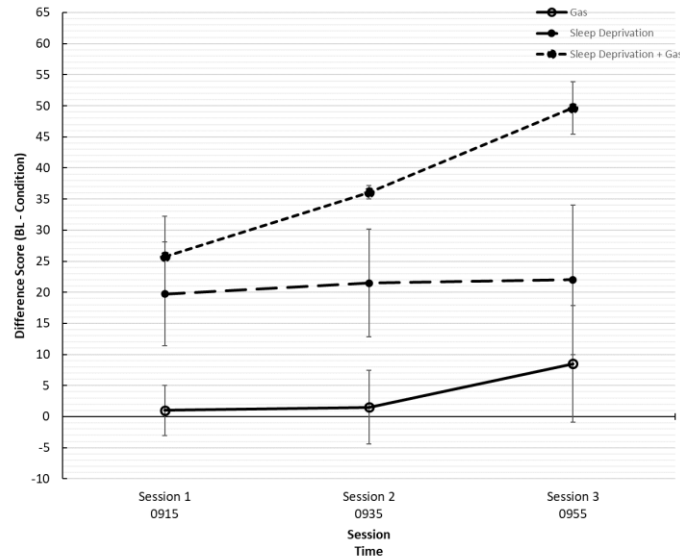


Figure 8. VAS Confidence scores; the graph represents the baseline difference scores for each condition at each session.

Energetic: There was not a statistically significant interaction between condition and session ($F(4, 12) = 0.46, p = .764, \eta_p^2 = .13$). There was a statistically significant main effect for condition ($F(2, 6) = 8.53, p = .018, \eta_p^2 = .74$). Post hoc analyses revealed a statistically significant difference between the BL-Gas and BL-SD conditions ($p = .061$) and between the BL-Gas and BL-SDGas conditions ($p = .058$). The means (*SEs*) for BL-Gas, BL-SD, and BL-SDGas are -8.33 (3.25), 30.50 (12.02), and 40.61 (15.85), respectively.

There was not a statistically significant main effect for session ($F(2, 6) = 0.12, p = .890, \eta_p^2 = .04$). The means (*SEs*) for sessions 1 through 3 are 22.52 (7.84), 22.78 (16.12), and 17.47 (8.93), respectively. These results also are illustrated in Figure 9.

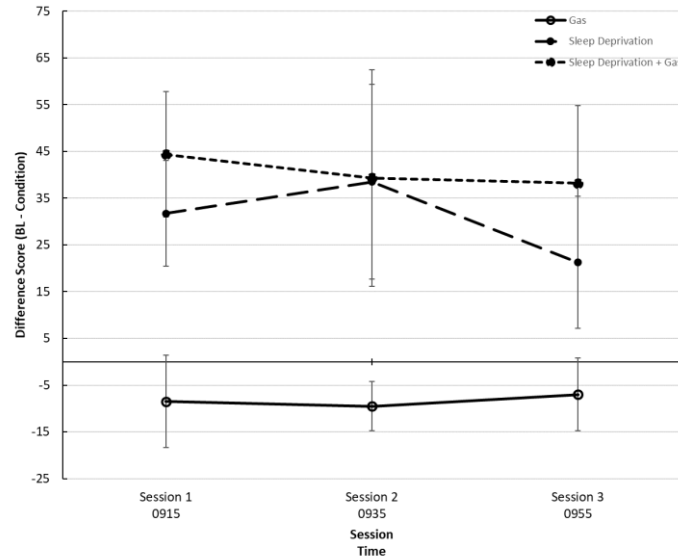


Figure 9. VAS Energetic scores; the graph represents the baseline difference scores for each condition at each session.

Irritable: There was not a statistically significant interaction between condition and session ($F(4, 12) = 0.47, p = .756, \eta_p^2 = .14$), nor a statistically significant main effect for condition ($F(2,6) = 0.18, p = .839, \eta_p^2 = .06$) or session ($F(2, 6) = 0.28, p = .766, \eta_p^2 = .09$). These results are illustrated in Figure 10.

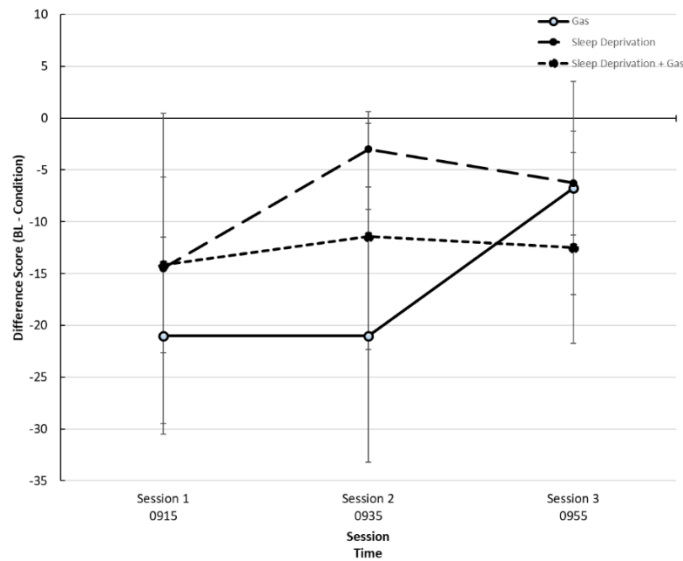


Figure 10. VAS Irritable scores; the graph represents the baseline difference scores for each condition at each session.

Jittery/Nervousness: There was not a statistically significant interaction between condition and session ($F(4, 12) = 1.95, p = .167, \eta_p^2 = .39$). There was no statistically significant main effect

for condition ($F(2, 6) = 0.33, p = .733, \eta_p^2 = .10$) or session ($F(2, 6) = 1.37, p = .324, \eta_p^2 = .31$). These results are illustrated in Figure 11.

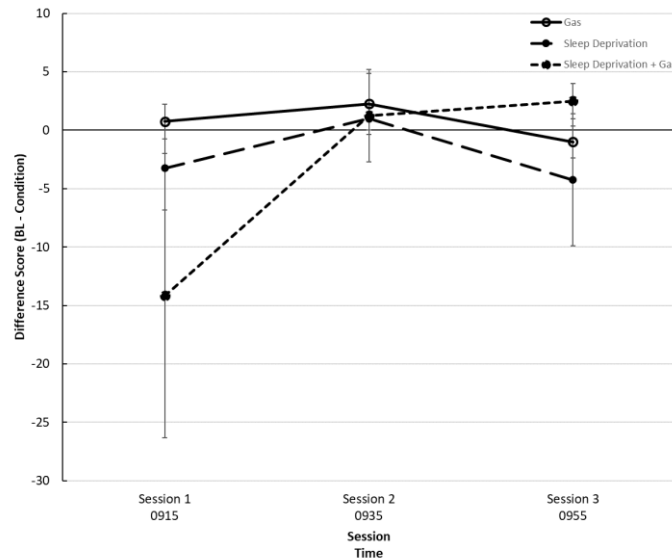


Figure 11. VAS Jittery/Nervousness scores; the graph represents the baseline difference scores for each condition at each session.

Sleepiness: There was not a statistically significant interaction between condition and session ($F(4, 12) = 1.04, p = .429, \eta_p^2 = .26$). The assumption of sphericity was violated for the condition main effect ($\chi^2(2) = 7.58, p = .023, \varepsilon = .51$). There was a statistically significant main effect for condition ($F(1.03, 3.09) = 10.91, p = .044, \eta_p^2 = .78$). Post hoc analyses revealed a statistically significant difference between the BL-Gas and BL-SD conditions ($p = .049$) and between the BL-Gas and BL-SDGas conditions ($p = .041$). The scores for the BL-SD and BL-SDGas conditions were significantly worse than the BL-Gas condition. The means (*SEs*) for the BL-Gas, BL-SD, and BL-SDGas conditions are 10.25 (5.67), -57.42 (17.49), and -53.03 (15.18), respectively.

There was not a statistically significant main effect for session ($F(2, 6) = 0.40, p = .688, \eta_p^2 = .12$). These results are illustrated in Figure 12.

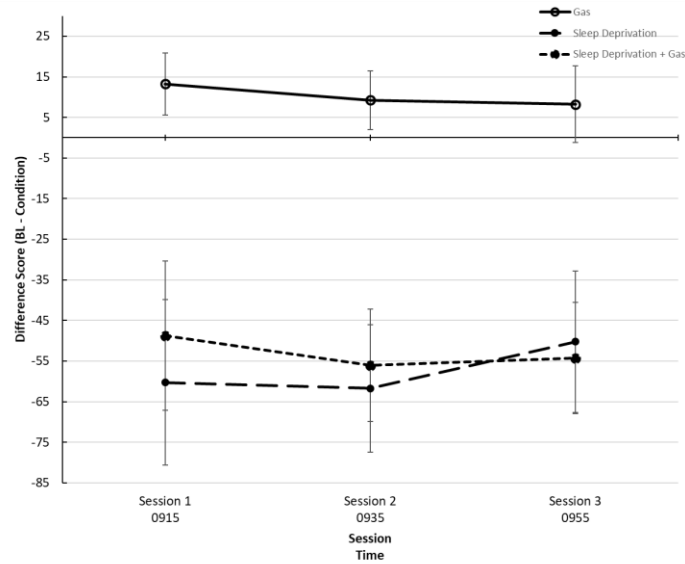


Figure 12. VAS Sleepiness scores; the graph represents the baseline difference scores for each condition at each session.

Talkative: There was a statistically significant interaction between condition and session ($F(4, 12) = 5.20, p = .012, \eta_p^2 = .63$). Analyses of simple effects indicated a difference between BL-SD and BL-SDGas at session 1 ($p = .049$) and session 2 ($p = .076$). These results are illustrated in Figure 13.

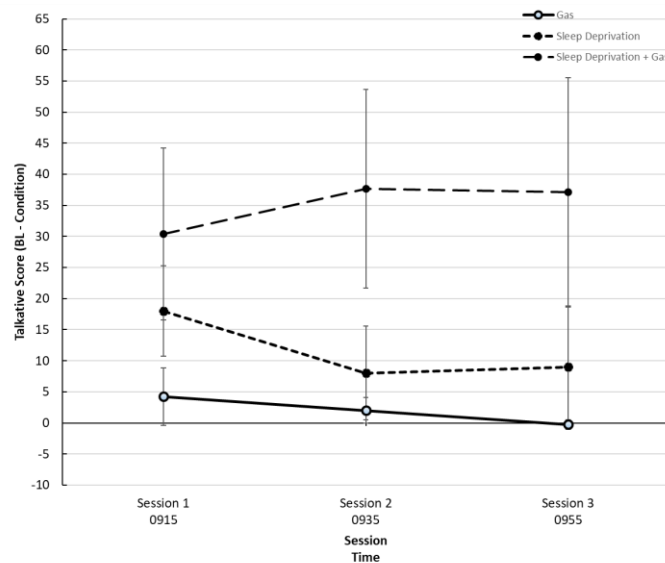


Figure 13. VAS Talkative scores; the graph represents the baseline difference scores for each condition at each session.

There was not a statistically significant main effect for condition ($F(2, 6) = 3.42, p = .102, \eta_p^2 = .53$) or session ($F(2, 6) = 0.18, p = .843, \eta_p^2 = .06$). The means (SEs) for BL-Gas, BL-SD, and

BL-SDGas are 2.00 (1.57), 11.67 (7.53), and 36.08 (16.01), respectively. The means (SEs) for sessions 1 through 3 are 17.56 (4.92), 15.89 (7.41), and 15.31 (9.09), respectively.

3.1.3 Side effects questionnaire (SEQ)

Responses for each adjective from the side effects questionnaire were tallied and presented in Appendix A. No statistical analyses were performed; this questionnaire was for safety monitoring only.

4.0 DISCUSSION AND CONCLUSIONS

The current study investigated the combined effects of increased CO₂ exposure and 28 hours of continuous wakefulness on memory. The combination of these two stressors were compared to a well-rested baseline and each stressor alone (3% CO₂ and 28 hours of continuous wakefulness). Due to the COVID19 pandemic restrictions, data collection was halted for many months; therefore, the final sample size was greatly reduced versus what was proposed or expected. A total of six participants enrolled in the study, with three completing all four phases and four completing at least three phases. The fourth phase for the participant who completed three phases was estimated using the condition mean for each session, resulting in a sample size of four. The data were baseline-corrected in order to have a sample size larger than the number of sessions (BL-condition). Due to the small sample size, the results of the study are considered preliminary.

Analysis of the memory task did not show a statistical difference among the conditions, but the number correct and number incorrect were better at session 1 than the other two sessions, regardless of the condition. Memory task performance during conditions involving sleep deprivation, however, tended to be worse than the gas alone condition, although not statistically significant.

The mood analyses did demonstrate differences among the conditions. Generally, the conditions involving sleep deprivation showed lower subjective levels of alertness, anxiety, confidence and energy; and higher subjective levels of sleepiness. When taken with increased CO₂ exposure, mood disturbances did not reveal a combined effect from the gas and sleep deprivation. Rather, sleep deprivation and combined sleep deprivation with 3% CO₂ conditions revealed a similar subjective experience. The lack of additional mood disturbances during CO₂ exposure highlights the gravity of sleep deprivation on subjective experiences.

Although these were not statistically analyzed, the side-effects questionnaire did indicate some endorsements among all the conditions with those indicating sleepiness, fatigue, etc. endorsed as severe or moderate in the conditions involving sleep deprivation. These effects reinforced the subjective mood questionnaire which also showed the conditions involving long hours of wakefulness had high levels of sleepiness and fatigue.

The present study was conducted in parallel with a rodent study in order to determine if similar results would be found between human and rodent exposure to CO₂ (Protocol F-WA-2019-0187-A). Since humans could not be exposed to high concentrations of the gas, a rodent study was

conducted that exposed rodents to an even higher level of CO₂ (10%) through a modified exposure chamber. The amount of sleep deprivation for the rodents was 4 hours which was achieved by continuous gentle handling to ensure the rodents were awake for the specified time. The human and rodent studies tested the same conditions: 1) rested, ambient CO₂; 2) sleep deprived, ambient CO₂; 3) rested, increased CO₂ exposure; and 4) sleep deprived, increased CO₂ exposure. The human study used a repeated measures design while the rodent study used a group design. Both studies used a task that assessed memory functions. The human study directly tested short-term memory in the Match-to-Sample task; the rodent study tested memory using the Novel Object Recognition (NOR) test that measured the time rodents interacted with a novel object versus a familiar one.

Rodent models, specifically rats, are widely used in basic physiology, pharmacology, toxicology, and neuroscience research. As such, the results of the animal “arm” of this study served to inform the human study. Research indicates that humans begin to experience negative effects at 3% CO₂ (Cutler *et al.*, 1964; Schaefer, Carey, Dougherty, Morgan, & Messier, 1979). Rats have a somewhat higher tolerance for CO₂ than humans (Krohn, Hansen, & Dragsted, 2003). It is not clear from the literature exactly what levels of CO₂ are required to induce behavioral effects, therefore ranges of both amount (5-10% CO₂) and time (15-60 minutes) were selected as part of an initial range-finding study to optimize the exposures used in a subsequent experiment. 30-minute exposures at a CO₂ level of 10% were then used during the second phase of the experiment involving rats that was completed in parallel with this human study. Analyses of the data from the rodent study did not reveal any effects of exposure to increased CO₂ or sleep deprivation with the NOR test, supporting some of the results of the human study that showed no effects of exposure to 3% CO₂ on memory.

Given the preliminary nature of this pilot study, a larger sample size may have shown differences among the conditions, particularly to determine if the combined effects of gas with long hours awake had a synergistic effect on either memory or mood. The 3% CO₂ exposure may have not been sufficient to affect memory. However, the inability of a higher level of CO₂ to show effects in rodent memory supports previous studies that memory is not affected significantly by these levels of CO₂ (Sayers *et al.*, 1987; Selkirk *et al.*, 2010). The human study did show effects of 28 hours of continuous wakefulness on both memory and mood which supports decades of research, but the additional exposure to 3% CO₂ did not lead to further performance or mood declines, supporting the prevailing paradigm.

In order to determine if the combination of exposure to increased CO₂ with long hours of continuous wakefulness continues to be of concern to the operational community, a future study could examine these effects in a larger sample and possibly a higher level of CO₂ and/or longer duration of exposure. The current study did not find effects of CO₂ exposure on memory, but did on other cognitive functions (e.g. reasoning). Future research could focus on various cognitive functions in addition to using a different memory task to determine if the combination of gas exposure with long hours of wakefulness has a synergistic effect on performance.

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Appendix A. Side Effects Questionnaire

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Trouble staying awake	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	3	3	4	4	3	0	0	0	0	0	0
Slight	0	1	1	0	0	1	1	0	0	1	1	0
Moderate	0	0	0	0	0	0	2	3	4	1	0	1
Severe	0	0	0	0	0	0	1	1	0	1	2	2

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Chills	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	3	4	4	3	3	4	4	2	3	3
Slight	0	0	1	0	0	1	1	0	0	1	0	0
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Loss of balance	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	3	3	3	3	3	3
Slight	0	0	0	0	0	0	1	1	1	0	0	0
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Numbness	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	4	4	4	3	3	3
Slight	0	0	0	0	0	0	0	0	0	0	0	0
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Dry mouth	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	2	3	3	4	4	4	3	3	3	2	2	1
Slight	2	1	1	0	0	0	1	1	1	1	1	2
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Nervousness	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	4	4	4	3	3	3
Slight	0	0	0	0	0	0	0	0	0	0	0	0
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Anxiety	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	4	4	4	3	3	3
Slight	0	0	0	0	0	0	0	0	0	0	0	0
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Stomach cramps	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	4	4	4	3	3	3
Slight	0	0	0	0	0	0	0	0	0	0	0	0
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Visual illusions	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	3	2	3	3	3	2
Slight	0	0	0	0	0	0	1	1	1	0	0	1
Moderate	0	0	0	0	0	0	0	1	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Drugged feeling	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	3	4	3	2	2	1	1	1
Slight	0	0	0	0	1	0	0	1	2	1	0	0
Moderate	0	0	0	0	0	0	1	1	0	1	2	2
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Light headed	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	4	4	4	2	2	2
Slight	0	0	0	0	0	0	0	0	0	1	1	1
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Excessive thirst	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	3	3	3	4	4	4	3	3	3	3	3	3
Slight	1	1	1	0	0	0	1	1	1	0	0	0
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Irritability	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	3	4	4	4	4	3	2	2	2
Slight	0	0	0	1	0	0	0	0	1	1	1	1
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Loss of coordination	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	3	2	3	1	2	2
Slight	0	0	0	0	0	0	1	2	1	2	1	1
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Irregular heartbeat	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	4	4	4	2	2	3
Slight	0	0	0	0	0	0	0	0	0	1	1	0
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Tremor	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	4	4	4	3	3	3
Slight	0	0	0	0	0	0	0	0	0	0	0	0
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
General discomfort	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	2	3	2	2	3	3	4	3	4	3	3	2
Slight	2	1	2	2	1	1	0	1	0	0	0	1
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Fatigue	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	2	3	2	3	2	2	0	0	0	1	0	0
Slight	2	1	2	1	2	2	2	0	1	0	1	0
Moderate	0	0	0	0	0	0	1	3	3	1	1	2
Severe	0	0	0	0	0	0	1	1	0	1	1	1

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Boredom	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	2	3	2	2	3	3	2	2	2	1	1	1
Slight	2	1	2	2	1	1	1	1	1	2	1	1
Moderate	0	0	0	0	0	0	1	1	1	0	1	1
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Drowsiness	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	3	3	2	3	2	2	2	1	2	0	0	0
Slight	1	1	2	1	2	2	0	1	0	1	1	0
Moderate	0	0	0	0	0	0	1	1	1	1	0	1
Severe	0	0	0	0	0	0	1	1	1	1	2	2

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Headache	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3*	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	4	2	3	2	2	2
Slight	0	0	0	0	0	0	0	1	1	1	0	0
Moderate	0	0	0	0	0	0	0	0	0	0	1	1
Severe	0	0	0	0	0	0	0	0	0	0	0	0

*Missing data point

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Nausea	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	4	4	4	3	3	2
Slight	0	0	0	0	0	0	0	0	0	0	0	1
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Difficulty concentrating	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	2	3	2	3	3	4	0	0	1	0	0	1
Slight	2	1	2	1	1	0	3	3	2	2	2	0
Moderate	0	0	0	0	0	0	0	1	1	1	1	1
Severe	0	0	0	0	0	0	0	0	0	0	0	1

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Mental depression	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	4	4	4	3	3	3
Slight	0	0	0	0	0	0	0	0	0	0	0	0
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Dizziness with eyes open	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	4	4	4	3	2	2
Slight	0	0	0	0	0	0	0	0	0	0	1	1
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Confusion	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	3	4	4	2	3	1
Slight	0	0	0	0	0	0	1	0	0	1	0	1
Moderate	0	0	0	0	0	0	0	0	0	0	0	1
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Vomiting	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	4	4	4	3	3	3
Slight	0	0	0	0	0	0	0	0	0	0	0	0
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Rapid heart beats	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	4	4	4	4	4	4	3	3	3
Slight	0	0	0	0	0	0	0	0	0	0	0	0
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

	Baseline			Gas Alone			Sleep Deprivation Alone			Sleep Deprivation + Gas		
Dyspnea	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3	Session 1	Session 2	Session 3
None	4	4	4	3	4	4	4	4	4	3	3	3
Slight	0	0	0	1	0	0	0	0	0	0	0	0
Moderate	0	0	0	0	0	0	0	0	0	0	0	0
Severe	0	0	0	0	0	0	0	0	0	0	0	0

N = 4 for Baseline, Gas Alone, and Sleep Deprivation Alone; N = 3 for Sleep Deprivation + Gas