Flashbang Effects: Stress Response

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Prepared for:
Shannon Foley, Ph.D.
Joint Intermediate Force Capabilities Office
Quantico, VA

Prepared by:

Ted Argo, Ph.D.
Greg Rule, P.E.
Nick Brunstad
Mark Espinoza

Applied Research Associates, Inc.
7921 Shaffer Parkway
Littleton, CO 80127

Monika Fleshner, Ph.D.
Robert S. Thompson, Ph.D.

University of Colorado Boulder

The University of Colorado Boulder
3100 Marine Street
Boulder, CO 80309

Nathaniel T. Greene, Ph.D.

University of Colorado Anschutz Medical Campus

The University of Colorado Anschutz
13001 East 17th Place
Aurora, CO 80045

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Ted Argo, Ph.D.; Greg Rule, P.E.; Nick Brunstad; Mark Espinoza (ARA)

Monika Fleshner, Ph.D.; Robert S. Thompson, Ph.D. (CU Boulder); Nathaniel T. Greene, Ph.D. (CU Anschutz)

E-Mail: targo@ara.com

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Applied Research Associates, Inc.
7921 Shaffer Parkway
Littleton, CO 80127

The University of Colorado Boulder
3100 Marine Street
Boulder, CO 80309

The University of Colorado Anschutz Medical Campus
13001 East 17th Place
Aurora, CO 80045

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14. ABSTRACT
Flashbang devices are widely used for degrading the response capability of individuals and small groups in situations such as tactical entry operations, but the required doses of acoustic, ocular, and tactile stimuli that result in effective suppression effects while minimizing risk of injury are not well understood. Each of these stimuli produce a physiological stress response which may cause deficits in performance. This study was designed to evaluate flashbang effectiveness through the impact of an acoustic overpressure at levels below temporary auditory injury thresholds on behavioral measures of stress with animal subjects. Specifically, animal behavior and performance for two acoustic overpressure levels and a control were evaluated under this protocol. Results for this testing show decreased performance for female subjects for a 155 dBP, 0.7 ms duration overpressure relative to control conditions or a 143 dBP, 0.7 ms duration overpressure. These results support recommendations for future flashbang research and development activities towards optimizing stimuli to achieve the desired suppressive effects while minimizing the risk of injury.

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Flashbang; Auditory Effects; Behavioral Effects; Startle

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Executive Summary

Introduction

Flashbang grenades are typically employed to distract and temporarily disable an adversary, gain surprise advantage in a physical confrontation, or disrupt communication. These devices are designed to emit high-amplitude acoustic signals, a bright light, and/or a concussive force depending on the design of the specific system. With continued development of new technologies, an understanding of the critical flashbang effects and their causal pathways is needed to optimize performance and deployment of these devices. Equally important is an understanding of the portions of the flashbang effects pathways, which may have a high risk of significant injury with the intent to mitigate this risk whenever possible. As a step toward exploring these pathways, this study is focused on observing the effect of acoustic overpressures on animal behavior. Our approach focuses on measuring the chain of effects caused by exposure to acoustic overpressure, then correlating that to the physiological response and stress-related behavioral outcomes in a rat model.

Experiment

To safely conduct experiments within a laboratory setting, flashbang stimuli were simulated. ARA routinely uses a compressed gas shock tube to simulate highly reproducible acoustic overpressures for hearing injury and hearing protection research. Use of the shock tube has been automated with custom electronic and pneumatic components operated from a computer interface. This stimulus was presented at multiple levels (Low – 143 dBP, High – 155 dBP) to determine stress-inducing mechanisms and the effect of the exposure magnitude. Experiments were performed in a state-of-the-art facility at the University of Colorado at Boulder that has extensive experience evaluating the stress response of animal models. In this study, each animal was surgically instrumented with a wireless physiological telemetry system which reported real time core body temperature, EKG, locomotor activity, and EMG. Using this telemetry system, the subject experience before, during, and after exposure was analyzed to successfully separate the stress response due to the overpressure stimulus from that induced by the Shuttle Box Escape, a behavioral learning task. The Shuttle Box Escape required a subject to learn to move to the opposite side of a cage and back when experiencing a foot shock to terminate the shock. This test gave an indication of the overall degradation in performance by humans on learning tasks which may be realized following a stress-inducing exposure. Terminal measures such as blood glucose levels and organ weights assured that a chronic stress condition was not induced by the study.

Results

A total of 114 subjects completed the protocol including 43 subjects who completed a series of pilot studies and a further 51 subjects randomly assigned to control, low level and high level exposures within the overall protocol. For each task, performance for each subject was benchmarked against unexposed controls with no acoustic exposure for that subject. Female subjects exhibited heightened recovery of heart rate toward baseline directly after High BOP exposures. Subsequent to the increased heart rate recovery, significantly degraded Shuttle Box Escape behavior is observed in this group. Additional support for the observed degradation is a significant difference in core body temperature and EMG voltage over the same time course.

Impact on Flashbang Effects Research

The stress response to transient acoustic exposure observed in this study serves to demonstrate that the behavioral response to flashbang exposure can be adequately assessed in a rat model. Specifically, increasing the magnitude of the stress response in terms of vital signs, vocalizations, and escape box performance with increasing levels of overpressure validates these pathways as having an increasing effect with proximity to a flashbang or increased source level. To further illuminate the causal model for flashbang effects, additional research must be conducted to improve the statistical power of these results and clarify the nature of the response curve through changes in pulse duration / impulse of the blast wave in addition to peak pressure. Additional research is justified to study the other flashbang effect pathways (e.g., visual, overpressure) to determine how these pathways may compare in strength to the auditory pathways tested in this study and may synergistically contribute to the disabling impact of the flashbang.

1. Introduction

Flashbang devices are widely used for degrading the response capability of individuals and small groups in situations such as tactical entry operations, but the exact doses of visual, auditory, and overpressure stimuli that result in effective, but not injurious, suppression effects are not well understood. The acute physiological stress response to the sudden and intense stimuli of a flashbang exposure impacts the sympathetic and parasympathetic nervous systems' "fight/flight/freeze" response (Jarczewski 2019). For military and law enforcement operations where a flashbang grenade is deployed, an understanding of both the acute and subacute physiological responses is needed to predict adversarial behavior while preventing acute injury and monitoring for longer-term detrimental physiological effects. We hypothesized that measurements of stress in a rat model before, during, and after simulated flashbang overpressure will show acute fight/flight/freeze behavior of variable duration correlated with a rise in physiological stress markers. Wireless *in vivo* biotelemetric measurement of heart rate, core temperature, and muscle contraction, ultrasonic vocalization, and terminal physiological stress metrics supported this understanding of the relative effect of the stimuli and their effects on both acute and near-term behaviors. These results will further our understanding of the physiological reaction to acute transient stress and will guide flashbang research and development activities to achieve optimal suppressive effects while minimizing the risk of significant human injury.

1.1. Background: Flashbang Effects

Flashbangs are typically employed with the intent to distract and temporarily disable an adversary, gain surprise advantage in a physical confrontation, or disrupt communication. These devices are designed to emit high-amplitude acoustic signals, a bright light, and/or concussive force depending on the design of the specific system. To predict effectiveness, new and existing weapons systems continue to be evaluated to quantify their output (e.g., Dayton 2016). Flashbangs are typically deployed with a desire to reduce the risk of significant injury from an operation relative to the use of firearms or other nonlethal weapons with a higher incidence of physical injury (Omer 2019). As such, we are interested in the impact of flashbang device output on humans such that permanent injury may be avoided.

Determination of the minimum thresholds for injury is not trivial for the effects generated by flashbangs such as permanent auditory threshold shift (PTS) and tympanic membrane rupture (TMR) (Cazares, 2016). Basic research into these injury thresholds for animal models continues to be funded (e.g., Casper, 2018) to narrow the range over which human risk may be realized. Similarly, models of injury risk for these effects, such as the AUDITORY model for hearing injury (Chan, 2012) and the TMR model for tympanic membrane rupture (Iyoho, 2017), have been developed to better predict risk from exposures. However, due to the range of settings in which the devices may be deployed and variability in detonation and biological response mechanisms, there is uncertainty in these injury models (Swallow, 2019) that must be addressed before they can be confidently used in high-risk operational scenarios.

As a step toward reducing the uncertainty in auditory injury models and supporting the optimization of operational device output, this study aims to better understand the impact of flashbang effects on the stress-related behavioral response of an animal model and infer the implications for human exposure to a flashbang. By identifying the flashbang stimuli that govern the desired physiological effect on the subject, devices may be developed and deployed that may reduce other injuries and collateral damage to the scene. As a framework for studying which flashbang effects are dominant, the causal path analysis methodology shown in Figure 1 was

developed previously (Madhavan, 2018). Each box in the path diagram indicates a link in the chained response to a flashbang exposure ranging from the flashbang source (yellow) to the physiological and psychological response (gray). Arrows in this diagram indicate causality between the different effects derived from a wide array of disciplines to create a framework through which understanding of flashbang effects can be studied. Although the elements in this framework represent a depiction of human response derived from empirical data, the validation of the interaction of these elements against human performance data is still largely unexplored.

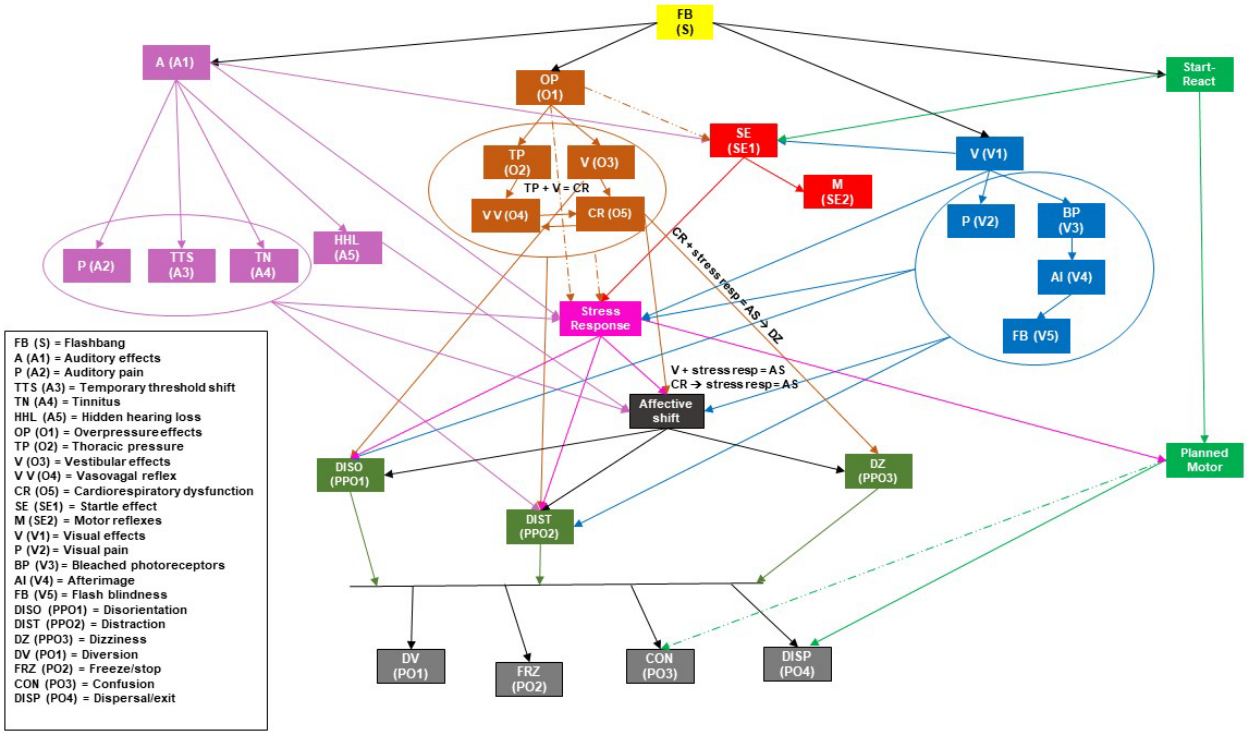

Figure 1. Causal model for flashbang effects on humans updated from Madhavan 2018.

1.2. Hypotheses

This study was designed to address a subset of the flashbang's full causal model. Shown in Figure 2, our study focuses on the auditory, startle effect, and StartReact components and downstream effects that lead to measurable performance outcomes. Components of the model we did not address in this study include impulse effects and visual effects, although a discussion of how these may be addressed is presented in Section 0. Following the pathways, we hypothesized that the stress response will impact behavior in measurable ways and play a role in flashbang effectiveness. Both physiological and performance measures of the stress response due to a transient acoustic stimulus were measured through relevant performance outcomes of escape box latency and physiological response in rat subjects.

In the escape box task, the following pathways may be evaluated:

1. O1/A1→SE1→[Stress Response + Affective Shift+SE2]→PPO2→Z
2. O1/A1(A3+A4)→[Stress Response + Affective Shift]→PPO1+PPO2 pathway→Z
3. StartReact→Planned Motor→Z

Under this effort, the O1→SE1→[Stress Response + Affective Shift+SE2]→PPO2→Z was primarily explored by ensuring stimuli were low enough in pressure to prevent A3 and A4 effects. This pathway was evaluated with the following metrics:

1. Escape Box latency, measured as time to complete the task. How is an individual's ability to respond to the shock stimuli as trained affected by the factors in the study.
2. Physiological Measurements. How do physiological parameters such as an individual's heart rate, heart rate variability, stress-induced hyperthermia, and resultant transient behavior affect the behavioral response in the study.

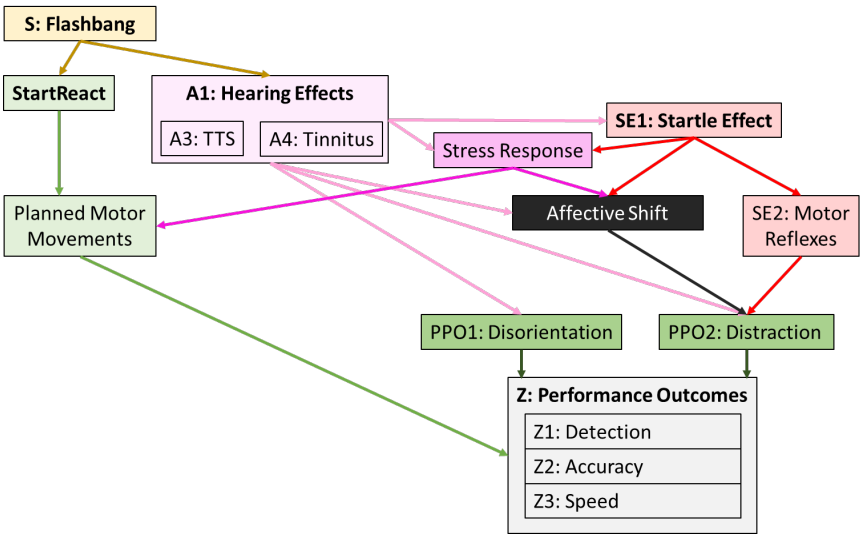

Figure 2. The portion of the causal model addressed by this study adapted from Madhavan 2018.

1.3. Objectives

The primary objective of this effort was to conduct preliminary studies investigating vital signs and escape box behavior as a function of acoustic pressure exposure in normal hearing rats to identify likely disorientation and distraction mechanisms. Using state-of-the-art *in vivo* biotelemetry and stress-sensitive behavioral measures of instrumental learning, this project will specifically test the following:

- The impact of a simulated overpressure flashbang stimuli (as a function of peak pressure) on *in vivo* measures of heart rate variability, core body temperature, electromyography of the dorsal neck muscle, and locomotor activity; ultrasonic vocalization; and terminal measures of stress physiology and the resultant transient behavior of a rat model.

- The nature of the relationships between *in vivo* stress physiology and behavioral task performance.

A secondary objective is to guide future research in flash bang effectiveness by improving upon the test methodology and recommending complementary research and translation of these methods to human subjects for validation.

The body of this report describes the experiments conducted to address these objectives. First, a literature review spanning the psychoacoustic and rat/human performance literature is presented, including technical rationale for our experimental approach. The equipment and

facilities designed for this test protocol are then discussed and a description of the test protocol is presented. This is followed by a description of the data collected and statistical analysis of that data to provide insight into the observed effects on human performance. A further discussion of the impact of these effects on flashbang use is provided including recommendations for research that could be conducted to further clarify the individual roles of pulse magnitude and duration. The report concludes with a discussion of the limitations of the data collected.

2. Experimental Design

In this section, we describe the organization of the behavior response tests. A brief overview of the animal subject protocol and screening process is presented to describe the measures taken to remove confounding variables like induced hearing loss from the study. We then describe the test location at the University of Colorado Boulder Campus. Finally, we present a description of the specific equipment and software used for control and measurement during the experiment.

2.1. Protocol

2.1.1. Approvals

The animal subjects research protocol was developed in close coordination with the Institute for Defense Analyses (IDA). We provided a draft protocol for review to IDA in March 2022 and addressed their comments in our subsequent submission of the protocol to the University of Colorado IACUC in April 2022. The University of Colorado IACUC approved the protocol on 18 May 2022. The protocol was subsequently submitted to BUMED for review and fully approved on 11 July 2022.

2.1.2. Overview

Male/Female Sprague Dawley rats (Harlan Laboratories) were housed with controlled temperature and humidity. Physiological measurements were taken using biotelemetry signals that were recorded 48 hours before, during, and 48 hours after experimental testing.

The behavioral testing was completed using the Shuttle Box Escape Test. Behavior during the acoustic stimuli exposure and shuttle box escape task was recorded using an ANY-maze automated video tracking system. Rat vocalizations convey the animal's psychological/physiological state and were recorded using GRAS Model 40 BP microphones prior to and during the exposure to the acoustic stimulus and escape tasks. Rat vocalizations in higher frequency bands that are centered near 50 kHz are indicative of a positive emotional state; whereas vocalizations in lower frequency bands that are centered near 22 kHz are indicative of anxiety and stress.

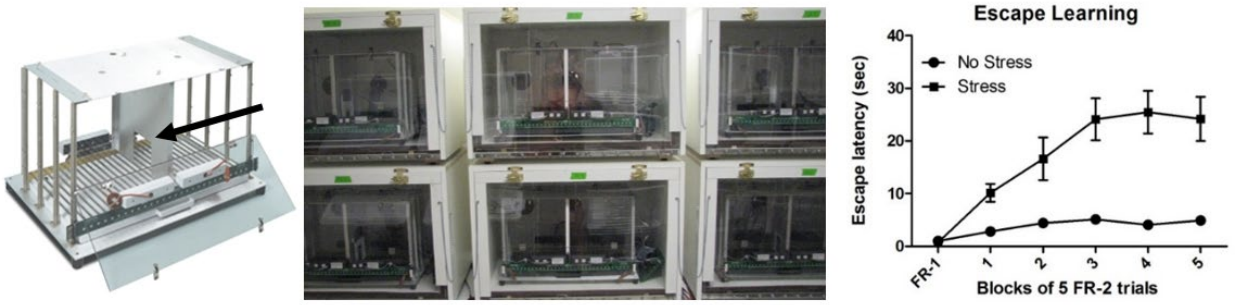

Figure 3. Left – The shuttle box has two chambers with a small opening between them and an electrified grid floor. Right – Performance in the standard Shuttle Box Escape task is shown to degrade when the subject is exposed to a stressor.

2.1.3. Test Matrix

1. No Injury Pilot Study: To ensure auditory injury was highly unlikely after exposure to the range of acoustic stimuli, we assessed the condition of the peripheral and central auditory system using distortion product otoacoustic emissions (dpOAEs) and auditory brainstem responses (ABRs) using a combination of commercially available and custom hardware and software, with a rat-specific earpiece.
2. Biotelemetry Pilot Study: The goal of this pilot study was to determine 1) the impact of two levels of non-injury level stimuli based on results from the no injury pilot study on the stress response; 2) if both Cluster A and Cluster B biotelemetric measures capture reliable and repeatable stress responses to a given level of acoustic stimuli; and 3) the nature (recorded behavior, acoustic vocalizations, biotelemetric), the degree of responses generated, and the time required to return to baseline. A subset of rats was used to determine feasibility of using lower foot-shock intensities and fewer FR2 trials than classically used in the learned helplessness shuttle box escape task.
3. Acoustic and Shuttle Box Escape Behavior: Rats were exposed to acoustic stimuli or placed in the shuttle box with no acoustic stimulus as controls. Immediately after the acoustic stimuli or no stimuli all rats were tested for performance of FR1 and FR2 trials in our modified shuttle box escape task.

2.2. Facilities and Equipment

A modular sound isolation enclosure was constructed to mitigate sound transmission from the experiment procedure room to adjacent rooms. The enclosure design included an opening for the shock tube used to produce the acoustic overpressure during experimentation. It also included observation windows, ports for microphones to record ultrasonic vocalization, and appropriate cable and ventilation ports. The specific wall construction was based on typical values of Sound Transmission Class (ASTM E413) for various wall panel assemblies. To ensure maximum isolation of the sound source to adjoining rooms, an interior wall assembly with an estimated STC of 50 was selected. The panel design included wooden studs, space for insulation within the wall, drywall, and sound absorbing foam lining the interior of the enclosure. The completed sound isolation box is shown in Figure 4.

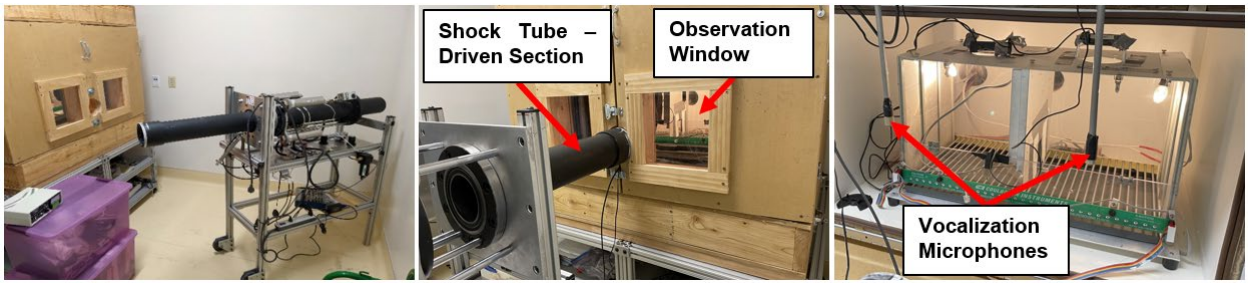

Figure 4. Left – The sound isolation box within the procedure room in preparation for testing. Center – Experimental configuration with the shock tube ported to sound isolation box. Right – The interior of the sound isolation box enclosed the shuttle box.

Table 1. Equipment used in this study

Equipment	Manufacturer/Model	Variable Measured	Variable Controlled	Placement
Implanted biosensor	Data Sciences International HD-S02	Locomotor activity (LA) + core body temperature (CBT) + electromyogram of the neck muscle (EMG) + either electroencephalogram (EEG) or heart rate-EKG (HR)		Implanted within rat abdomen
Microphone	GRAS 40BP	Ultrasonic Vocalizations		Mounted on each side of the shuttle box ceiling
Camera		Escape speed, behavior		One on either side of the shuttle box aimed from outside an acoustic isolation box
ANSI S12.42 Shock tube	B/C Precision Tool (modified)		Acoustic overpressure	Aimed at the shuttle box within an acoustic isolation box
Electroshock Escape Box	Coulbourn	Behavior	Foot shock magnitude	In the procedure room, in an acoustic isolation box

2.2.1. Acoustic Source

Existing literature on blast exposure levels was reviewed to develop a reasonable scale to translate rodent impulse noise exposures to known human data. Unfortunately, few studies have conducted noise exposure studies in rats, instead preferring to conduct noise exposures in guinea pigs, or more often, chinchillas, in part due to the closer similarity in audiometric thresholds to humans (Henderson & Hamernik 1986). The few studies that have investigated impulse noise & blast exposure in rats have used high level impulse noise that is far above the threshold of inducing either temporary or permanent threshold shifts (TTS or PTS), or even tympanic membrane rupture (Escabi et al. 2019, Holt et al. 2019). Anatomical differences include substantial differences in middle ear geometry resulting in substantially lower stapes displacement in rats (~20 µm) than chinchillas (~80 µm; Peacock et al. 2018) or humans (~150 µm; Greene et al. 2017) in response to low frequency tones (Figure 5; Walilko et al. 2018). Additionally, rats have right shifted audiograms (Gould 1941), with a higher low frequency limit of hearing (520 Hz), compared to humans (at 20 Hz), indicating higher baseline hearing thresholds at lower frequencies, but extended hearing at higher frequencies (Heffner et al. 2001).

To provide a noise exposure comparable to blast exposures in humans, Chan (Chan 2012) (Auditory 4.5) implemented a 28 dB scaling factor from human to chinchilla, whereas Walilko (Walilko 2018) estimated a more modest ~20dB chinchilla scaling factor for noise over <165 dB. Observation suggests positive phase duration greater than 2 ms will not have equivalent effects on rats as on humans. Since the middle ear shows greater limiting of high level noise transmission in rats than in chinchillas based on peak stapes displacement measurements, we anticipate that the scaling factor for the rat model will be greater than the chinchilla model.

The OSHA noise exposure limit for impulse noise is 140 dBP SPL (OSHA 1910.95(a)) due to a substantial risk of hearing loss for higher level exposures. We therefore predicted that the

threshold of inducing temporary threshold shift (TTS) will be at least 20 dB higher, or 160 dBP, but were unable to find a direct measurement of rat TTS threshold induction in the literature.

In summary, since we wish to minimize the confounding effect of permanent hearing loss, while maximize the stress induced by these impulse noise exposures, we determined that maximizing impact and translation of findings to human equivalent loading will require the high impulse noise input to the rat model be approximately 160 dBP with a positive phase duration of <2ms at 500Hz. However, we further determine that we should directly assess the threshold of TTS induction in rats when exposed to impulse noise with these characteristics in a pilot study, prior to beginning the full study.

The suitability of the scaling factor was considered before finalizing the protocol to determine the appropriate stimuli to elicit the desired stress response.

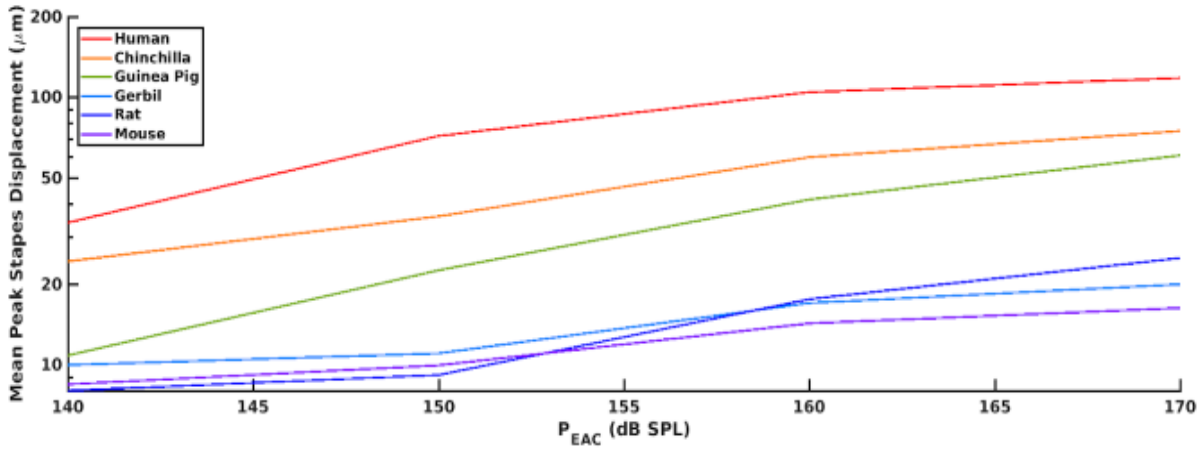

Figure 5. Mean peak stapes displacement is less for rat and chinchilla compared to humans for increasing pressure intensity at ear canal.

During initial testing of the startle source, data from a shock tube test with no subject present is shown in Figure 6. As expected, a transient acoustic pulse was observed followed by a decay (~0.2 s) before returning to ambient pressure levels. In the frequency domain, a resonance was observed near 57 Hz with odd numbered harmonics which is consistent with dimensions of the shock tube acting as a quarter wavelength resonator. The amplitudes of the harmonics decrease with increasing frequency and do not contribute significantly to the received signal over 10 kHz.

The spectrogram shows background noise indicated as horizontal striping in the spectrogram and as a harmonic series above 10 kHz in the frequency domain. This noise is present at equal amplitudes both pre- and post-trigger and is indicative of systemic electronic interference. Additional testing will be conducted to determine the source of the noise and/or mitigate the noise.

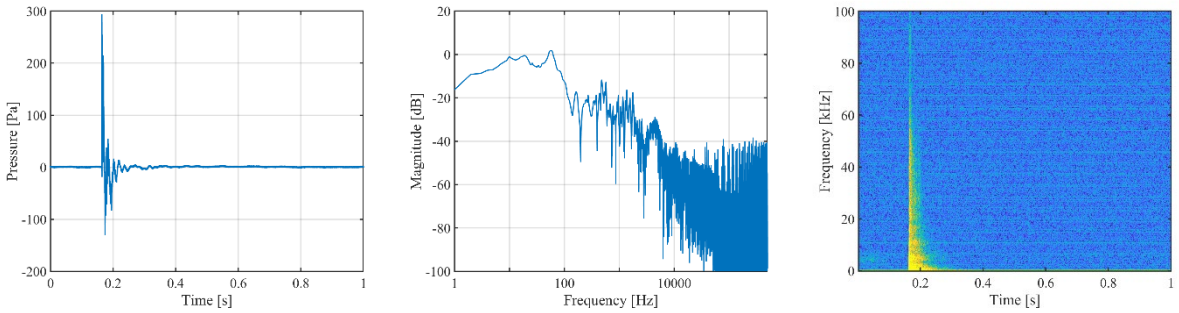

Figure 6. Left – Acoustic response measured inside the sound isolation box. Center – Frequency response of the sound isolation box. Right – Spectrogram showing background noise as narrow horizontal bands.

2.2.2. Implanted Biosensor

The biotelemetry transmitters (Data Sciences International, St. Paul Minnesota.) shown in Figure 7 were implanted into animals for in vivo telemetry. The small HD-S02 implant is capable of continuously recording two biopotential channels simultaneously (EEG, EMG and/or EKG) depending on lead placement location. Animals were fully anesthetized and unresponsive following administration of ketamine (i.p. 75.0 mg/kg), and medetomidine (i.p. 0.5 mg/kg). Animals were shaved and prepped for surgery. A midline incision was made approximately 5.0 cm in length on the ventral abdominal wall. The transmitter was placed on the intestines, the biopotential leads were passed through the ventral abdominal wall and then the F40-EET transmitter was sutured to the ventral abdominal wall. Once the transmitter was sutured into place, the EMG leads were positioned to measure neck muscle electrical activity and the EKG cardiac leads were positioned to measure heart rate. Immediately following surgery rats were given meloxicamER (i.p. 4.0 mg/kg) for analgesia after which they recovered on a heating pad at 37° C until ambulatory. Once rats were self-ambulatory, they were placed back into clean home cages.

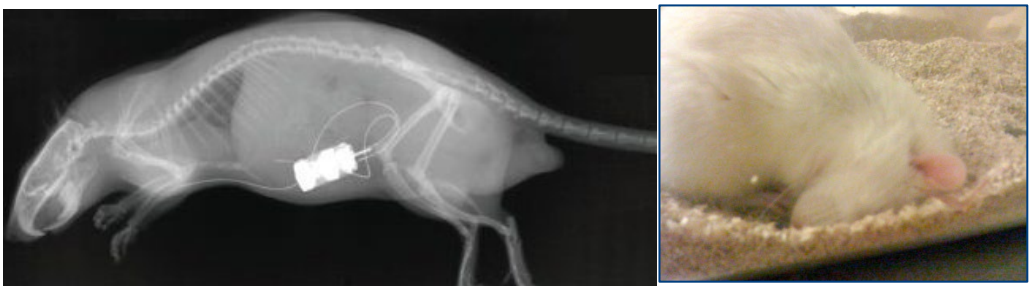

Figure 7. The implanted telemetry device (left) is designed to measure an array of physiological variables while minimally impacting performance or behaviors such as sleeping (right).

2.3. Experiment Control Software

Biotelemetry recordings were acquired/analyzed using Ponemah Software System (Data Sciences International, St. Paul, MN). This system allows real-time continuous measurement/recording of locomotor activity, core body temperature, and two biopotential channels from the HD-S02 biotelemetry device. Once recorded, the Ponemah Software System allows us to export waveform data as either raw EDF files, moment-to-moment parameter data, or automatically mark features of the different waveforms collected.

3. Test Administration

Details of the experiments are provided in this section including the overall test matrix, description of the three pilot tests to refine the experimental process, and a full description of the Escape Box test protocol.

3.1. Pilot Tests

The Test Readiness Review was completed on 19 July 2022; the finalized test matrix and number of completed subjects are shown in Table 2.

Table 2. Final test matrix showing the initial pilots and the remaining test conditions.

Test Series	Content	N		Date	
		Target	Completed	Start	End
Pilot 1	Source level evaluation	18 (+2)	15	9/19/2022	10/25/2022
Pilot 2	Biotelemetry configuration	32 (+6)	22	9/12/2022	10/28/2022
Pilot 3	Learning task shock level	4 (+2)	22	8/15/2022	9/23/2022
Full Study	Shuttle box escape under stress	96 (+2)	51	10/7/2022	02/24/2023
		150 (+12)	110		

3.1.1. Pilot 1: Source Level Evaluation

To determine the presence of hearing injury (Temporary Threshold Shift, TTS) resulting from exposure to the blast overpressure (BOP) generated by the shock tube, anesthetized rats were exposed to one of four BOP stimuli levels. The positioning of the rat during testing is shown, with a microphone to measure the exposure level placed coincident with the rat position, is shown in Figure 8 alongside the position of the reference source level microphone. Visual inspection of the external ear, head, and neck, as well as auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) measurements were performed pre- and post-exposure to assess for gross injury as well as temporary threshold shifts (TTS). Results were collected in 13 rats (two additional rats did not survive the anesthesia), of both sexes, and are presented for 9 of these animals (5 males, 4 females), for a total of 18 ears tested.

First, tone evoked auditory brainstem responses (ABRs) were collected at various levels for 4 kHz, 8 kHz, 16 kHz, and 24 kHz in order to construct an ABR audiogram for each ear simultaneously. Responses were averages of 600 stimulus presentations per condition. The level was initially set to some suprathreshold value, and the ABR waveform identified visually in real time. The level was then decreased (in 10 dB steps) in order to identify the lowest level at which the ABR was visible above the noise. The measured signal is an evoked electrical signal derived from auditory brainstem structures. The primary outcome measure is the ABR threshold (in dB SPL) as a function of stimulus frequency (in kHz). A secondary outcome measure may be the amplitude and latency of specific ABR waveform features (e.g. wave III peak amplitude in µV or latency in ms).

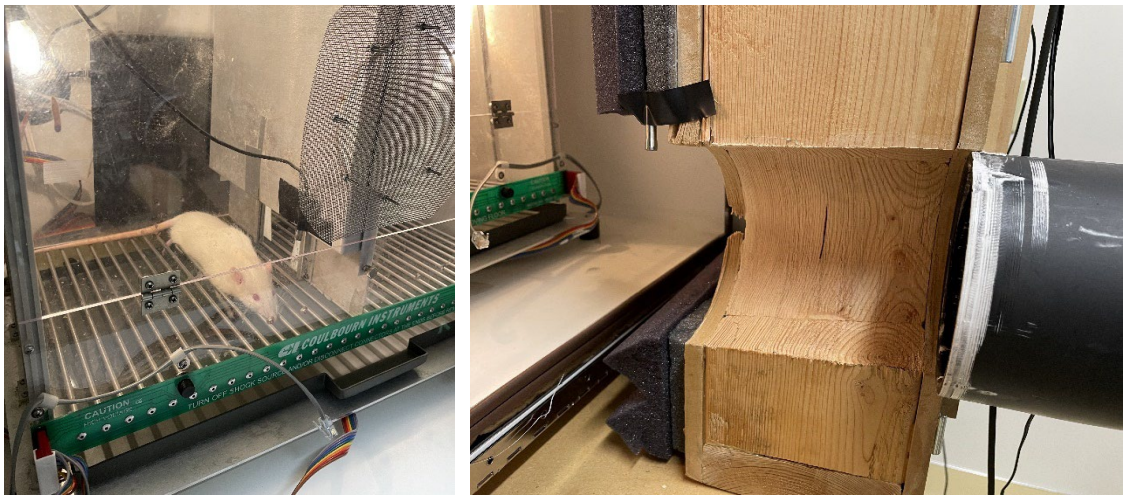

Figure 8. Positioning of the anesthetized rat and exposure level microphone (left) and source level microphone (right). Peak impulse pressures were measured by the microphone and pencil probe housed in the sound booth during exposures, as well as by a microphone at the position of the animal's head after all exposures were completed.

Second, distortion product otoacoustic emissions (dpOEAs) were measured for pairs of tones (f_1 and f_2) in each ear. Tone pairs were set with a relationship of $f_2/f_1 = 1.22$ and generate a distortion product in a healthy ear at a frequency of $f_{dp} = 2f_1 - f_2$. The recorded measure is the recorded level (in dB SPL) of f_{dp} and is reported as a function of the f_2 frequency (in kHz), and the combined output level of f_1 and f_2 (in dB SPL). Responses are always shown relative to the noise floor at that frequency, in that measurement (as only measurements above the noise floor shall be valid). The primary outcome measure will be OAE threshold (which is rarely visible in these measurements due to low thresholds, and elevated background noise), and secondary measures will include dpOAE amplitude and trajectory.

An example set of recordings from one animal exposed to the lowest BOP level are shown in Figure 9, example dpOAE responses in one ear from that same animal are shown in Figure 10, and the average difference in measured ABR thresholds were calculated as the TTS are summarized in Table 3. dpOAE thresholds were typically not quantifiable due to hardware limitations, thus are excluded from this table.

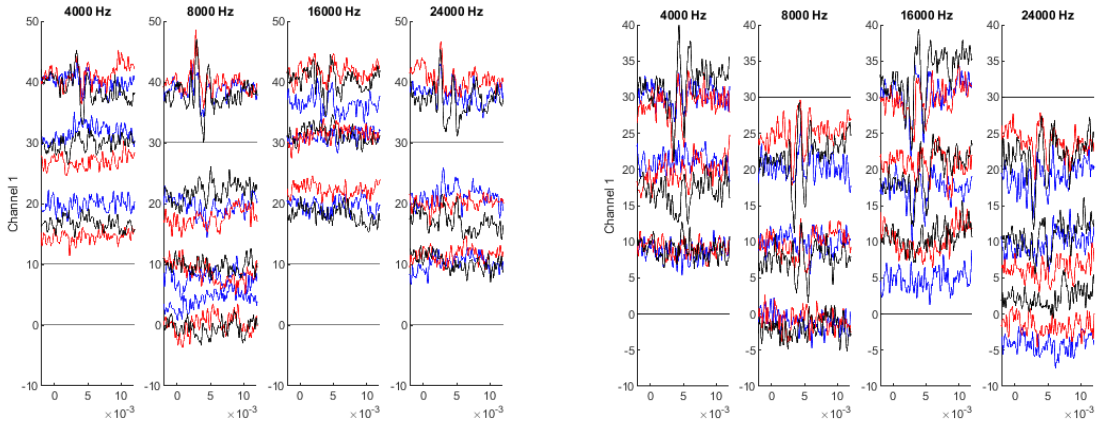

Figure 9. Example ABR waveforms from one rat, recorded pre (left) and post (right) a 148 dBP BOP exposure. Responses are shown for test tones presented in the left (blue), right (red), and both (black) ears, for each of the four frequencies (columns), and at several sound pressure levels (rows). Threshold was determined by identifying the lowest level at each frequency with an identifiable waveform.

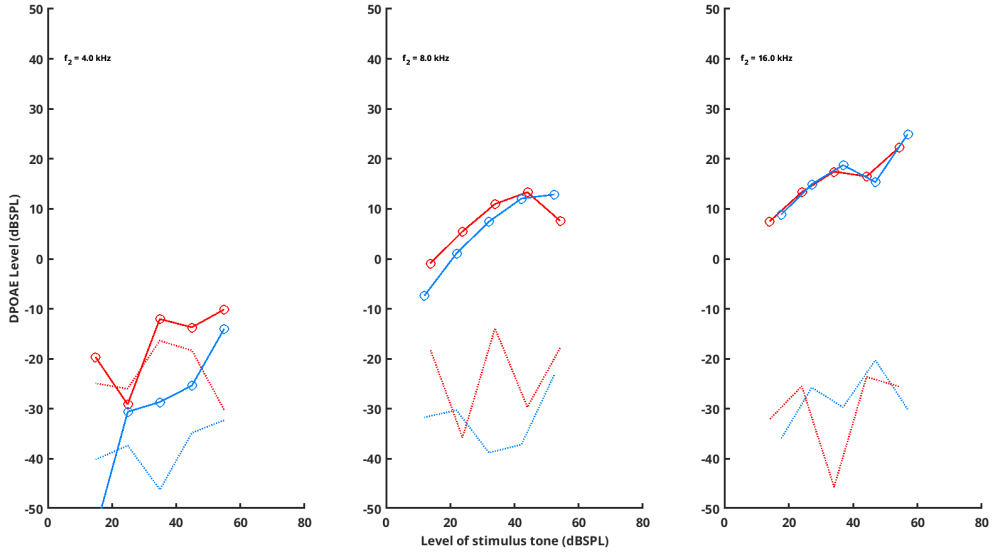

Figure 10. Example dpOAE responses from the left ear in one rat (same as in Fig. 11), recorded pre (red) and post (blue) a 148 dBP BOP exposure. Responses (solid lines, circles) are the SPL of the evoked OAE in the ear canal, for the three test tones (columns), at several sound pressure levels of the eliciting tone pair (x-axis), along with the background noise (dashed lines) present during that recording.

Table 3. Result of injury level evaluations in anesthetized rats

Exposure Level at Tube Exit [dBP]	Exposure Level at Subject [dBP]	N [total ears]	Mean TTS – ABR [dB, mean ± std.dev]			
			4 kHz	8 kHz	16 kHz	24 kHz
165	148	4	-2.5 ± 4.3	-7.5 ± 8.3	-10 ± 0.0	0 ± 10.0
173	155	6	0 ± 8.2	3.3 ± 4.7	1.7 ± 3.7	3.3 ± 4.7
181	163	8	2.5 ± 4.3	8.75 ± 7.8	13.75 ± 8.6	8.75 ± 6.0

In all four ears exposed to 148 dBP, thresholds shows comparable or slightly decreased ABR thresholds post exposure, suggesting that no TTS was induced, and that these differences observed were the result of test-retest variability. Similarly, dpOAE responses were typically nearly identical at most elicitor levels, again suggesting no TTS; however, we often could not identify threshold due to limitations in lowest levels at which we could present the elicitor tone pair (≥15 dB SPL), and the lowest levels detectable by the OAE microphone.

ABR and DPOAE thresholds for 155 dBP exposures similarly showed small threshold shifts that varied between ±10 dB in all six ears, and the average ABR threshold shift was small (< 5 dB), though more often positive, suggesting that this exposure level may be at or just below the threshold of TTS induction in these rats. Similarly, dpOAE responses were consistently very comparable pre and post exposures. Note, these results are presented from three animals. A previous set of measurements in 4 animals at this exposure level showed significantly elevated baseline thresholds, suggesting this cohort of animals had existing hearing loss, or that these recordings were otherwise unreliable, thus results from those 8 ears have been excluded.

ABR and DPOAE thresholds for 163 dBP exposures showed somewhat larger changes post exposure than either of the lower exposures. ABR thresholds were consistently elevated, and dpOAE levels were slightly, though consistently suppressed post exposure, suggesting some TTS was induced in these animals. The degree of this TTS is not large, ABR threshold shifts only reached 13.75 ± 8.6 dB at 16 kHz, suggesting this exposure level was slightly above the injury threshold of TTS induction in these rats.

To identify an appropriate TTS threshold, we referred back to the US Army's Aeromedical Research Lab's walk up study (Johnson and Patterson 1997), which used a temporary threshold shift of 25 dB as their criteria for an unacceptable change in hearing for each condition:

> "If a volunteer had more than 25-dB TTS (determined 2-6 minutes post exposure), he was considered to have an unacceptable TTS for that exposure condition as well as all conditions with the same number of shots and higher peak levels. Also, he was considered to have unacceptable TTS for conditions with the same peak level and a greater number of shots. That is, all more energetic conditions were considered unacceptable. Conversely, when an exposure condition resulted in TTS less than 25 dB, all conditions for the same number and lower levels (less energetic conditions) were considered acceptable."

Both measures of hearing were relatively unchanged in these rats. Slight threshold elevation in ABRs, and slight decreases in dpOAE levels were noted in some specific conditions; however, both effects were small. In particular, the threshold elevation was less than the 25 dB TTS criterion used by the Albuquerque walk-up study, indicating that the TTS induced by the noise exposure was mild, and would have been considered an 'acceptable' change TTS for continued participation in the study.

While we did not explicitly inspect the ear canal or middle ear for injury, the minimal changes in ABR thresholds and dpOAE levels suggests that the middle ear was not injured, and the tympanic membrane was not perforated by the noise exposure. This result is consistent with prior reports that only observed tympanic membrane perforation at much higher (16 psi; 195 dBP) peak pressure levels (Wang et al. 2020). Similarly, the minimal change in dpOAE levels suggest that cochlear Outer Hair Cell (OHC) function was minimally affected, and the minimal change in ABR thresholds suggests Inner Hair Cell (IHC) and spiral ganglion/auditory nerve fiber function was minimally affected.

Overall, since all threshold shifts observed were less than 25 dB, we conclude that the TTS observed in these rats was low, would have been acceptable in the walk-up study, and a shock wave exposure of this magnitude or lower will not cause a significant TTS, or measurable PTS, in exposed rats at any level tested, but especially at the two lower exposure levels.

Conclusion: Exposure levels of 155 dBP and 143 dBP as measured at the subject were selected for the full study.

3.1.2. Pilot 2: Biotelemetry Configuration

A total of 18 subjects underwent testing to evaluate the two potential configurations of the telemetry system. The two sensor clusters as proposed are:

- Cluster A: Activity, Core Body Temp, EKG, and Heart Rate
- Cluster B: Activity, Core Body Temp, EEG, and EMG

A preliminary example of data for Core Body Temperature in 6 Males is shown in Figure 11 for control (blue) and BOP exposure (red). A common baseline temperature of 37.2°C is shown in their home cages (Figure 12) which increases to 38.1°C upon handling and introduction to the shuttle box. As the subjects acclimatize to the shuttle box, the core body temperature begins to decrease. In the control animals, this decrease is shown until handling and return to the home cage. For the BOP-exposed animals, the hyperthermia is immediately increased upon exposure to the stimulus. Upon returning to the home cage, both control and BOP-exposed animals decrease in temperature with the BOP-exposed animals remaining hyperthermic relative to the controls.

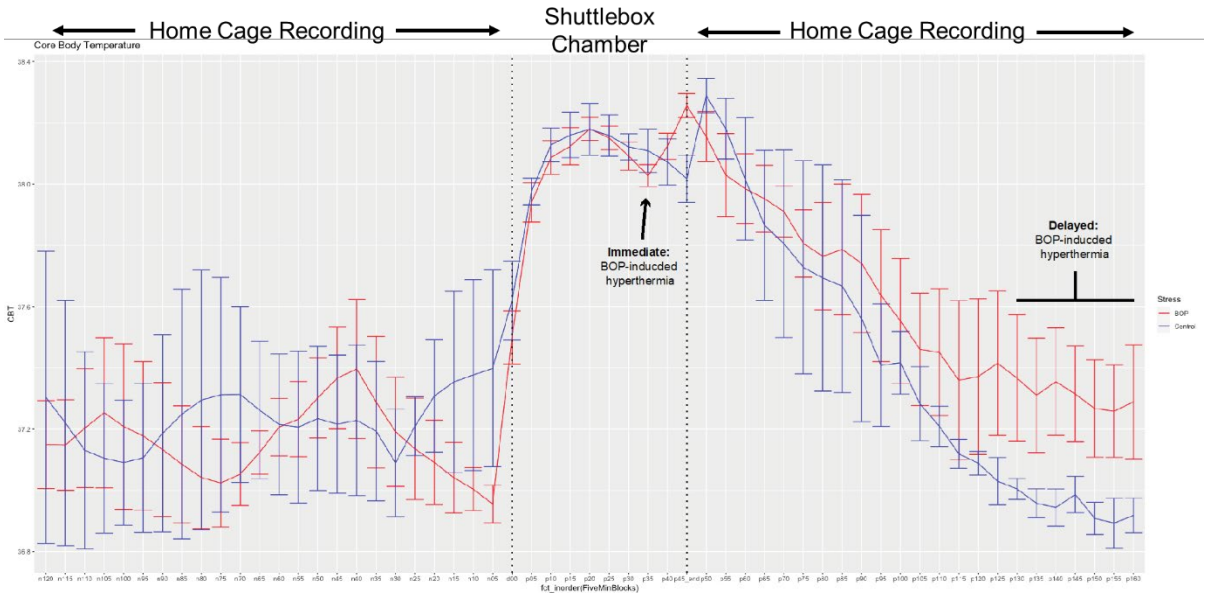

Figure 11. Core Body Temperature (N=6) for a BOP-only condition.

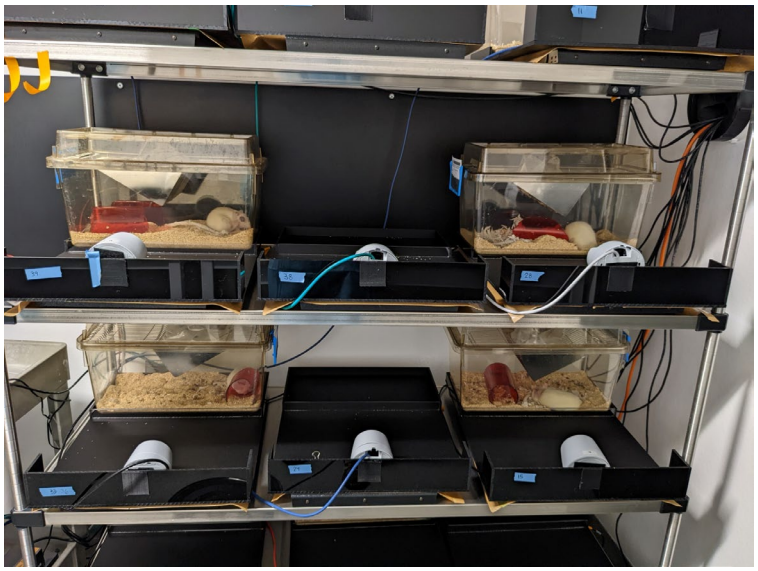

Figure 12. Rats in their home cages with telemetry recordings active.

All proposed measures from the sensor modules in both configurations have been collected successfully. EEG data is primarily of use during the analysis of sleep patterns and may not be as useful as other measures in determining the subject response to the transient stressor. Heart rate and EKG are strong indicators of transient stress, as is core body temperature (shown above). EMG measurements are useful to measure the duration and magnitude of any freeze/startle response and allow for potential comparison to human subject data collected in previous studies.

Conclusion: Due to the compressed test matrix, we plan to implement a hybrid sensor cluster consisting of activity, core body temp, heart rate/EKG (one channel only), and EMG (neck muscle). This suite will provide the most relevant data for transient stressors. Typically, the research team uses two channels for heart rate/ECG to ensure more accurate measurements; this will be decreased to one lead in favor of the EMG measurement for comparison to previous human subject data collected. This is the only compromise seen by the research team in taking this approach.

3.1.3. Pilot 3: Learning Task Shock Level

To conduct pilot testing while the sensors and rats were being acquired, the Pilot 3 scoping exercise to determine the optimal level of foot shock required to elicit a measurable response was conducted on non-naïve rats. These rats were made available by the CU IACUC after completing other studies to ensure subjects are of maximal scientific value. Additionally, they provided an opportunity to ensure the shuttle box procedures were optimized. Although these rats were larger than the size to be used in this study, they were still able to provide data to indicate the appropriate shock intensity.

The shuttlebox learning task in male rats was optimized and shuttlebox learning behavior was consistent with the published literature (Jacobs 1985). The goal of this optimization of the shuttlebox escape task allowed us to determine that the overpressure is sufficient to disturb performance of the learned task.

Figure 13. Pilot 3 testing with non-naïve rats was conducted to scope the shock level used in the behavioral task.

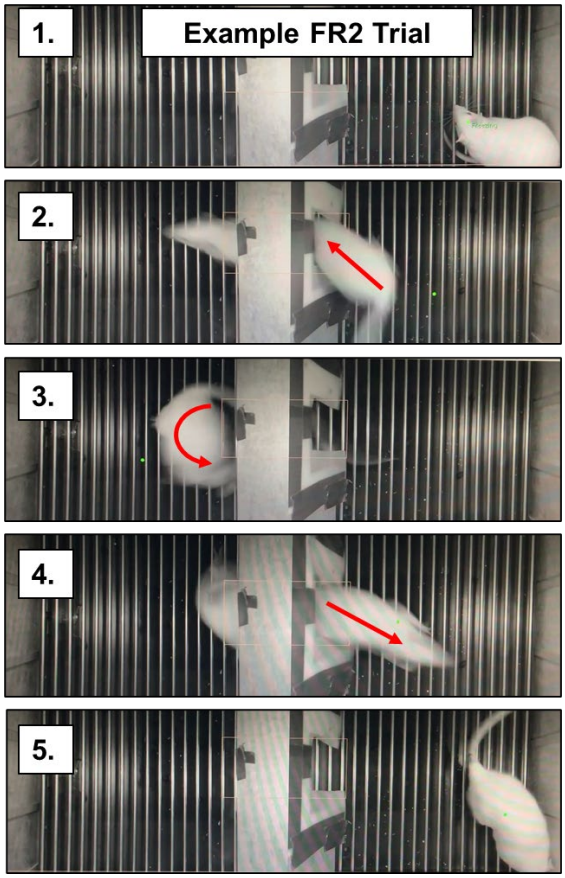

Figure 14. View of rat from pilot testing of the escape test. The rat runs fully through the doorway and back to terminate foot shocks. The task is repeated 25 times with random time intervals between each task. Task completion time (Escape Latency) is recorded during each trial to track learning.

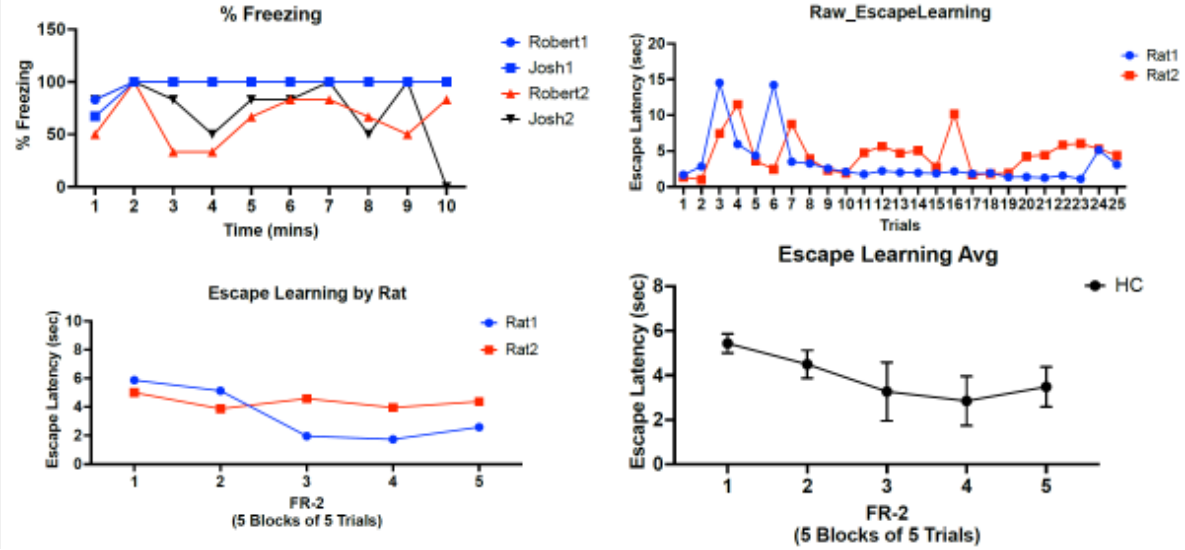

Figure 15. Preliminary data from pilot testing of the escape test to ensure female and male rats have comparable behavioral patterns. Top left – Observed freezing behavior after the FR1 learning task. Top Right – Escape performance for two rats on 25 FR2 trials. Bottom Left – Escape performance aggregated into blocks of five trials. Bottom Right – Aggregate performance across all rats.

Preliminary data from the testing is shown in Figure 15. Traditional escape learning published in the literature is collapsed into 5 blocks of 5 trials (25 total trials) for simplified data presentation. Due to the potential transient nature of BOP on escape learning we determined data presentation across each trial with greater temporal resolution was a more accurate depiction of the behavioral responses post BOP. Based on the individual rat performance shown (Bottom Left) and subsequent measurements not shown here, it was determined that the acoustic stimulus should be presented between Block 3 and Block 4 of the FR2 tests. It is at this point that the subject performance begins to demonstrate a floor effect. The stress due to the acoustic exposure was expected to cause an increase in escape time (worse performance) for the subsequent tests.

It was noted, however, based on the first five subjects, that the female rats were potentially performing differently (faster) than their male counterparts. Therefore, additional rats were allocated (N = 15) to ensure the measurements were consistent among the males versus females. As shown in Figure 16, a difference greater than one standard deviation can be seen.

Finally, to establish the minimal level of foot shock required to motivate the subjects, the time to complete the task (escape) shown in Figure 17 was averaged over blocks of five trials. Two shock current levels, 0.65 mA and 0.45 mA were tested. The 0.45 mA level was evaluated in both the control (HC) and experiment (BOP) rooms to ensure consistency. No strong difference was noted between the more standard 0.65 mA shock level and the lower 0.45 mA shock level, so the 0.45 mA shock level was used for all testing.

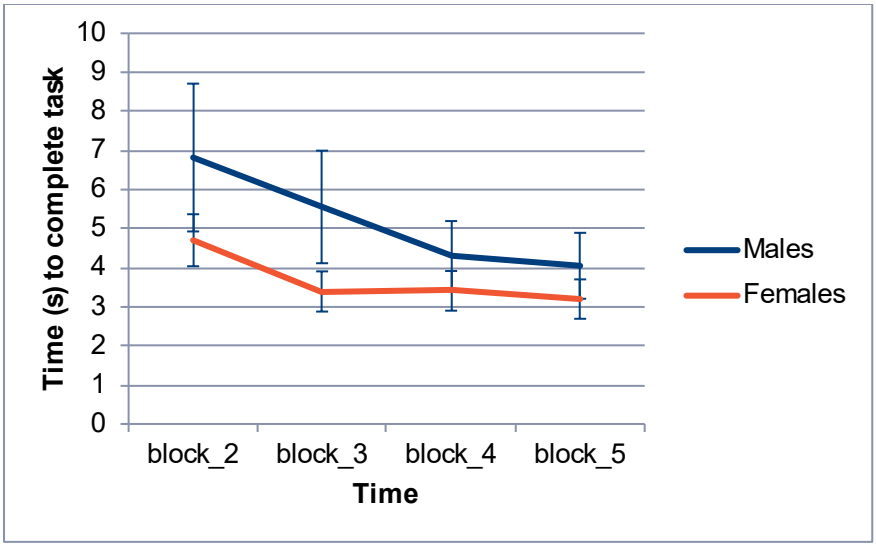

Figure 16. Pilot 3 testing with naïve rats was conducted to verify the foot-shock level used in the behavioral task collected from non-naïve rats.

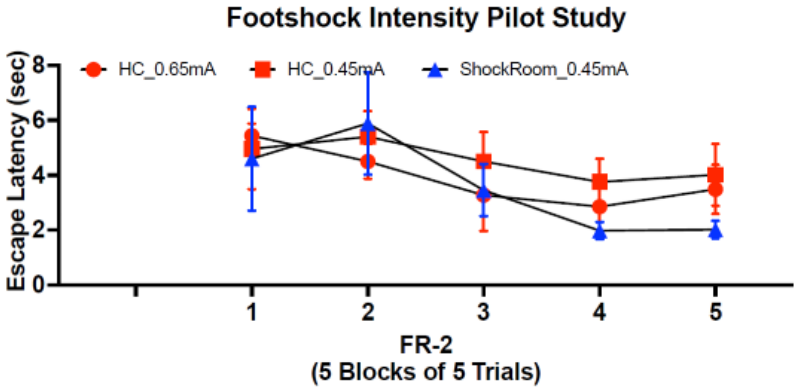

Figure 17. Measurements of escape time versus foot shock level were used to establish a lower bound on the foot shock level sufficient to motivate the subjects.

3.2. Full Protocol

3.2.1. Walkthrough of a Trial

Subjects proceeded through the final protocol based on the hybrid sensor configuration, lower-level foot shock, and pressures defined through the pilot studies. The sequence of events for a subject following the protocol is:

1. Animal receipt and housing acclimatization
2. Biotelemetry surgery
3. Recovery
4. 48 hours baseline home cage telemetry measures and behavior video
5. Shuttle Box Training
6. Shuttle Box Testing with Overpressure/Control Stimulus
7. 48 hours home cage biotelemetry measures and behavior video recording
8. Sacrifice and harvest for analysis

3.2.2. Final Test Plan

A total of sixty subjects were split evenly among control/low BOP/high BOP conditions and Male/Female sex in the revised protocol. Twenty remaining subjects were intended to improve data quality for any test conditions where additional testing would be of use. The protocol (biotelemetry surgery, recovery, 48 hrs baseline home cage biotelemetry measures and behavior video recording, Shuttle box testing + Overpressure stimulus with biotelemetry, video and ultrasonic vocalization measurements, 48 hours home cage biotelemetry measures and behavior video recording) was completed for all subjects from December 2022 through February 2023.

Table 4. Test plan summary for the full study

Sensors	Control (M/F)	Low BOP (143 dBP)	High BOP (155 dBP)	Reserve
EKG/EMG/CBT/LA	10/10	10/10	10/10	10/10

Table 5. Schedule for the full study

Cohort #	Sex	Completion Date	Cumulative N
1	M	12/03/2022	8
2	M	12/09/2022	16
3	F	12/23/2022	24
4	F	12/30/2022	24
5	M	01/13/2023	44
6	M	01/20/2023	52
7	F	02/03/2023	64
8	F	02/10/2023	73
9	M	02/24/2023	83
10	F	02/28/2023	95

4. Results

In this section, a description of the overall data collected is provided. The extent of the dataset, types of data collected for analysis, and example data are shown with indications of how the time-resolved data relates to each subject's progress through the protocol.

4.1. Extent of Dataset

We completed 69 subjects over the course of the full protocol. We were able to collect complete data sets on 51 subjects. The subject coverage is shown in Table 6 below.

Table 6. Summary of the dataset collected under this protocol

Total N	Control	lowBOP	highBOP
Male	14	12	14
Female	10	9	10

Final N*	Control	lowBOP	highBOP
Male	10	8	9
Female	8	8	8

*14 rats failed to learn

4 rats had unusable telemetry signals

2 rats had immune complications related to surgery

4.2. Data Types Analyzed

Wireless DSI Biotelemetry produced the following data:

- Locomotor Activity (LA). A measure of the whole-body motion of the subject. Sampled at 1 Hz. Units are arbitrary since distance traveled can not be calibrated.
- Core Body Temperature (CBT). A measure of body temperature and associated change. Sampled at 10 Hz. Units are degrees Celsius.
- Electromyogram (EMG). A measure of contraction of the primary neck muscle. Sampled at 10 Hz. Units are Volts.
- Electrocardiogram (ECG/EKG). A measure of heart muscle contractions. Sampled at 1 kHz. Units are Volts. Data is typically converted to a heart rate in beats per minute (BPM).

Ultrasonic Vocalizations (UV) were recorded as a measure of stress. Sampled at 100 kHz. Units are pressure in Pa with respect to time in seconds and frequency in Hz.

Escape Latency is scored by the experimenters and represents the time it takes for a subject to traverse the shuttle box gate twice. Collection is performed using cameras. Units are seconds.

Terminal Measures were collected two days post experiment to evaluate whether the subject exhibits any chronic stress. Adrenal/Thymus/Spleen weights and blood glucose reflect the stress state of the sympathetic/parasympathetic stress response.

Analysis for the purposes of this report was limited to approximately one hour during which the subject was within the shuttle box. Additional analysis may be performed on the wireless telemetry and video collected in the subject home cages.

4.3. Typical Data

Examples of each data type are provided in the following sections. Each data type was collected for each subject in the full protocol. Example data presented here is typical of each data type.

4.3.1. Biotelemetry

The DSI biotelemetry systems implanted within each subject wirelessly transmitted data on four physiological measures. An example of the raw recording from one subject is shown in Figure 18. Each portion of the test protocol is visible in the telemetry:

- Acclimation, T = 0 – 600 s: Each rat was allowed to explore the enclosure the day before test administration in an attempt to reduce stress due to transportation and handling on subsequent days. The discontinuity in the CBT at T = 600 s represents when the data from the day of the test begins.
- Exploration, T = 600 – 1200 s: Rats are allowed to explore the enclosure before testing commences.
- FR1 Trials (2), T ≈ 1200 – 1400 s: Two FR1 trails are conducted as evidenced by the voltage spike in the EMG and ECG data and the transient LA.
- Freezing, T ≈ 1400 – 1800 s: The freezing behavior of the rat is recorded as evidenced by the lack of LA data.
- FR2 Trails (25), T ≈ 1800 – 3800 s: The onset of each trial is visible as an increase in voltage for the ECG and EMG data. Evidence of each trial is also visible in the LA recording.

Further descriptions of each data type are provided in the following sections.

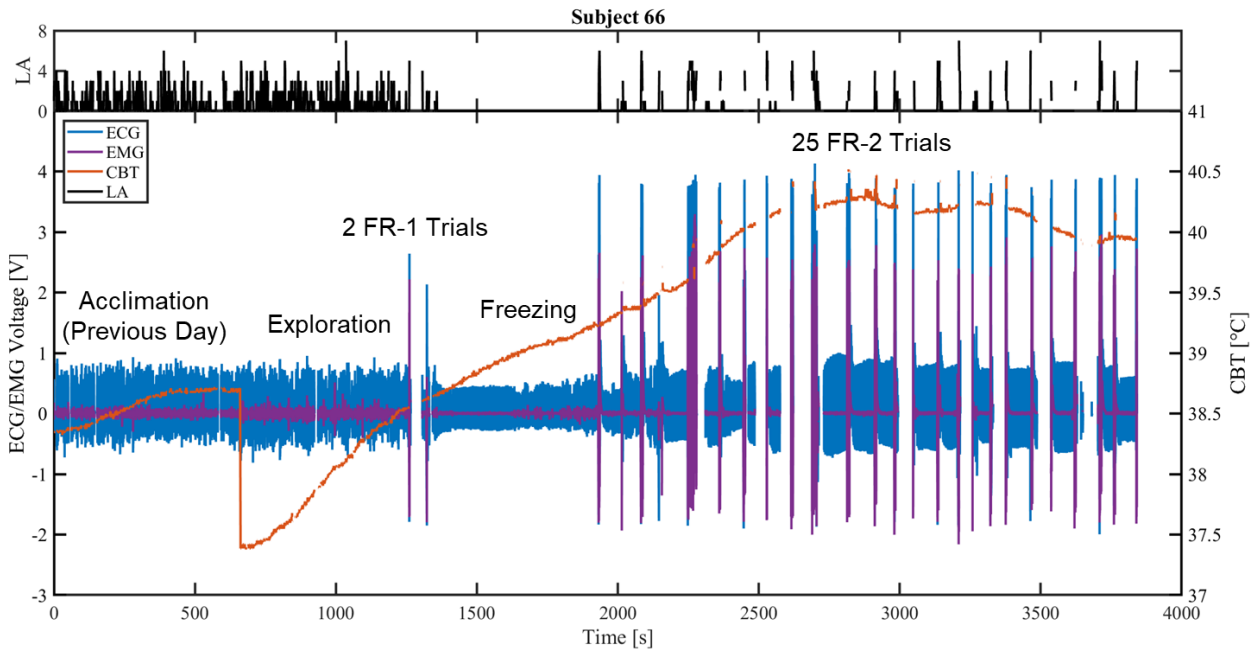

Figure 18. Typical wireless biotelemetry received during a test. Each portion of the test protocol may be observed. EMG/ECG voltage (axis left) is contrasted with CBT (axis right) and LA (above).

4.3.1.1. Locomotor Activity (LA)

LA is a measurement of body motion versus time. Units for LA are arbitrary since no absolute measure of distance is recorded. Briefly, the telemetry device inside the animal is in a position relative to the orientation of the receiver. When the device moves in relationship with the stationary receiver a "count" is recorded and locomotor activity is present. An example recording is shown in Figure 19. Exploration is a continuous series of movements whereas each shuttle box trial is visible as a discrete movement event.

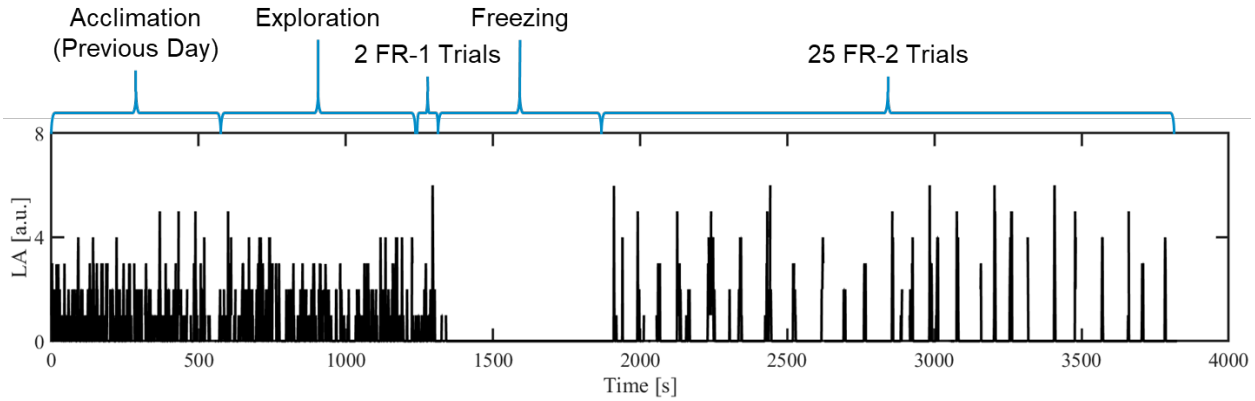

Figure 19. Typical locomotor activity recorded using the DSI biotelemetry system. Each portion of the test protocol may be observed.

4.3.1.2. Core Body Temperature (CBT)

Core body temperature is the measurement of internal body temperature. Units are degrees Celsius. An example recording is shown in Figure 20. Vertical lines represent the onset of FR2 trials with green lines representing measurements before a BOP is administered and red lines representing measurements after a BOP is administered. In general, CBT increases during the exploration, FR1, and FR2 phases of the protocol due to body motion during performance of the shuttle box escape task. In general, temperatures reach a plateau during the FR2 trials before the BOP is administered and may change post-BOP.

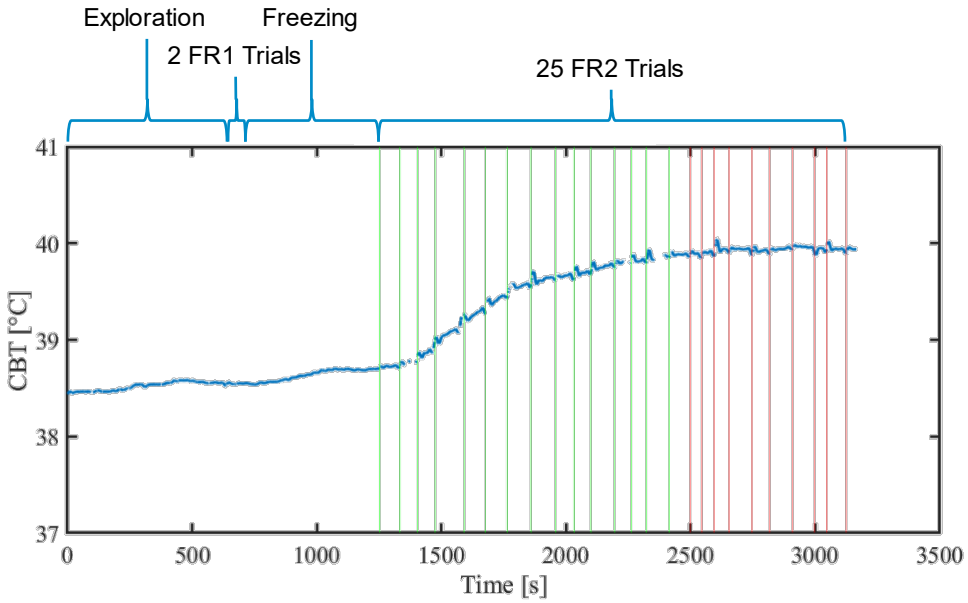

Figure 20. Core body temperature increases slowly during early phases of the protocol and more rapidly at the onset of the FR2 trials (green) and remains stable post-BOP (red).

4.3.1.3. Electromyogram (EMG)

EMG measurements were recorded on the dorsal neck muscle. During the trial period, EMG data in Volts was recorded from each rat and analyzed independently, as illustrated in Figure 21, to obtain a clear signal for each section of the trial. The data selection process involved identifying intervals in the trial where no external stimuli, artifacts or noise were present in the EMG signal, to obtain clean and reliable data for subsequent analysis.

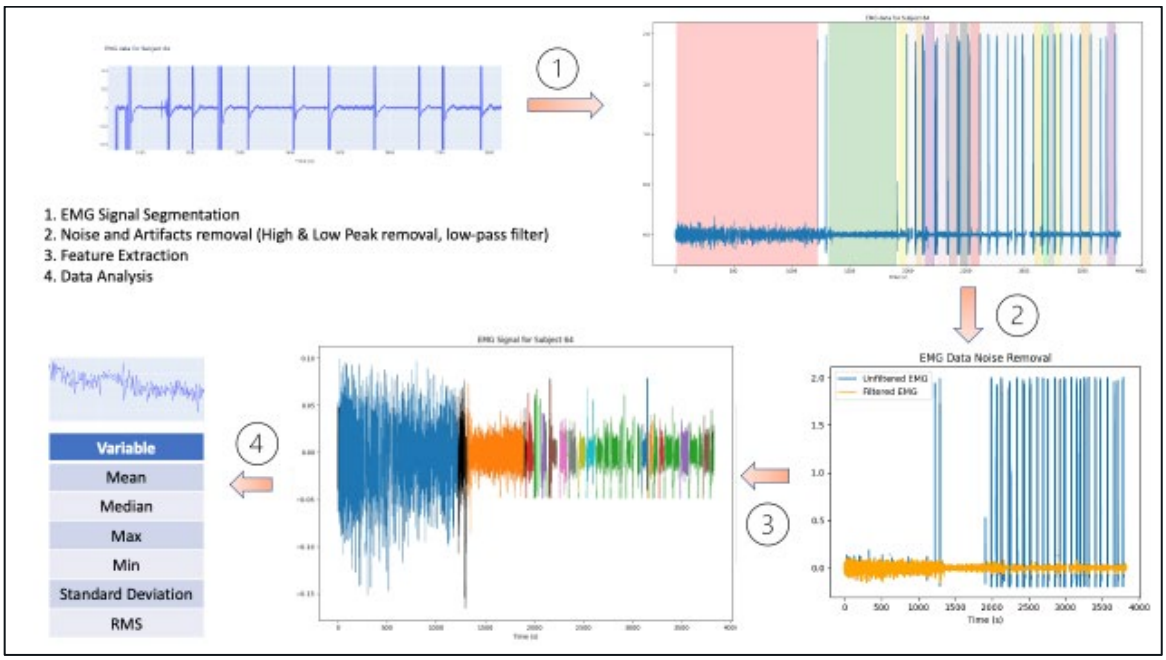

Figure 21. EMG signal processing steps (1- 4) was based on signal segmentation, cleaning, and analyzing data based on trial times (exploration, post FR1s, interment (i) and FR2s, post-Bop FR2s).

The clean extracted EMG signals where then analyzed based on defining muscle activity during each section by calculating the root mean square (RMS) amplitude for each interval defined. The RMS is a useful measure for detecting changes in muscle activity over time and can be used to compare activity across different conditions or timepoints (i.e., exploration, post FR1's, High Bop v Low Bop). RMS is calculated using the following equation:

$$RMS = \sqrt{\frac{1}{n}\sum x^2}$$

Provided that n is the number of data points in the interval and x is the amplitude of the EMG signal at each data point.

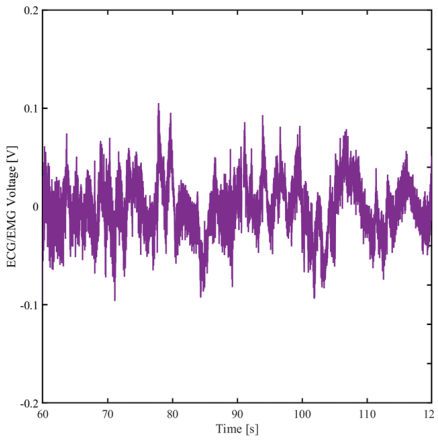

Figure 22. Clean electromyogram signals demonstrate contraction of the dorsal neck muscle.

With RMS defining total energy of the EMG signal, which is proportional to muscle activity it reflects the overall level of muscle activity. Higher values of RMS indicate active or an increase in muscle activity, while a lower RMS value is indicative of less muscle activity or inactivity.

4.3.1.4. Electrocardiogram (EKG/ECG)

Electrocardiogram is the measurement of activation of the cardiac muscle. Units of ECG are Volts. Various components of the ECG waveform may be analyzed such as the Q-R-S Complex and the heart rate. Heart rate (HR) was selected as the measurement of interest under this program. Figure 23 – Left shows a one second long recording of heart rate with the peaks of each muscle

contraction highlighted by red circles. Finding the difference in time of subsequent peaks yields the HR in beats per minute (BPM). Figure 23 – Right shows the HR derived from the difference in time of subsequent peaks for one subject. Vertical black lines are FR1 and FR2 trials with the red line indicating the onset of Trial 16 coincident with the BOP. Blue circles indicate raw peak-peak time difference and orange circles indicates data smoothed to remove volatility in the measurement and remove points which exhibit a non-physical instantaneous change in HR.

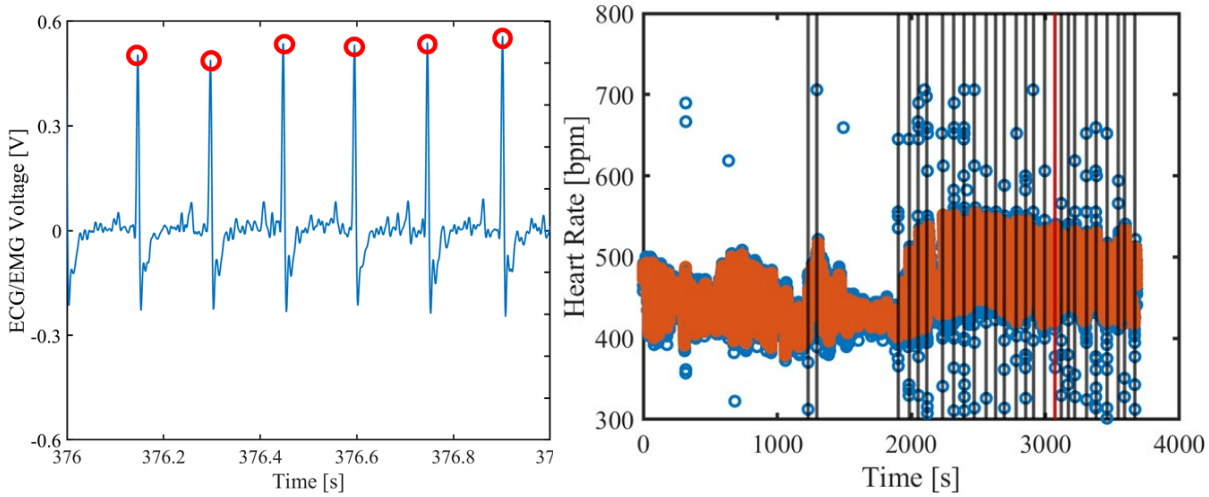

Figure 23. The time difference of the peaks from the ECG (left) may be converted to heart rate (right). Blue circles are raw calculations. Orange circles are filtered data. Black lines represent trial times with the trial succeeding the BOP shown in red.

4.3.2. Escape Behavior

The time to run back and forth through the doorway is recorded as a time, the Escape Latency, for each of the 25 Trials. Figure 24 shows the aggregate learning behavior for the 51 subjects used across all measures. Trials 1-3 are not included in analysis since these trials serve to orient the subject to the task and are not yet representative of learning behavior. Behavior was shown to asymptote to an escape latency around five seconds. Comparison of the performance between the Control and BOP subjects was analyzed after the BOP was administered before Trial 16 and is presented in Section 5.

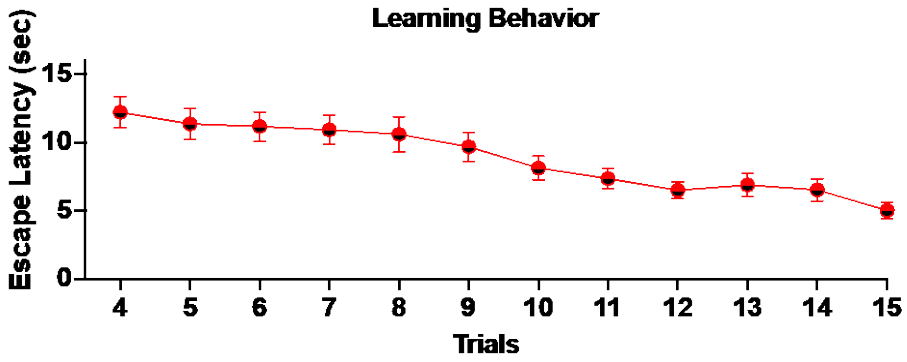

Figure 24. The aggregate learning behavior for all subjects under this protocol. Escape latency decreases as the subject learns the task.

4.3.3. Vocalizations

Ultrasonic vocalizations were recorded for each subject throughout the shuttle box protocol; an example of the types of vocalizations detected are shown in Figure 25. Individual calls are shown as chirps with the dominant frequency decreasing from near 30 kHz to near 23 kHz. Few rats produced vocalizations under this protocol, however, the duration and quantity of calls for each rat that did vocalize were calculated for each FR2 trial.

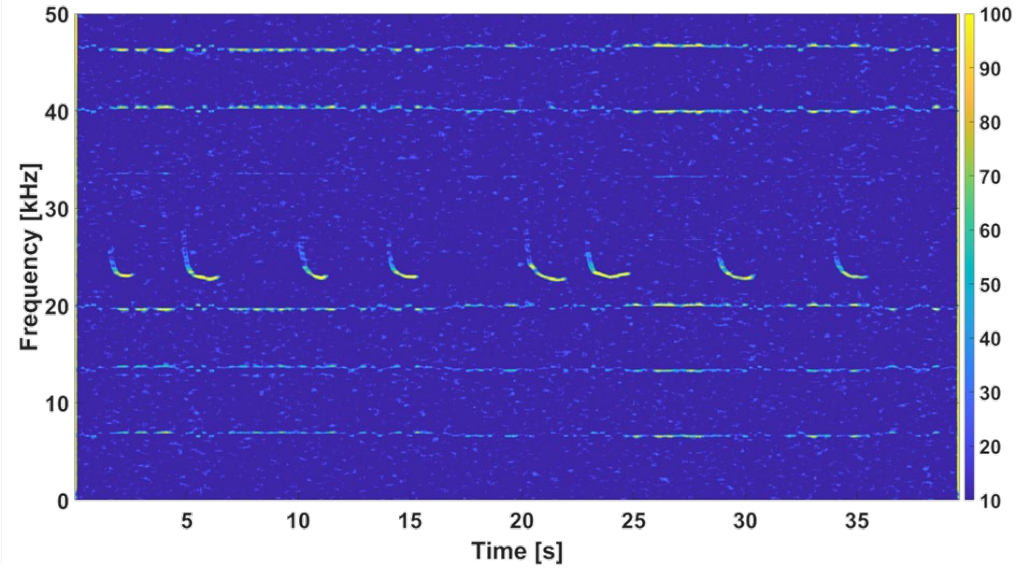

Figure 25. Vocalizations show as short downward chirps between 23 and 30 kHz.

4.3.4. Terminal Measures

Terminal measures of physiological stress were recorded to observe whether chronic stress was induced in each subject following the shuttle box escape tests. Two days after the test protocol was completed, the subjects were sacrificed and the weights of the thymus, spleen, and adrenal gland, organs associated with chronic stress indicators, and blood glucose, a biochemical marker of stress, were collected. Data for the full population is shown in Figure 26. The only differences observed is related to size; male rats are overall larger than female rats. Therefore, it was concluded that no chronic stress was induced and the observed stress could be contributed solely to this specific test protocol.

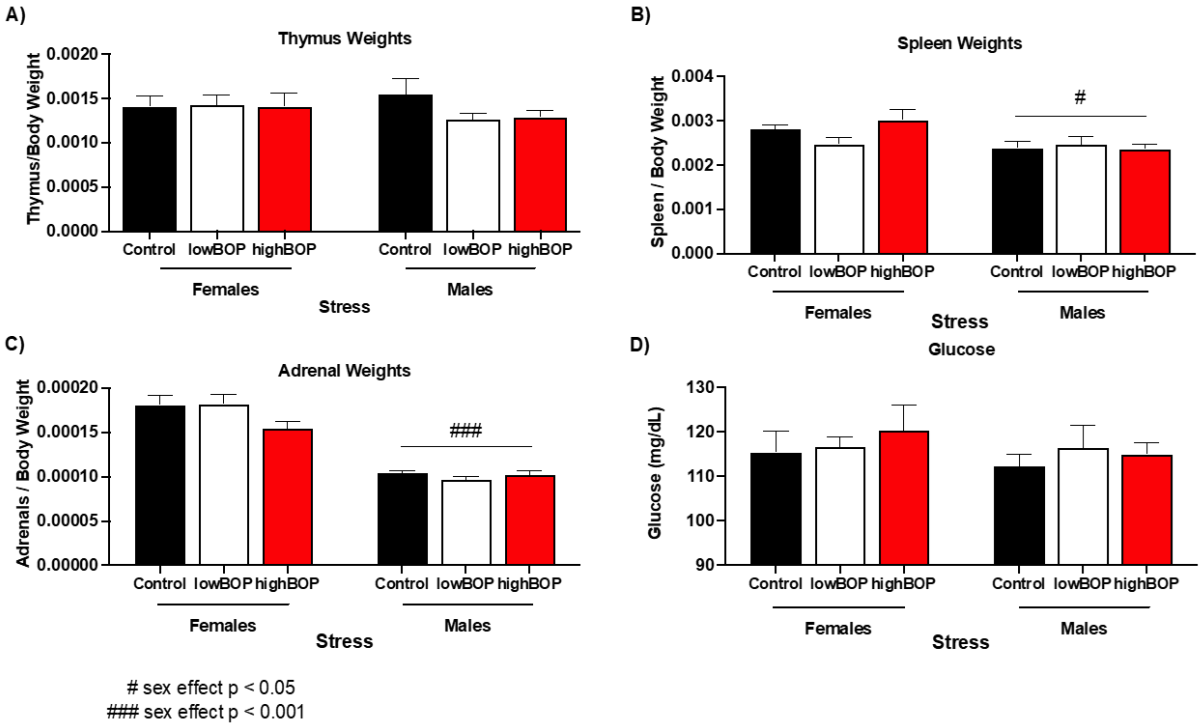

Figure 26. All terminal measures indicate no chronic stress was induced through the experiment. Only sex-based effects are observed.

5. Analysis

In this section the aggregate analysis of the data collected across all subjects is presented. Statistical analysis performed on each data type is shown with conclusions regarding the sequence of effects observed in both sexes and three test groups.

5.1. Statistical Approach

Escape latency data and heart rate correlation coefficient data were analyzed as repeated measures ANOVA across the 25 trials both before blast overpressure exposure (Trials 4-15) and after blast overpressure exposure (Trials 16-25). Telemetry data were also analyzed with repeated measures ANOVA across 1-min intervals. When necessary, pair-wise comparisons were examined using a bonferroni correction. All statistically significant findings are displayed embedded within the figures.

5.2. Statistical Outcomes

Analysis of each of the biotelemetry measures, escape behavior, and vocalizations were performed. Statistics were calculated where possible to determine whether observed effects were significant for the different test groups. Results for each data type are presented in the following sections.

5.2.1. Escape Behavior

Escape latency, defined as the time required to transit the shuttlebox door twice, was scored by the experimenters and recorded for subsequent analysis, if needed. The escape latency for each Trial was recorded and the performance was analyzed for pre-BOP (Trials 4 – 15) and post-BOP (Trials 16 – 25) regimes taken as a whole for each subject. Statistics for each individual Trial were not calculated.

Analysis of the escape latency is shown in Figure 27 for (top to bottom) all subjects, females, and males. Rats were randomly assigned to each experimental condition. Because there was no statistical difference between the groups in shuttle box escape latency prior to the BOP, shuttle box learning was averaged across the groups pre-BOP. Exposure to the High BOP, but not Low BOP, impacted performance of the learned behavior. Immediately post-BOP (FR-16), animals' latency to perform the task was fast, possibly due to the immediate startle response. Females, however, displayed a clear reduction in performance at FR-17. Interestingly, the nature of the impact of BOP exposure observed in the males was different than females, with males displaying a trend for performance deficits in later trials, although these changes were not significant due to the larger variability in performance across the Control and Low BOP conditions.

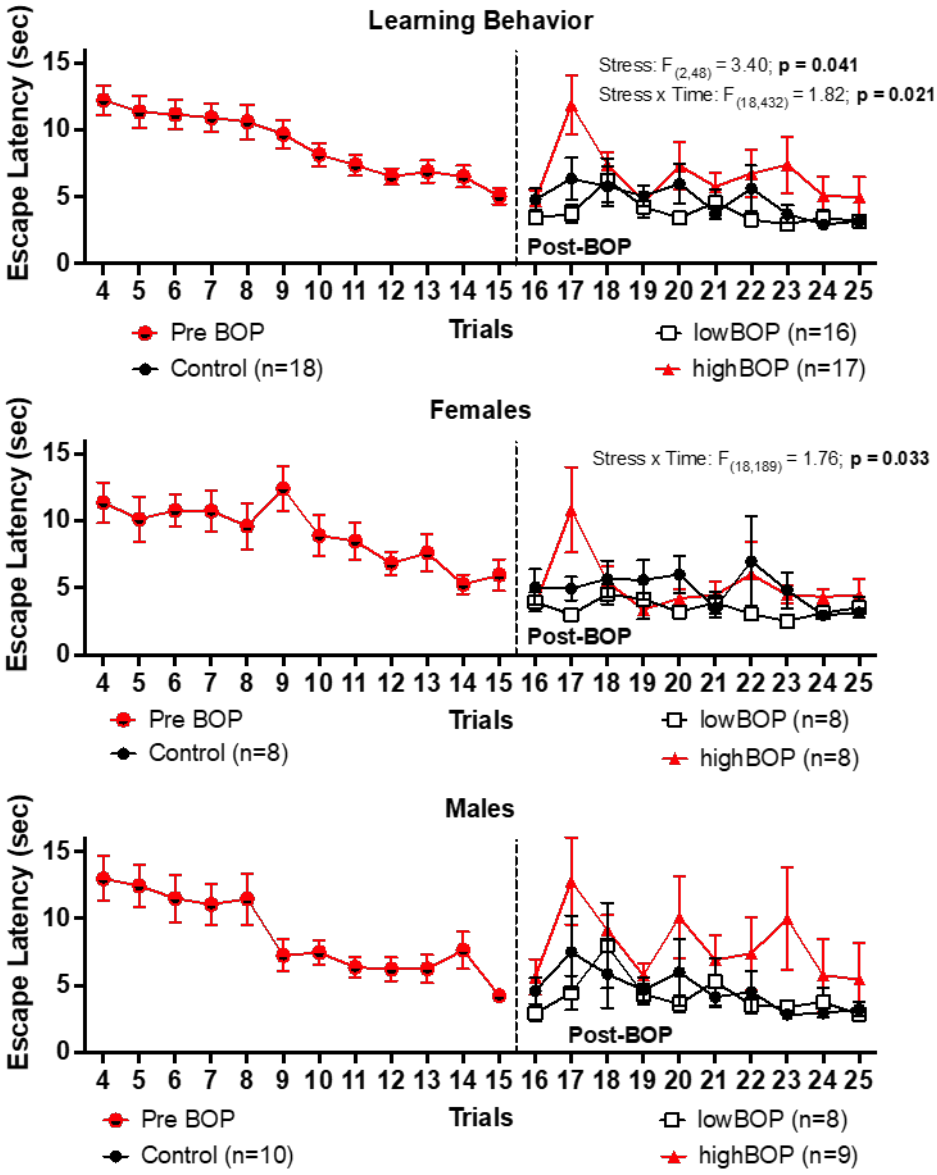

Figure 27. Escape latency versus trial number is shown for aggregated subjects, females, and males. Females exhibited a significant decrease in performance for Trial 17 for the High BOP condition. All other trials and conditions do not exhibit a significant change.

5.2.2. Locomotor Activity

Locomotor activity, defined as a period of motion within the shuttlebox, was analyzed for both sexes as shown in Figure 28. Data spanning the test protocol is shown in (A) for females and (B) for males. The different time regimes are:

- T < -40 min: Measurements in the home cage.
- -40 min < T < 0 min: Exploration, FR1, and pre-BOP FR2 trials (Trials 1-15).
- T = 0 min: Beginning of Trial 16 and BOP exposure for the Low BOP and High BOP conditions.
- 0 min < T < 20 min: post-BOP FR2 trials (Trials 16-25).
- T > 20 min: Measurements in the home cage.

Similar trends in LA were observed pre-BOP for both female and male subjects. On average, female subjects exhibited a greater level of LA for each condition. Following the BOP, a greater amount of variability is observed in the female High BOP group compared with the other groups. Although not statistically significant, this increased variability, shown in Figure 28 C) and D) with an expanded range, may be clearly observed in the minutes directly after the BOP with the exception of the time bin centered at four minutes which corresponds to the time of Trial 17.

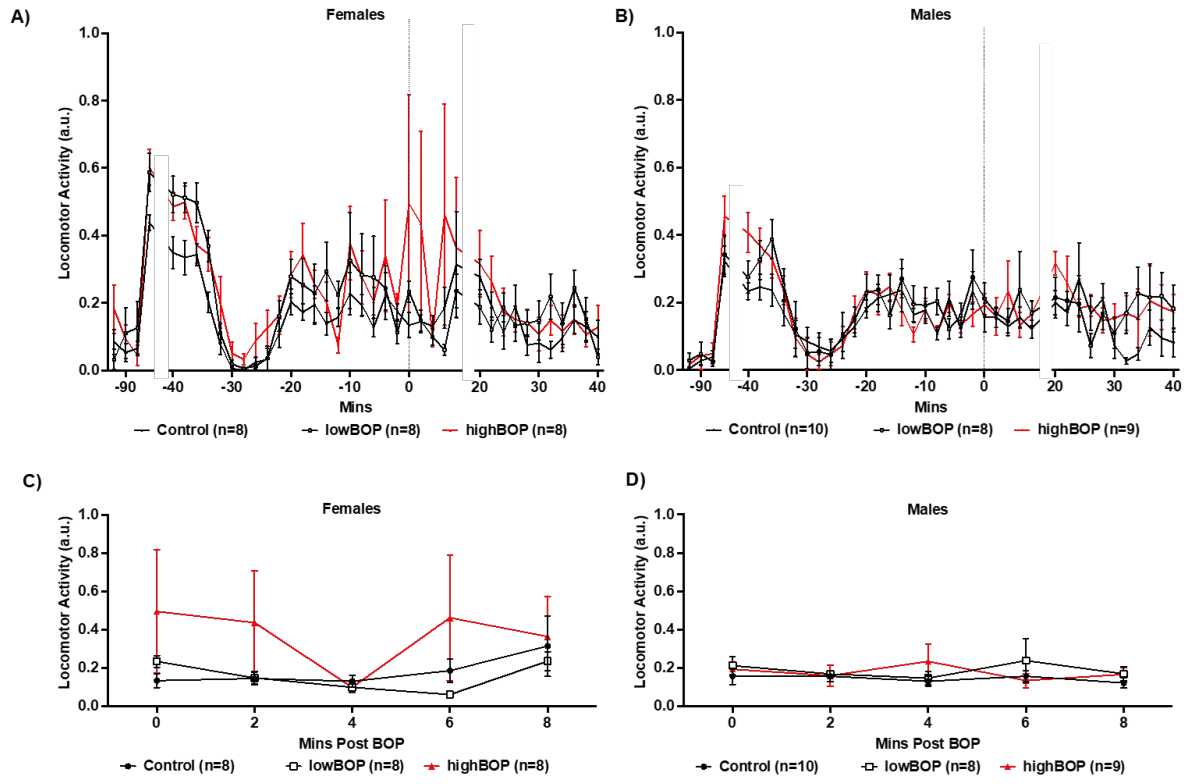

Figure 28. Locomotor activity (LA) for female (A,C) and male (B,D) subjects. Following the BOP, female subjects exhibited a much higher level of activity when compared with all other conditions.

5.2.3. Electromyograpy

The EMG of the dorsal neck muscle is representative of head motion in the subject. The root-mean-square voltage from the EMG is presented for females and males in Figure 29. EMG measurements were not significantly different before the BOP and were aggregated for the exploration (Exp.), FR1, and first fifteen FR2 Trials. Further, data for Trials 13-15 (pre FR2s) and Trials 16-18 (post FR2s) are presented. The post FR2 EMG was significantly lower for the High BOP condition than for the Low BOP or control conditions for the female subjects.

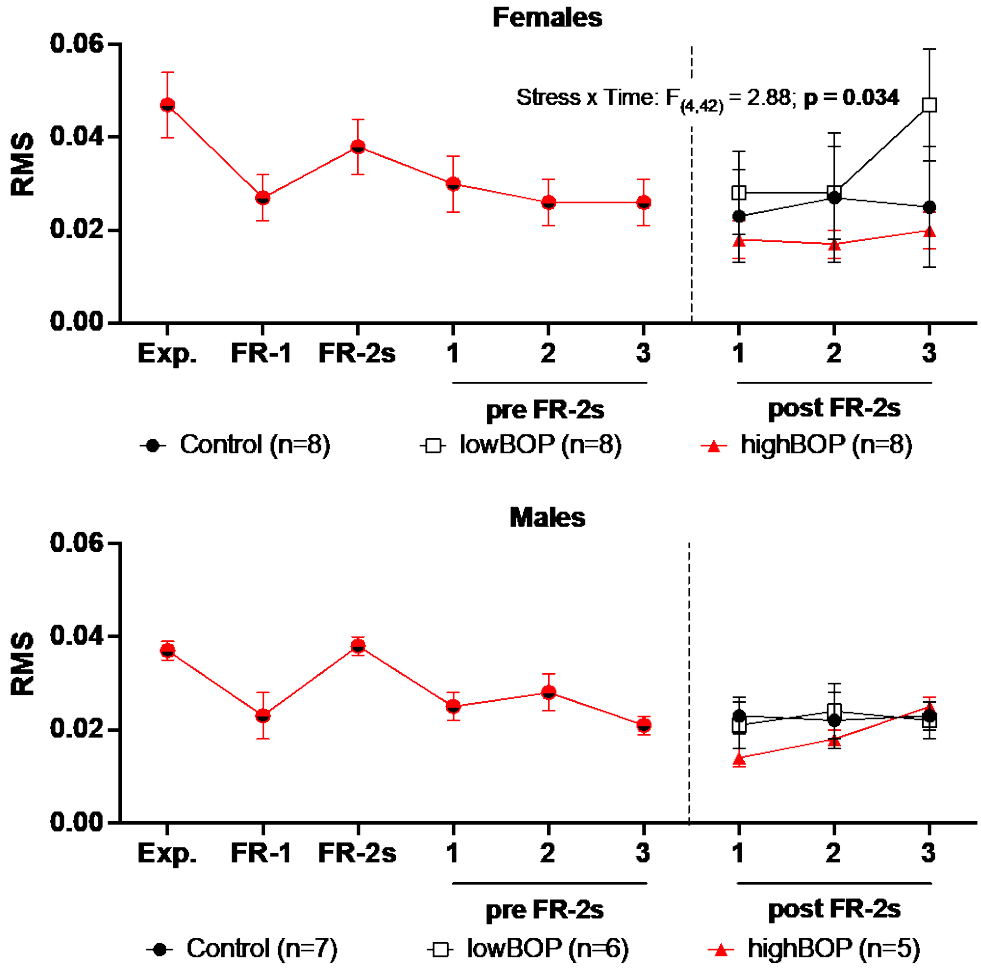

Figure 29. RMS EMG voltage for female (top) and male (bottom) subjects. FR-2 Trials are aggregated with Trials 1-12 collected under "FR-2s"; Trials 13, 14, and 15 under "pre FR-2s" 1, 2, and 3; and Trials 16, 17, and 18 under "post FR-2s" 1, 2, and 3. Males and females displayed no significant change in EMG between pre-BOP FR-2 Trials 13-15 and post-BOP FR-2 Trials 16-18. The high BOP condition females did show a significant difference between pre-BOP FR-2 Trials and post-BOP FR-2 Trials (p=0.034), indicating some reduction in neck muscle activity post-BOP.

5.2.4. Heart Rate Recovery

The rate of HR recovery is indicative of the rate at which the body processes the transient increase in HR due to motion and biochemistry. Figure 30 – Left shows an example of the HR for FR-2 Trial 19 for all female rats in the High BOP condition. Each HR is centered about its mean for the purposes of analysis. All subjects exhibit a similar trend in the decay rate of the HR toward a baseline.

To analyze HR recovery, the HR for each FR-2 trial was fit using

$$HR = A\,t^{B}$$

where t is time in seconds, A is the maximum heart rate for the trial in BPM, and B is the rate of decay of the heart rate in BPM/second. Due to individual variation in absolute HR, only the rate of decay, B, was analyzed further to examine the effects of stress on each subject population.

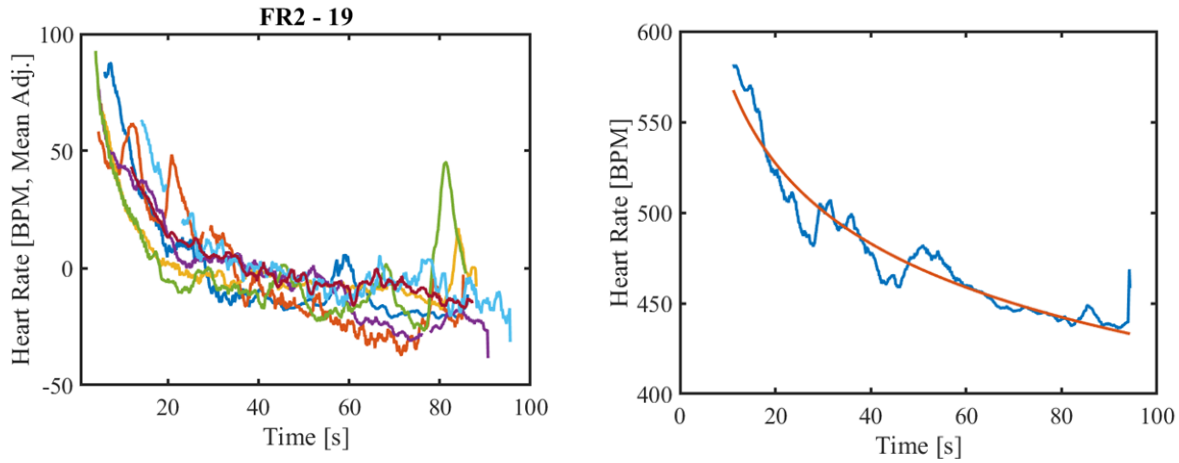

Figure 30. (Left) The heart rate for each subject for Trial 19 of the Female High BOP condition is shown. Each HR is adjusted relative to its mean HR for purposes of comparison. (Right) An example regression performed on the HR for each Trial was used to observe HR recovery rate. Data is in blue and the exponential fit is in orange.

As shown in Figure 31, no difference was found in the HR decay coefficient across each study condition pre-BOP and all data was aggregated. No significance was found between conditions for the combined male/female data or the male data in isolation post-BOP. Similarly, there was no observed effect in the Low BOP data relative to the control. The female data exhibited a significantly higher (more negative) decay rate indicating a faster change in overall HR toward the baseline.

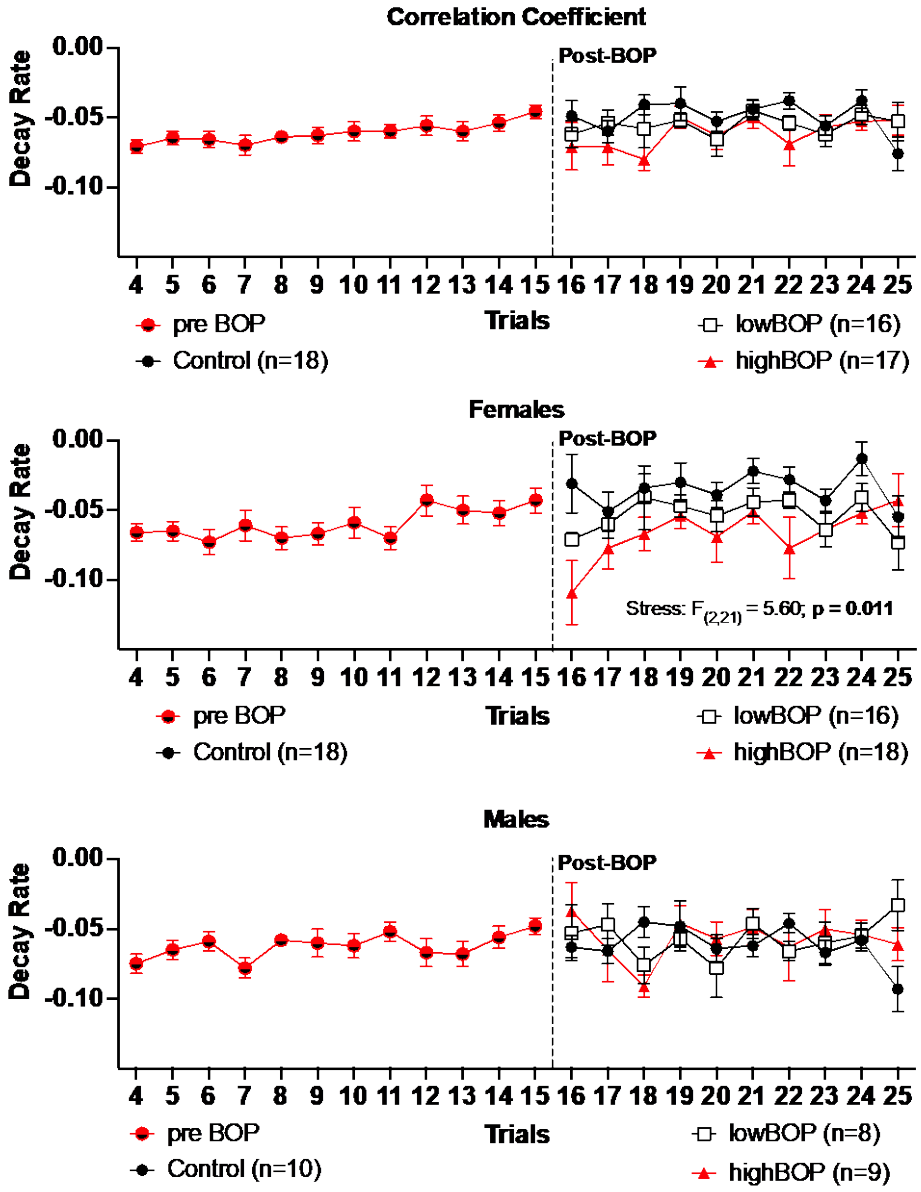

Figure 31. Heart Rate decay rate versus trial number is shown for aggregated subjects, females, and males. Females exhibited an increased decay rate for Trial 16 for the High BOP condition. All other trials and conditions do not exhibit a significant change.

5.2.5. Core Body Temperature

Core body temperature measurements are shown in Figure 32. Similar trends in CBT are shown for both female (A, C) and male (B, D) subjects. The different time regimes are:

- T < -40 min: Measurements in the home cage.
- -40 min < T < 0 min: Exploration, FR1, and pre-BOP FR2 trials (Trials 1-15).
- T = 0 min: Beginning of Trial 16 and BOP exposure for the Low BOP and High BOP conditions.
- 0 min < T < 20 min: post-BOP FR2 trials (Trials 16-25).
- T > 20 min: Measurements in the home cage.

For the time immediately following the BOP (Figure 32 C-D), the female subjects exhibited mild, yet significant, hypothermia for the High BOP condition which is consistent with a stress response. No significance was found for the other test conditions.

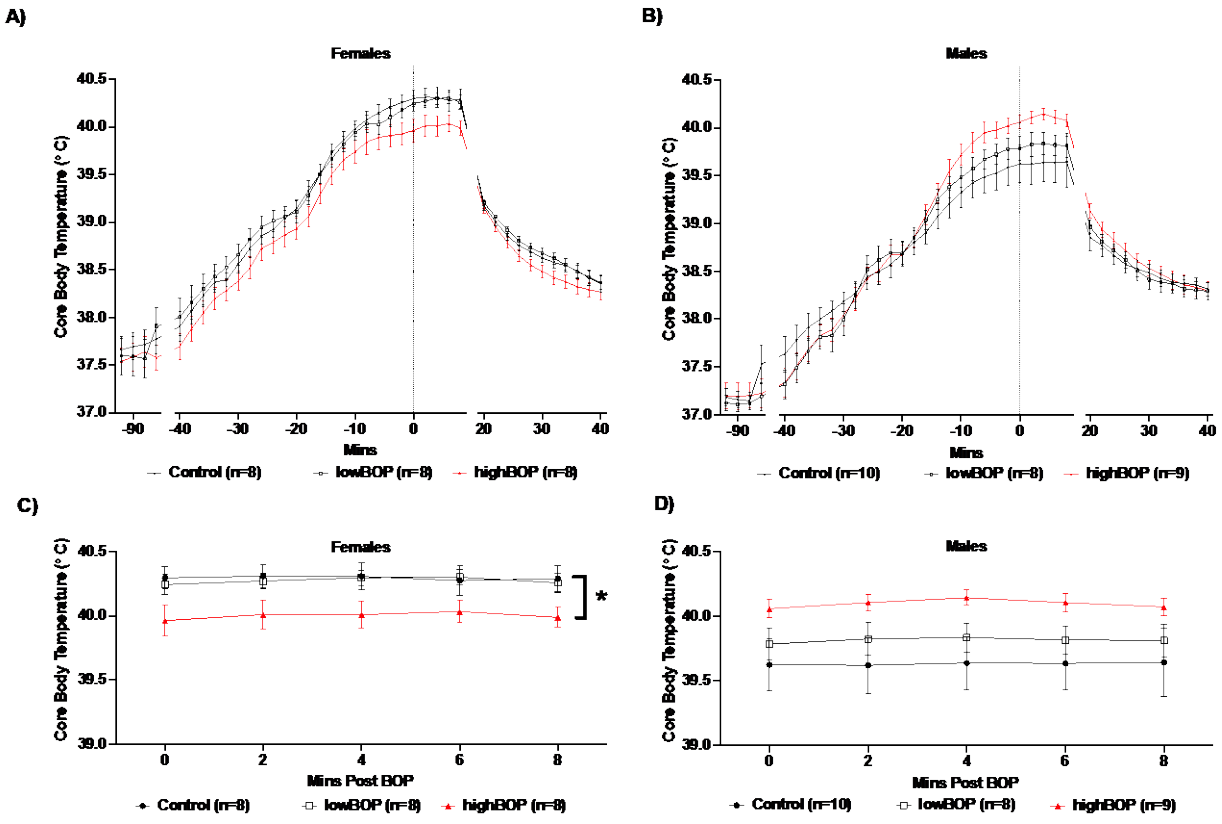

Figure 32. Core Body Temperature (CBT) for Female (left) and Male (right) subjects. Top: Time-resolved CBT is referenced to the time of BOP at t = 0 seconds. The break in the axis before T = 20 seconds is due to relocation from the test enclosure to the home cage. Bottom: Females exhibited a significant decrease in CBT post-High BOP.

5.2.6. Vocalizations

Based on the evidence provided by the biotelemetry data, analysis of ultrasonic vocalizations was limited to female subjects in the Control and High BOP conditions. Of these, only four of the Control and two of the High BOP subjects produced vocalizations. Due to the low number of subjects, statistical analysis could not be performed. However, the number of vocalizations versus trial number are shown in Figure 33. The two High BOP subjects produced few vocalizations across trials whereas the Control subjects produced a highly variable number of vocalizations across trials with the mean number of vocalizations decreasing as the subject proceeded through the protocol.

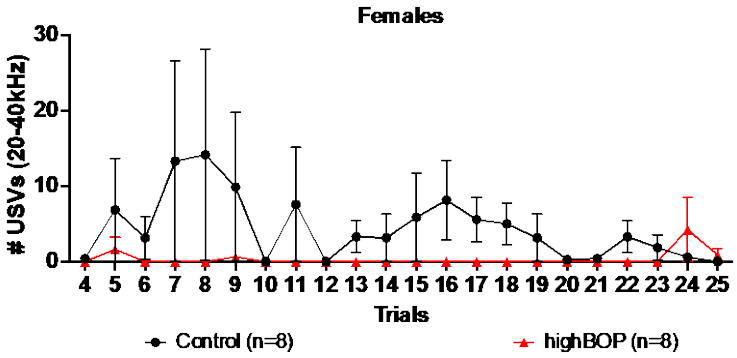

Figure 33. Ultrasonic vocalizations were produced by few subjects. For the subject population, control subjects produced more calls on average across trials than the High BOP subjects, though formal statistics could not be performed.

5.3. Analysis Summary

Based on significant historical data, the rat model is a reasonable first step in the development of a human model of flashbang behavioral response. Peak acoustic exposure levels for rats and humans are comparable, despite the significant difference in body mass. Furthermore, rats can be quickly trained to perform a variety of learned tasks on demand that provide a useful cognitive measure of the effects of the acoustic startle.

The physiological data (heart rate and heart rate recovery rate, core body temperature, EMG) and the escape latency demonstrate a sequence of events that, when taken together, indicate a causal relationship for female subjects in the High BOP condition. Immediately after BOP and FR-2 15, heart rate rapidly increases. Between FR-2 15 and 16, the rate of heart recovery is accelerated relative to low-BOP or controls and performance in the next trial (FR-2 17) is disrupted. In fact, the escape latency for females post-high BOP approximately doubles in Trial FR-2 17 before returning to pre-BOP levels. Following the BOP, transient effects on physiology are also present in the Core Body Temperature which increases during learning and in the EMG which shows decreased neck motion. These effects are not visible in the Control or Low BOP conditions indicating that the exposure levels selected statistically bound the onset of the stress effect on performance in a rodent model. These measures demonstrate a transient, stress-related response to the acoustic startle before the subjects return to baseline. After 48 hours post-BOP, organ weights demonstrate that there is no chronic stress condition induced by the exposure.

Male rats did not show a statistically significant difference in response post-BOP. This may be due to different physiology, or their large size and body mass require a higher exposure threshold than females.

6. Summary and Conclusions

6.1. Summary

To characterize the auditory effects of flashbang devices, the effects of acoustic startle on rat performance of a shuttlebox escape task was evaluated. Pilot studies were performed to optimize experimental parameters such as the strength of the foot shock stimulus and the placement of implanted biotelemetry systems. For the full protocol, a total of 95 subjects were enrolled with 69 completing the full protocol, however, 12 of those failed to learn the task and 4 had unreliable telemetry signals, and 2 had immune reactions around the implanted telemeter devices. Due to unforeseen complications, primarily in female rats, around 26 animals were lost due to difficulty with recovery from surgical implantations. Thus, 51 subjects successfully completed the protocol, were healthy, and had good data.

For each task, performance was benchmarked against a control condition with no overpressure stimulus. For the escape task, the overpressure stimulus followed literature trends by decreasing performance (increasing time) for the high (155 dB) exposure level. Similarly, multiple measures of stress (vital signs and locomotion) indicated increased levels of stress following higher exposure levels.

6.2. Limitations of Results

The results of this pilot study may have limitations due to:

- **Small sample size.** The statistical power of the data is low due to the small number or animal subjects successfully processed through the test protocol. Under the protocol, this study was designed to evaluate 80 rat subjects split among the two exposure levels, and both sexes to ensure at least ten subjects of each sex completed testing for each condition. More statistical power may be desired as the results of this study are interpreted.

- **Limited test conditions.** The pilot study incorporated two levels of BOP exposure and one level of impulse. The exposure levels selected give an impression of the trend in performance with increasing effect, but more intermediate effect strengths need to be exercised to understand limiting cases.

- **Limitations on noise exposure.** The level of the acoustic startle stimulus used in this protocol was selected to reliably elicit a stress response in the rat subjects while remaining intrinsically safe. However, this source is below even temporary injury threshold and will not incorporate potentially synergistic effects between multiple causal pathways shown to induce a stress response.

6.3. Next Steps in Analysis and Experimentation

Further studies of flashbang effectiveness can be broken into two categories: (1) studies that extend the findings from this study to a greater number of subjects to increase the statistical significance and to allow for more detailed analysis and (2) studies that expand and contrast our understanding of the auditory effects with other pathways from Figure 1 (Madhavan, 2018).

6.3.1. Extension of the Stress Effects Protocol

Further Understanding the Stress Effects Study

Due to the limited scope of this pilot study, the results show only preliminary trends in the data at a low statistical power. A larger study would allow for an increase in statistical power of the results by increasing the number of subjects and the number of conditions each subject could perform. As an example, more subjects following the same protocol with additional pressure levels would help to determine whether the peak pressure or other factors may be responsible for increased stress-related degradation in performance in the shuttle box escape test.

Effect Threshold Study

Two peak overpressure levels and a single source duration were a part of the current test protocol. The levels for these stimuli were chosen to prevent the activation of the tinnitus/TTS pathways and performance degradation was still observed in female subjects. However, the threshold for onset of performance degradation is unknown. A study specifically focused on spanning a range of TTS or exposure levels would allow for the development of a dose-response curve for degradation in performance. This would be analogous to an 'effect risk criteria' in contrast with a typical 'injury risk criteria,' where the probability of causing a 30% degradation in performance capability with a specific device could be determined.

Additional Stress Metrics

In addition to expanding the test matrix to enable development of a dose-response curve, the results of this study have demonstrated several gaps in the rat behavioral model related to the study protocol performed herein. These studies would clarify some of the performance differences noted in the data presented above.

- The protocol only included a single BOP duration for both the low and high BOP exposure groups. Historical blast exposure studies have demonstrated that the total energy in a BOP exposure has a significant effect on injury risk. By examining the effect of acoustic duration on behavioral and physiological outcomes we can clarify if the duration effects extend to these low-level blast exposures.
- The males did not demonstrate the same change in behavior post-BOP as did the females. Among the possible explanations is that sympathetic (SNS) and parasympathetic nervous system (PNS) drive during shuttle box learning (FR2 4-15) and shuttle box performance (FR2 16-25) are different in males and females. Consequently, High BOP exposure results in a PNS burst that may contribute to the transient disruption of performance of a learned behavior to one sex and not the other. By extending the exposure levels to higher pressure levels for the males, we can determine if this mechanism is the determining factor in the sex difference.
- The footshock administered to motivate the rats to perform their behavioral task is in itself a stressful event which can impact the physiological measurements. Consequently, the rats are already in a stressed state at the time of the BOP exposure. Given it is well-established in the human literature that the behavioral impacts of BOPs are impacted by subject state of stress or arousal, it is important to compare the innate behavioral (startle, freezing) and physiological responses (HR, CBT, EMG) to BOP in the absence of shuttle box testing. A test series with BOP exposure in the absence of footshock to compare physiological responses would characterize the physiological responses caused by the footshock task.

6.3.2. Expansion of the Stress Effects Protocol to Additional Pathways

Add Flash for Synergistic Auditory/Visual effects

In a similar manner as this pilot study, additional pilot studies looking at other pathways could be performed to gain similar insight into the dominant performance disruption mechanisms. For example, evaluation of the V1/V3/V4/V5 portion of the causal diagram could be conducted using the same experimental design as the auditory effects study by using an LED panel or flashlamp in addition to the acoustic stimuli. The study team believes this study is an important next step due to the similarity in processing paths for the visual and auditory signals in the brain and included the use of such a flash in the approved human subject protocol in the event this study expanded to include this stimulus.

Analysis of the Stress Response in Humans

In the causal pathway analysis, the stress response is a nexus for the full range of auditory, visual, and overpressure effects. Study of this specific component is critical to determining how the stress response incorporates these different stimuli as it meditates the human psychological, physiological, and behavioral response. Human subjects can be considered for study of the auditory and visual effects, though studies in which stress is intentionally inflicted would need to be carefully designed to gain maximum information from the fewest trials.

6.3.3. Translation of the Stress Effects Protocol to Human Relevance

This test protocol was designed to use pressures just below the TTS threshold for the subjects. For the shock tube source, this corresponds to Sound Exposure Levels of 105 dB (Low) and 117 dB (High). The 50th percentile 1 dB TTS threshold for humans predicted by AUDITORY 4.0 is approximately 117 dB. Therefore, tests in this protocol were conducted at the same threshold for TTS in both rats and humans. Scaling for effect may require changes to the duration of the acoustic exposure for humans similar to the methods where blast exposure durations are scaled based on body weight.

Since there is consistency in the injury thresholds for this exposure, impacts of the stress response on human performance may be inferred from the stress response of the rat model. Task performance may:

- Be unaffected immediately following exposure
- Decrease significantly minutes after exposure before returning to baseline

Other mechanisms may further affect performance in humans. An additional factor in comparing the response of humans to an acoustic startle from the previous human auditory effects pilot study to rats in the current rat study is that the response task for the humans was an auditory trigger, whereas the response task for the rats was a tactile trigger (e.g., non-injurious foot shock). Since the trigger and startle occur using different neurological mechanisms, there may be an intrinsic difference in response based on the separate neurological pathways used for each. This could likely affect the delay in response seen in the rats versus humans. Further testing can verify this pathway question between humans and rats by using a haptic trigger for the response task in the human study, as there are no validated acoustic triggers available for the rat model.

6.4. Concluding Remarks

The degradation in performance observed in this study serves to clarify the role of the overpressure (O) and startle (SE) pathways within the causal model. Specifically, increasing degradation in performance with increasing BOP levels, although expected, validates these pathways have an increasing effect with closer proximity to a flashbang or higher source level. The overpressure proved to critically decrease performance in female subjects, who were delayed in completing the learned task, thereby demonstrating the relative strength of this pathway in isolation.

To further illuminate the causal model for flashbang effects, additional work needs to be performed both to validate the results of this program and further evaluate the different pathways to determine the dominant effects and interactions driving human response to flashbangs. A further study focusing on strengthening our understanding of the differences and synergies between BOP exposure levels and impulse level could be performed. Further studies to examine the individual overpressure and visual response pathways (and any synergistic effect when combined with auditory stimuli) would continue to validate the causal model and deepen our understanding of human and animal response to these events. In the end, study of the synergistic effects of the dominant mechanisms of flashbang effectiveness will optimize injury risk analyses and specialize device development using this causal framework.

6.5. Acknowledgements

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