AFRL-RH-WP-TP-2014-0061

Evaluation of Jet Fuel and Noise-Induced Hearing Loss in Rats (Rattus norvegicus)

David R. Mattie
Bioeffects Division
Molecular Bioeffects Branch

Brian A. Wong
Shawn M. McInturf
James E. Reboulet
Pedro A. Ortiz
Naval Medical Research Unit – Dayton
Wright Patterson Air Force Base OH

O'neil W. Guthrie
Loma Linda Veterans Affairs Medical Center
Loma Linda CA
School of Medicine, Loma Linda University Medical Center
Loma Linda CA

May 2014

Final Report for October 2010 to October 2013

STINFO Copy

Air Force Research Laboratory
711th Human Performance Wing
Human Effectiveness Directorate
Bioeffects Division
Molecular Bioeffects Branch
Wright-Patterson AFB OH 45433-5707

NOTICE AND SIGNATURE PAGE

Using Government drawings, specifications, or other data included in this document for any purpose other than Government procurement does not in any way obligate the U.S. Government. The fact that the Government formulated or supplied the drawings, specifications, or other data does not license the holder or any other person or corporation; or convey any rights or permission to manufacture, use, or sell any patented invention that may relate to them.

Qualified requestors may obtain copies of this report from the Defense Technical Information Center (DTIC) (http://www.dtic.mil).

The experiments reported were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

(AFRL-RH-WP-TR - 2014 - 0061) has been reviewed and is approved for publication in accordance with assigned distribution statement.

MATTIE.DAVID.R.1230101880

Digitally signed by MATTIE.DAVID.R.1230101880
DN: c=US, o=U.S. Government, ou=DoD, ou=PKI, ou=USAF, cn=MATTIE.DAVID.R.1230101880
Date: 2014.12.04 14:47:30 -05'00'

DAVID R. MATTIE, Work Unit Manager
Molecular Bioeffects Branch

POLHAMUS.GARRETT.D.1175839484

Digitally signed by POLHAMUS.GARRETT.D.1175839484
DN: c=US, o=U.S. Government, ou=DoD, ou=PKI, ou=USAF, cn=POLHAMUS.GARRETT.D.1175839484
Date: 2014.12.17 08:18:14 -06'00'

GARRETT D. POLHAMUS, DR-IV, DAF
Chief, Bioeffects Division
Human Effectiveness Directorate
711th Human Performance Wing
Air Force Research Laboratory

This report is published in the interest of scientific and technical information exchange, and its publication does not constitute the Government’s approval or disapproval of its ideas or findings.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
13-05-2014	Final	Oct 2010 – Oct 2013

4. TITLE AND SUBTITLE
Evaluation of Jet Fuel and Noise-Induced Hearing Loss in Rats (*Rattus norvegicus*)

5a. CONTRACT NUMBER
FA8650-10-2-6062

5b. GRANT NUMBER
NA

5c. PROGRAM ELEMENT NUMBER
62202F

6. AUTHOR(S)
Mattie, David R.*; Wong, Brian A.[1]; McInturf, Shawn M.[1]; Reboulet, James E.[1]; Ortiz, Pedro A.[1]; Guthrie, O'neil W.[2,3]

5d. PROJECT NUMBER
OAFW

5e. TASK NUMBER
P0

5f. WORK UNIT NUMBER
OAFWP001/**H04R**

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
[1]NAMRU-D, 2729 R St, Bldg 837, WPAFB OH 45433-5707
[2]Loma Linda Veterans Affairs Medical Center, 11201 Benton St, Loma Linda CA 92357
[3]School of Medicine, Loma Linda University Medical Center, 11175 Campus Street, Loma Linda CA 92350

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Air Force Materiel Command*
Air Force Research Laboratory
711th Human Performance Wing
Human Effectiveness Directorate
Bioeffects Division
Molecular Bioeffects Branch
Wright-Patterson AFB OH 45433-5707

10. SPONSOR/MONITOR'S ACRONYM(S)
711 HPW/RHDJ

11. SPONSORING/MONITORING AGENCY REPORT NUMBER
AFRL-RH-WP-TR-2014-0061

12. DISTRIBUTION AVAILABILITY STATEMENT
A: Approved for public release (PA); distribution unlimited. PA Case No: 88ABW-2014-3358 Date Cleared 14 July 2014

13. SUPPLEMENTARY NOTES

14. ABSTRACT
This study investigated whether JP-8 jet fuel contributes to hearing loss when combined with noise below the exposure limit for noise injury. Two whole body 28-day inhalation exposures were conducted to determine the combined effect of noise plus JP-8 on hearing. Both studies included control, JP-8 only, noise only, and noise + JP-8 groups (10 male and 10 female rats, N=20 per group). The JP-8 exposure was 1000 mg/m3 and the noise dose was an 8 kHz octave band noise at 85 dB sound pressure level; exposures lasted 6 hours per day, five days per week for 4 weeks. The first study used 80 Long Evans (LE) rats, while the second study utilized 80 Fischer 344 (F344) rats. Four weeks post-exposure, LE and F344 rats exposed to noise and JP-8 separately and in combination displayed no permanent peripheral hearing impairment at JP-8 exposure levels within the range that previously showed changes in F344 rats with higher sound levels. Auditory function was not different between groups, as assessed by distortion product otoacoustic emissions (DPOAE) and compound action potential (CAP) testing of the cochlear nerve. Inner ear hair cell loss was also not affected. Peripheral JP-8 ototoxicity may depend on the presence of damaging (higher dB) levels of noise. However, a pilot assessment indicated a central auditory processing dysfunction (i.e., impaired brainstem encoding of stimulus intensity) among LE rats exposed to JP-8 alone and JP-8 with noise; further research of this effect is warranted.

15. SUBJECT TERMS
JP-8, noise, hearing loss, Long Evans rats, Fischer 344 rats

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			David R. Mattie
U	U	U	SAR	39	19b. TELEPONE NUMBER (*Include area code*) NA

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI-Std Z39-18

THIS PAGE INTENTIONALLY LEFT BLANK.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

THIS PAGE INTENTIONALLY LEFT BLANK.

PREFACE

Funding for this project was provided through Defense Health Program (DHP) from the Surgeon General's (SG) Air Force Medical Support Agency (AFMSA) (SG5I/Medical Research and Innovations/Force Health Protection). This research was conducted under contract FA8650-10-2-6062 with the Henry M. Jackson Foundation for the Advancement of Military Medicine and NAMRU-D work unit number 61062. The program manager for the contract was David R. Mattie, PhD (711 HPW/RHDJ), who was also the technical manager for this project. The additional electrophysiology was supported by a CDA-2 (C7600-W) Award from the Rehabilitation Research and Development Service of the Office of Research and Development United States Department of Veterans Affairs-Veterans Health Administration.

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at both Wright-Patterson AFB (F-WA-2012-0136-A) and the Loma Linda Veterans Affairs Medical Center (0003/967). The animal protocols were in compliance with the following regulations: 1) Title 9 Code of Federal Regulations, Chapter 1, Subchapter A: Animal Welfare; 2) Department of Defense (DoD) Instruction 3216.01; 3) Air Force Manual (AFMAN) 40-401; and 4) the 2011 Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council.

The authors would like to acknowledge Ms. Teresa R. Sterner for assistance in preparing this report for submission to the SG AFMSA, as well as final preparation for publication.

THIS PAGE INTENTIONALLY LEFT BLANK.

1.0 SUMMARY

The objective of this research is to study exposure conditions to noise and JP-8 jet fuel that can increase the risk of hearing loss so that this disability can be reduced in the military. The hypothesis is that jet fuel contributes to hearing loss when combined with noise exposure that is below the occupationally recognized limits for noise-induced auditory injury. Our study design, developed and reported previously, involved exposure to noise 5 dB below the Occupational Safety and Health Administration (OSHA) standard of 90 dB and to jet fuel at a concentration where changes in hearing were observed in previous studies with damaging noise levels.

In order to determine the combined effect of noise plus JP-8 on hearing, two 28-day industrial toxicity inhalation exposures were conducted. In both studies, animals were exposed to noise plus JP-8 for 6 hours per day, five days per week over 4 weeks for a total of 20 exposures. The first study used 80 Long Evans (LE) rats, while the second study was performed with 80 Fischer 344 (F344) rats. In both studies, rats were divided into four groups. Each study included a control group, a JP-8 only group, a noise only group, and a noise plus JP-8 group; each group consisted of 10 male and 10 female rats, with an N of 20 per group. The JP-8 exposure was 1000 mg/m3 and the noise dose was an 8 kHz octave band noise (OBN) at 85 dB sound pressure level (SPL). All groups of rats were treated at the same time using a different whole body inhalation chamber for each group.

Permanent hearing loss was determined four weeks after exposure to allow time for any temporary hearing loss to recover. Peripheral auditory function was assessed by performing distortion product otoacoustic emissions (DPOAE) testing and electrophysiologic recordings of neural thresholds to frequency specific stimuli (compound action potential (CAP) testing). A microscopic examination of the inner ear (cochlea) was conducted to determine the percentage of receptor (outer hair cell) loss in the inner ear (cochleograms). DPOAE data, CAP data and cochleograms for LE and F344 rats exposed to noise and JP-8 jet fuel separately and in combination did not find hearing impairment at JP-8 concentrations that were within the range that previously showed changes in F344 rats, when combined with higher (102 dB) noise levels. These data suggest that accelerated hearing damage caused by JP-8 to the peripheral sensory hearing process may require the presence of damaging levels of noise.

In an additional pilot study, central auditory nervous system (CANS) function assessments were conducted on five rats from each group. The assessments were centered on auditory brainstem (ABS) evoked potential recordings. Preliminary results from the first study with LE rats are briefly discussed and reveal the presence of a central auditory processing dysfunction (CAPD), shown as impaired brainstem encoding of stimulus intensity in response to sound, in rats exposed to JP-8 alone and JP-8 combined with noise. The results of the second study with F344 rats are not available at this time and will be reported in a separate publication. Based on preliminary pilot study results, further research of the effect of JP-8 on the central auditory hearing loss is warranted.

2.0 INTRODUCTION

Individuals who work within and around aircraft are at risk for developing hearing loss. Studies have demonstrated that pilots, aircrew, aircraft technicians and mechanics have high rates (32 to 47 percent prevalence) of hearing loss when compared to audiologically normal populations from large databases (Fitzpatrick, 1988; Jaruchinda *et al.*, 2005). For instance, aircraft technicians and mechanics in the commercial aviation industry develop hearing loss at younger ages (30 to 40 years) compared to age matched populations (Smedje *et al.*, 2011). Additionally, Air Force F-111 fuel tank maintenance workers were shown to have lower than expected hearing thresholds when compared to published data from otologically normal populations (Guest *et al.*, 2010). Furthermore, military fighter pilots and helicopter pilots are known to be at high risk for developing hearing loss (Raynal *et al.*, 2006). The hearing loss typically assumes a sloping high frequency configuration, even with the use of hearing protection including ear plugs, headsets or helmets (Jaruchinda *et al.*, 2005).

Noise over-exposure is a prominent factor in the development of aerospace human hearing loss. For instance, the measured noise level (91 to 110 dBA) within the cockpit and around the aircraft may exceed the values for Occupational Safety and Health Administration's (OSHA) permissible exposure limit (PEL) of 90 dBA for an 8 hour workday and the National Institute for Occupational Safety and Health's (NIOSH) recommended exposure limit (REL) of 85 dBA for an 8 hour workday (Jaruchinda *et al.*, 2005). The occupational exposure limit for JP-8 is 200 mg/m^3 for an 8 hour workday. Preliminary epidemiologic analyses on aircraft maintenance personnel have concluded that jet propulsion fuels (JP-8 or JP-4) may interact with noise exposures to further induce or accelerate hearing loss (Kaufman *et al.*, 2005). Recent animal studies have confirmed this conclusion by revealing that exposure to JP-8 combined with noise may result in the loss of pre-neural cochlear sensitivity as shown by suppression of distortion product otoacoustic emissions (DPOAE) and depletion of cochlear sensory cells as evidenced by cytocochleograms that plot the percentage of missing outer hair cells (Fechter *et al.*, 2007, 2010, 2012). Interestingly, the effect of combining the exposures (jet fuel plus noise) was greater than that of the individual exposures. These findings are particularly important because military and government regulations regarding toxic exposures are often based on exposure to a single agent and less is known about combined exposure to jet fuel and noise.

The goal of the present project is to determine whether or not combined exposure to low levels of jet fuel and non-damaging noise interact to induce permanent peripheral auditory dysfunction. A previous study on Fischer 344 rats showed that 1000 mg/m^3, 6 hours per day, 5 days per week for 4 weeks (20 total exposures) of JP-8 and 102 dB of noise for 15 minutes each hour (90 minutes total exposure) did not induce hearing loss when individually applied, but combined exposures were ototoxic to the peripheral auditory system (Fechter *et al.*, 2012).

In the current project, the exposure design of Fechter *et al.* (2012) was used (standard industrial toxicity inhalation exposures of 6 hours per day, 5 days per week for 4 weeks, 20 total exposures), except for a lower continuous noise level of 85 dB during the exposure. The first study used Long Evans (LE) rats and the second utilized Fischer 344 (F344) rats. A previous study was performed with Fischer 344 rats to link the results to kinetic data available for this strain (Fechter *et al.*, 2012); Long Evans rats had also been used for hearing loss studies (Fechter

et al., 2007 and 2010). This project was designed to investigate potential strain differences. A noise level of 85 dB for 6 hours per day is considered legally safe according to OSHA and meets NIOSH's REL as well. Auditory function was assessed by performing DPOAE testing and electrophysiologic recordings of neural thresholds to frequency specific stimuli. A microscopic examination of the inner ear (cochlea) was conducted to determine the percentage of receptor (outer hair cell) loss in the ear.

3.1 METHODS

3.2 Animals

Eighty pigmented Long-Evans rats served as subjects in the first study and eighty albino Fischer 344 rats were used in the second study. Half the animals were males and the other half were females; animals were 4-5 weeks old at the beginning of the study. The animals were acquired from Charles River Laboratories (Wilmington MA) and housed at the 711HPW RHDV animal facility at Wright-Patterson Air Force Base (WPAFB) OH. The animals were given one week to acclimate to the animal facility and had free access to food and water. In order to identify animals with compromised auditory function, a baseline screening of DPOAE levels was conducted on each animal. All the animals exhibited normal DPOAE levels as determined by normative values established in our laboratory for the Long-Evans and F344 rat strains (Guthrie *et al.*, 2011). The animals were then randomized into four experimental groups, where each group consisted of 10 males and 10 females; groups consisted of a control group (n = 20), a noise-only group (n = 20), a jet fuel-only group (n = 20) and a jet fuel plus noise group (n = 20).

The goal of this project was to evaluate permanent loss of peripheral auditory function following exposure. Each experimental group received their respective exposure at the Navy Medical Research Unit – Dayton (NAMRU-D) inhalation facility at WPAFB. During the week following the final exposure, all rats were shipped overnight to the vivarium at the Loma Linda VA Medical Center (Loma Linda CA) for further auditory assessments. At the Medical Center, the animals were given four weeks to recover from any temporary hearing loss due to the exposures or the shipping. Permanent loss of peripheral auditory function from jet fuel, noise or combined jet fuel plus noise has been shown to occur at four weeks post-exposure in Long-Evans (Fechter *et al.*, 2007, 2010) and F344 rats (Fechter *et al.*, 2012). After four weeks, all the animals were evaluated for evidence of peripheral auditory dysfunction by measuring DPOAEs, recording puretone-auditory brainstem responses (ABRs) and constructing cytocochleograms of outer hair cells. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at both WPAFB and the Medical Center. Furthermore, the animal protocols were in compliance with the following regulations: 1) Title 9 Code of Federal Regulations, Chapter 1, Subchapter A: Animal Welfare; 2) Department of Defense (DoD) Instruction 3216.01; 3) Air Force Manual (AFMAN) 40-401; and 4) the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council (NRC, 2011).

3.3 JP-8 Exposure

The fuel was supplied from a stock maintained by the AFRL Fuels and Energy Branch (AFRL/RQTF) at WPAFB OH. The fuel consisted of a blend of petroleum-derived Jet A fuels obtained from various refineries (POSF 4658). The fuel was converted to JP-8 by the additive package, consisting of diethylene glycol monomethyl ether to inhibit ice formation plus static and corrosion inhibitors. A single lot of fuel was used to complete all of the studies described here.

Both the fuel generation and exposure systems for JP-8 have been described previously (Fechter *et al.*, 2012). The 1000 mg/m^3 concentration in this project used a Sonomist HSS600-1 nebulizer (Misonix, Inc., Farmingdale NY), no orifice plate, and a 0.5-inch line to the chamber. To eliminate problems with occasional malfunction due to liquid jet fuel accumulation around the nebulizer, the nozzle was positioned pointing down. To eliminate accumulating too much jet fuel at the drain ports, auxiliary air was added to the nebulizer chamber.

The rats were exposed to JP-8 using a whole-body system consisting of 690-L Toxic Hazard Research Unit (THRU) chambers operated by NAMRU-D. Each chamber was operated with a total flow of 180 lpm consisting of the combination of jet fuel generator input and the main airflow. The main airflow was supplied by two vortex blowers (model VB030SB-012 Spencer Turbine Company, Windsor CT); one provided input air and one handled exhaust flow. The exhaust air flow was adjusted to maintain a slightly negative pressure (1 to 2 inches of water below ambient) inside the chamber, as measured with a magnehelic pressure gauge (Dwyer Instruments, Champlain NY) attached to the upper plenum of the chamber. Airflow through the chambers was controlled with mechanical valves, which were adjusted to obtain the desired flow rate. Flow rate was monitored on the input side of the chamber using a Hastings (model LSD58D, Teledyne-Hastings, Hampton VA) laminar flow unit and the signal level was measured using a Hastings (model 40) monitor. The back of the chamber has nine ports, which can be used for various sampling devices. Attached to one port was a Nicolet Fourier-transform infrared spectrophotometer (FTIR) (model IS10, Thermo Fisher Scientific Inc., Waltham MA) equipped with a 2-m path length gas cell for high concentrations. Prior to entering the FTIR, the aerosol portion of the sample was removed using a small high efficiency particulate air (HEPA) filter. Sampling by the FTIR was controlled using a computer software macro that averaged every 10 spectrums collected, displayed the average concentration of jet fuel on the screen, and saved the data to a file. The system was programmed to collect and save one sample per minute for the entire six-hour exposure period.

3.4 Noise Exposure

The noise exposure selected was designed to produce a just observable permanent impairment in auditory function, such that additive or potentiating effects of JP-8 exposure could also be detected (Fechter *et al.*, 2012). Computer software (Audacity version 1.3 freeware; Audacity Development Team, audacity.sourceforge.net) installed on a laptop computer was used to generate a precisely filtered white noise file. A high-pass filter with a 48 dB per octave roll-off was applied within the software to attenuate frequencies below 5.6 kHz, followed by a low-pass

filter with the same roll-off value to attenuate frequencies above 11.3 kHz. The filter produced a finished file of one octave band noise (OBN), centered at 8 kHz. The filtered file was then played through electrodynamic shakers that induced vibration from the outside in the metal plenums at the bottom of each exposure chamber. During exposures, the sound intensity was measured inside the chambers at a central reference point using a Puma data acquisition system (Spectral Dynamics, San Jose CA). Noise exposure was summarized in Fechter *et al.* (2012) and is fully explained in Stubbs (2010). This method used to produce the noise in the chambers had a pathway for mechanical vibration of the cages. There is an unknown and immeasurable mechanical vibration that could possibly reach the rats' auditory system via bone and tissue conduction and potentially contribute to the noise exposure they received in these studies.

3.5 Distortion Product Otoacoustic Emissions

DPOAEs assess pre-neural peripheral cochlear activity, particularly the nonlinear transducer function of the outer hair cells in response to acoustic stimulation. These sound-sensing hair cells are particularly sensitive to jet fuel or noise exposure in that the level of the DPOAE is reduced after exposure (Fechter *et al.* 2007, 2010, 2012). Therefore, the level of the DPOAE was measured in 0.1 octave increments across frequencies that ranged from 3.2 to 63 kHz. The animals were anaesthetized with ketamine/xylazine (75/5 mg/kg, respectively, intramuscular (i.m.)), then placed ventrally on a 7" x 15" surgical table with built-in temperature control to maintain normal body temperature (37° ± 1° C). All measurements were obtained in a double-walled sound-isolation chamber (Industrial Acoustics Company Inc., Bronx NY).

A probe assembly was physically and acoustically coupled to the external auditory meatus via an ER3-34 infant silicon tip (Etymotic Research, Elk Grove Village IL). The position of the probe in the external auditory meatus was standardized by spectral analyses of the *in situ* output sound pressure level (OSPL) from each transducer before each measurement (Martin *et al.*, 2006). The probe assembly consists of two polyethylene tubes coupled to two separate Realistic® dual radial horn tweeters (Radio Shack, Tandy Corp., Ft Worth TX). These tweeters were used to present two stimulus puretones: f1 and f2. These puretones were acoustically mixed in the external auditory meatus to avoid artifactual distortion. The probe assembly also consisted of a pre-amplifier microphone cable coupled to an ER-10B+ emission microphone (Etymotic Research). This allowed for the detection and amplification of acoustic emissions and the recording of background noise in the external auditory meatus. All elements of the probe assembly were controlled through a customized signal presentation, acquisition and analysis algorithm written in LabVIEW version 7.1 (National Instruments, Austin TX). This LabVIEW algorithm was also used to drive a PCI-4461 computer-based digital signal processing board (National Instruments).

The DPOAE was recorded with stimulating puretones: f2 and f1, where f2 is mapped basally to f1 along the cochlear spiral. The stimulating puretones were presented at an f2/f1 ratio of 1.25. The sound pressure level (SPL) for the f1 was 55 dB SPL (L1) and that for the f2 was 35 dB SPL (L2) with a level ratio of 1.57 (L1/L2). These combined frequency and level ratios were selected to maximize the 2f1-f2 SPL recorded from the external auditory meatus (Whitehead *et al.* 1995a, 1995b, 1995c). The noise floor was computed by averaging SPLs from the external auditory

meatus for frequency bins above and below the 2f1-f2 bin (± 3.75 Hz). A DPOAE is considered to be present when the SPL exceed the noise floor by at least 3 dB.

A 0.2 cm^3 hard-walled cavity that approximates the rat's external auditory meatus was used to monitor the quality of the DPOAE recordings, identify nonbiogenic distortions and check the level of the noise floor. These quality measurements were free of artifacts and did not produce distortions that exceeded the noise floor.

3.6 Electrophysiology

The auditory brainstem response allows for simultaneous evaluation of the peripheral cochlear nerve and the ascending auditory brainstem pathway in a single recording. Therefore, an abnormal brainstem response in the presence of normal peripheral nerve activity can be ascertained within an individual animal (internal control). Wave I of ABR recordings corresponds to the compound action potential (CAP) used in previous studies (Fechter *et al.*, 2012). The CAP recordings were obtained under general anesthesia (ketamine/xylazine, 75/5 mg/kg respectively, i.m.). Each animal was ventrally positioned on a 7" x 15" surgical table with built-in temperature control. Core body temperature was monitored with a rectal probe attached to a 43TD telethermometer (Yellow Springs Instrument Company, Inc., Yellow Springs OH) and maintained at 37° ± 1° C. All recordings were obtained in the double-walled sound-isolation chamber referenced above. A five electrode montage was used to conduct two-channel differential recordings. Two non-inverting electroencephalographic needle electrodes (0.4 mm, platinum/iridium alloy, VIASYS NeuroCare, Madison WI) were placed on the vertex, another two below the right and left mastoids (inverting), and one electrode (common) was placed in the dorsum close to the tail.

The presentation of calibrated stimuli, signal acquisition and manipulation, equipment control and data management was accomplished with Intelligent Hearing System's hardware driven by the 3.94b version of the SmartEP Windows USB Software (Intelligent Hearing Systems, Miami FL). The acoustic stimuli were digitally synthesized and consisted of 512 puretones (1.56 milliseconds Blackman envelope) and clicks (100 µs rectangular voltage pulse). The puretones ranged from 2 to 32 kHz in octave (2 to 4 kHz) and ½ octave (6 to 32 kHz) intervals and were presented at a rate of 10 per second. A Sound Booster Box with high pass filter was used to drive a high frequency transducer (Intelligent Hearing Systems) for puretone stimulation of the right ear. The electroencephalographic responses to the puretones were amplified (100 K), bandpass filtered (100-1500 Hz), parsed with 31 µV artifact rejection over a 1.3 to 13.1 milliseconds rejection region of the recording epoch (16 milliseconds) and line filtered to reduce any possible electrical interference (e.g., 60 Hz).

To uncover possible deficits in synaptic efficiency in the brainstem, a click stimulation rate of 100 per second was presented to the left ear (Backoff and Caspary, 1994). The electroencephalographic responses to the clicks were amplified (100 K), bandpass filtered (100 to 3000 Hz), parsed with 31 µV artifact rejection over a 1.0 to 10.5 milliseconds rejection region of the recording epoch (12.8 milliseconds). The clicks were presented through ER-3A transducers (Etymotic Research, Elk Grove Village IL). The transducers were physically and

acoustically coupled to the external auditory meatus via silicon tubing (24.76 cm) with a corrected (subtracted from response latency) acoustic delay of 0.9 millisecond. The transducer diaphragm was driven in alternating phase for both the clicks and puretones.

The biogenic origins (as opposed to artifacts from electrical input to the transducers) of the recordings were verified in four separate procedures: 1) uncoupling the transducer tubing from the ear-bar; 2) pinching the sound delivery tube; 3) coupling the transducer into a 2 cm^3 hard-walled cavity; and 4) obtaining recordings from a rat cadaver. These procedures were made with the electrodes in place and the animal staged for collecting ABR recordings. In all cases the ABR was absent from the recordings.

It is known that ABR thresholds exhibit a statistically significant linear correlation with thresholds from the most sensitive single neuron from the peripheral cochlear nerve (Ngan and May, 2001). Therefore, frequency specific sensitivity of the peripheral nerve was assessed through puretone thresholds of the compound action potential (Wave I or W1). Furthermore, the compound sensitivity of the nerve was assessed through click thresholds of Wave I. All thresholds were measured using a modified Hughson-Westlake threshold search sequence and the waveforms were displayed on a normalized scale (Jerger *et al.*, 1959; Zhou *et al.*, 2006). The lowest stimulus intensity to elicit a visually detectable Wave I was scored as the threshold. This threshold was bracketed by a visually undetectable Wave I at 5 dB below threshold and a visually detectable Wave I at 5 dB above threshold.

In addition to thresholds, click amplitude/latency-intensity functions were obtained using a deductive method, where a response to 100 dB SPL was recorded first. This initial recording served as a reference for identifying and tracking all five ABR waves. Then subsequent recordings were made in descending 10 dB steps down to 10 dB. The absolute amplitude (µV; peak-to-forward-trough) and latency (milliseconds) of each wave was measured. The amplitude/latency ratio (µV/millisecond) was then plotted as a function of stimulus level (called growth function). Amplitude and latency measurements tend to exhibit high inter-subject variability due to factors such as skull thickness, head size and/or volume conductance (Rowe, 1981). Therefore, the slope of the growth functions were calculated (slope = $\Delta Y/\Delta X$). Unlike absolute amplitude and latency, slope has been shown to be stable over time and more resistant to interfering subject and recording variables (Hunter and Willott, 1987). Furthermore, in humans, slope correlates with auditory processing capacity (Gopal and Kowalski, 1999; Hunter and Willott, 1987; Wible *et al.*, 2004).

3.7 Cytocochleogram

It is known that rats exposed to significant levels of noise or jet fuel plus noise may exhibit a loss in the percentage of outer hair cells along the cochlear spiral as revealed by the cytocochleogram (Fechter *et al.*, 2012). Therefore, cytocochleograms were plotted for animals in each experimental group at the end of the studies (after all physiologic recordings were collected). The animals were anesthetized with a ketamine/xylazine mixture (75/5 mg/kg, i.m.) and euthanized per an American Veterinary Medical Association (AVMA, 2007 and 2013) approved method. A 1 ml, 25 gauge syringe (Becton, Dickinson and Company, Franklin Lakes NJ) was

used to perfuse 0.5 ml of 4 percent formaldehyde from the round window through the cochlear spiral to exit at the oval window (intracochlear perfusion). The whole procedure to access and perfuse each cochlea was accomplished in less than 51 sec. The specimens were then submerged in 4 percent formaldehyde for overnight fixation at 22° C.

After overnight fixation, the specimens were washed and stored in phosphate-buffered saline (PBS, 10 mM, pH 7.4). Under a stereomicroscope (Nikon Instruments Inc., Melville NY), the bulla was further resected in PBS to reveal the entire cochlear portion of the osseous spiral labyrinth. The superficial osteal layer of the cochlea was carefully removed and the membranous spiral labyrinth, including the modiolus and the VIIIth cranial nerve were removed en masse. The specimen was then demineralized in 10 percent formic acid (Guthrie, 2008). This was followed by gross transverse sectioning of the specimen into apical, middle and basal whole-mount coils. Each coil was then mounted with glycerol on microscope slides. An N-PLAN 40x/0.65 objective lens on a Leica DM2500 upright microscope (Leica Microsystems, Inc., Bannockburn IL) was used for Nomarski microscopy. A gradicule positioned in the objective lens was used to measure the length of the apical, middle and basal coils and to record the number of outer hair cells present. The outer hair cells were counted as present if the outline of the cells were clearly visible, inter-cell distances were less than the width of an outer hair cell and there were no scar tissues within rows 1, 2 or 3. The percentage of present outer hair cells as a function of percent distance along the neurosensory epithelium from the helicotrema (apical end) was plotted as cytocochleograms.

3.8 Statistical Analyses

Statistical analyses were conducted with Prism 5, version 5.03 (GraphPad Software, Inc., La Jolla CA). Data from separate one and two factor designs were analyzed. ABR click thresholds were treated with one factor analysis of variance (ANOVA). The DPOAE, threshold shift, rate-level and gain-level data were treated with a split-plot ANOVA followed by Bonferroni pair-wise contrasts and Dunnett's post hoc testing. F-tests were conducted to determine differences between slopes, with the level of significance accepted as $p < 0.05$.

4.1 RESULTS OF STUDY 1 USING LONG EVANS RATS

4.2 Jet Fuel Exposure – LE rats

The jet fuel exposures had a target concentration of 1000 mg/m^3. The average nominal concentrations, the percent nominal and the actual measured concentration from the FTIR for the JP-8 alone chamber and the combined JP-8 and noise chamber are shown in Table 1. The table also shows the aerosol concentration from the filters, the mass median aerosol diameter (MMAD) and the geometric standard deviation (GSD) for both chambers.

Table 1. JP-8 in the Chambers with Jet Fuel Exposure for Study 1

		JP-8 Alone*	JP-8 plus 85 dB*
Vapor + Aerosol	**Nominal (mg/m^3)**	896.8 ± 18.9 (20)	1049.4 ± 19.8 (19)
	Percent Nominal	113.2 ± 2.3 (19)	96.5 ± 1.3 (19)
	FTIR (mg/m^3)	967.2 ± 8.2 (20)	965.1 ± 10.1 (20)
Aerosol	**MMAD (µm)**	2.31 ± 0.25 (5)	1.80 ± 0.24 (5)
	GSD	1.97 ± 0.15 (5)	1.99 ± 0.12 (5)
	Filter Concentration (mg/m^3)	58.0 ± 7.0 (5)	25.1 ± 6.9 (5)

* mean ± standard deviation (n)

4.3 Noise Exposure – LE rats

The two "with sound" exposure chambers had a target concentration of 85 dB. The "no sound" chambers contained only a background level of noise. The average decibels for the daily reading in each chamber are shown in Table 2.

Table 2. Noise Data in all Four Chambers with and without Jet Fuel Exposure for Study 1

	No sound 1000 mg/m^3	85 dB alone	85 dB + 1000 mg/m^3	No sound (air control)
Mean ± Standard Deviation	45.67 ± 1.02	85.54 ± 0.33	85.37 ± 0.57	46.23 ± 0.46
n	20	20	20	20

4.4 DPOAE – LE rats

This testing measures the function of auditory receptor cells in the cochlea. If the noise exposure, fuel exposure or noise plus fuel exposure altered cochlear function, then DPOAE levels would be reduced when compared to levels from the control (chamber only exposure) group. The DPOAE testing was performed 4 weeks after the exposures to assess permanent changes in cochlear function. Both male and female LE rats were included in the study. Therefore, the DPOAE data were separated by gender.

Figure 1 reveals the DPOAE data for the male LE rats. The results demonstrate that the exposures (noise, fuel or combination of noise plus fuel) did not reduce DPOAE levels relative to that of the control group. Support for this conclusion was also evident in statistical analyses. A one-way ANOVA was conducted; no significant differences were found between study groups ($F[3, 172] = 0.5578$; $p > 0.05$). Furthermore, Dunnett's multiple comparison post-hoc testing revealed no significant differences ($p > 0.05$) between the control group and the other three groups.

Figure 2 depicts the DPOAE data for the female LE rats. Again, exposures (noise, fuel or combination of noise plus fuel) did not reduce DPOAE levels relative to that of the control group. Results from a one-way ANOVA indicate that there were no significant ($F[3, 172] = 0.8202$; $p > 0.05$) differences between the groups. No significant differences ($p > 0.05$) were found between the control group and the other three groups using the Dunnett's multiple comparison post-hoc test.

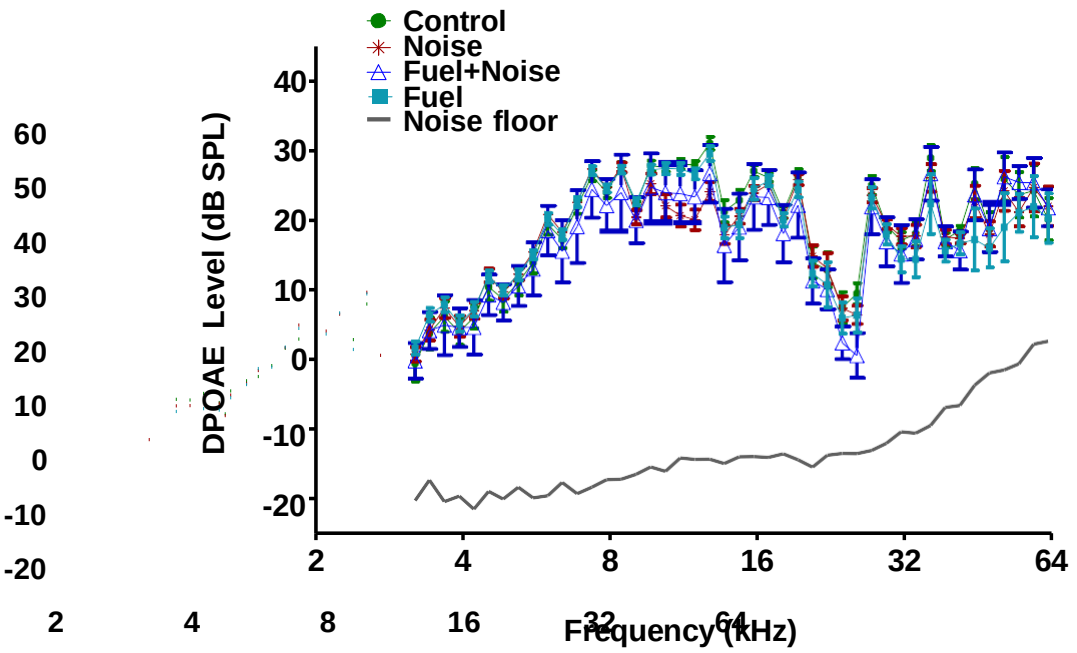

Figure 1. DPOAE Results for Male LE Rats. DPOAE levels as a function of frequency are shown for each treatment group: Control (air + background sound), Noise (air + noise), Fuel (fuel + background noise) and Fuel + Noise. Primary levels of 55/35 dB SPL were used to stimulate DPOAE responses. The noise exposure was an 8-kHz octave band at 85 dB SPL for 6 hours/day for 4 weeks. The jet fuel exposure was a 1000 mg/m^3 dose of JP-8 for 6 hours/day, 5 days/week for 4 weeks. The noise floor (solid gray line) represents biological and instrumental background activity. Error bars indicate standard errors of the means. Note that there are no significant differences between groups.

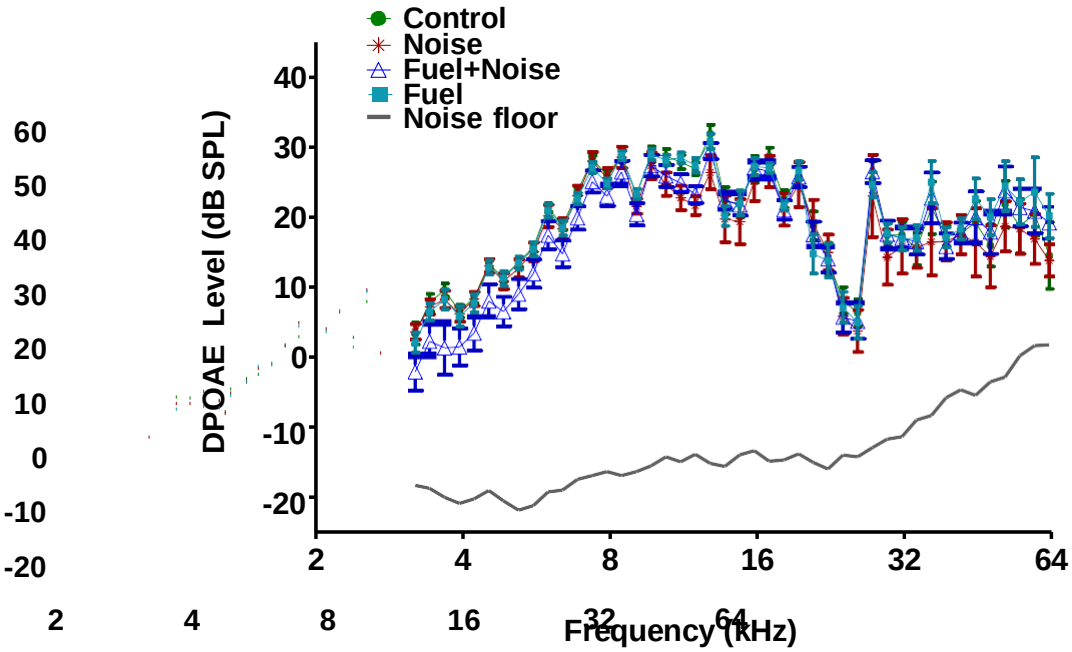

Figure 2. DPOAE Results for Female LE Rats. DPOAE levels as a function of frequency are shown for each treatment group: Control (air + background sound), Noise (air + noise), Fuel (fuel + background noise) and Fuel + Noise. Primary levels of 55/35 dB SPL were used to stimulate DPOAE responses. The noise exposure was an 8-kHz octave band at 85 dB SPL for 6 hours/day for 4 weeks. The jet fuel exposure was a 1000 mg/m^3 dose of JP-8 for 6 hours/day, 5 days/week for 4 weeks. The noise floor (solid gray line) represents biological and instrumental background activity. Error bars indicate standard errors of the means. Note that there are no significant differences between groups.

4.5 Electrophysiology – LE rats

The sensitivity of the auditory nerve (Wave I) was monitored in a series of sound evoked waves representing neural CAPs in the cochlea and brainstem. If the noise exposure, fuel exposure or noise plus fuel exposure altered the sensitivity of the auditory nerve, then neural thresholds would increase when compared to thresholds from the control (non-exposed) group. The electrophysiologic recordings were performed 4 weeks after the exposures to assess permanent changes in neural sensitivity. Since both male and female LE rats were included in the study, the electrophysiologic data were separated by gender.

Figure 3 reveals thresholds (CAP) for the male LE rats. The results indicate that the exposures (noise, fuel or combination of noise and fuel) did not increase thresholds relative to that of the control group. A one-way ANOVA was conducted; there were no significant ($F[3, 28] = 0.4689$; $p > 0.05$) differences between the groups. Furthermore, the Dunnett's multiple comparison post-hoc test revealed no significant differences ($p > 0.05$) between the control group and the other three groups.

Figure 4 depicts the thresholds (CAP) for female LE rats. The results demonstrate that the exposures (e.g., noise, fuel or combination of noise and fuel) did not increase thresholds relative to that of the control group. One-way ANOVA results show that there were no significant ($F[3, 28] = 1.704$; $p > 0.05$) differences between the groups. No significant differences ($p > 0.05$) between the control group and the other three groups were found with the Dunnett's multiple comparison post-hoc test.

An interesting observation from the threshold data is that the animals from all groups (and both genders) had an elevation in threshold at around 8 kHz, relative to historic controls tested in our laboratory. DPOAE levels in all groups at 8 kHz are normal (not depressed).

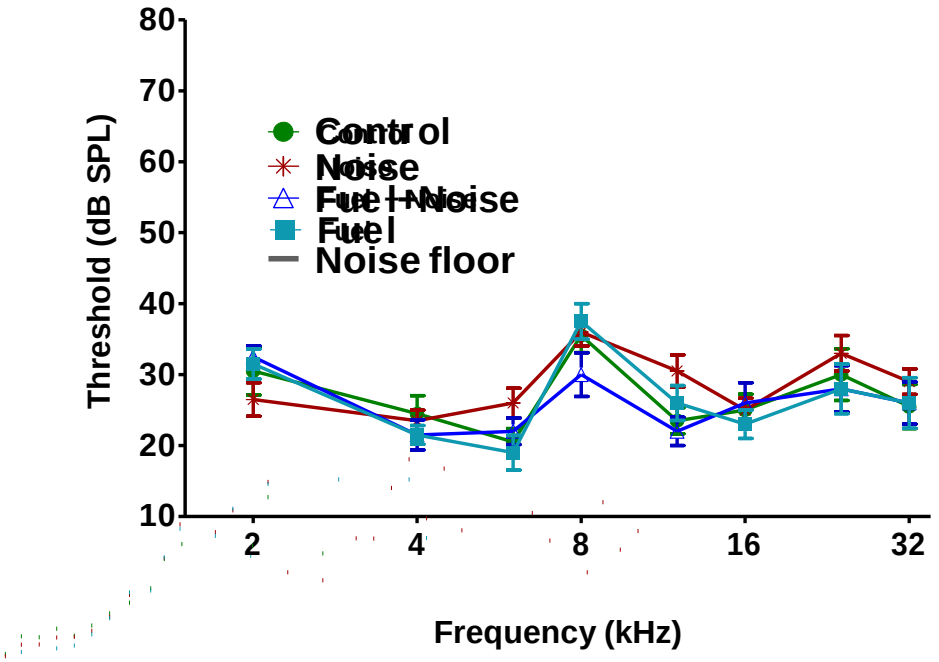

Figure 3. Threshold (CAP) Results for Male LE Rats. Threshold in dB SPL as a function of frequency are shown for each treatment group: Control (air + background sound), Noise (air + noise), Fuel (fuel + background noise) and Fuel + Noise. The noise exposure was an 8-kHz octave band at 85 dB SPL for 6 hours/day, 5 days/week for 4 weeks. The jet fuel exposure was a 1000 mg/m^3 dose of JP-8 for 6 hours/day, 5 days/week for 4 weeks. Errors bars indicate standard errors of the means. Note that there is no significant difference between the groups.

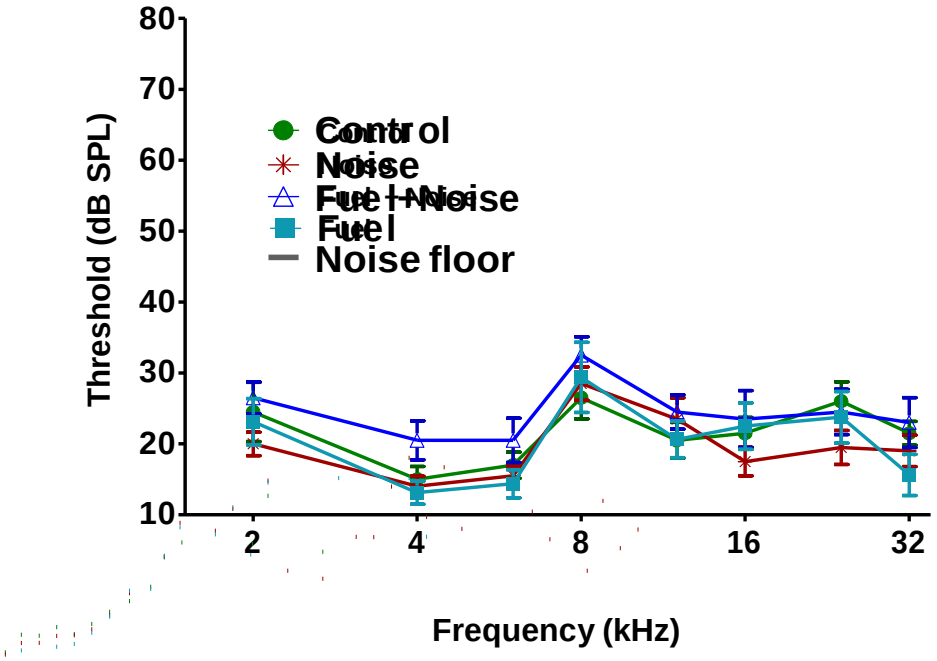

Figure 4. Threshold (CAP) Results for Female LE Rats. Threshold in dB SPL as a function of frequency are shown for each treatment group: Control (air + background sound), Noise (air + noise), Fuel (fuel + background noise) and Fuel + Noise. The noise exposure was an 8-kHz octave band at 85 dB SPL for 6 hours/day, 5 days/week for 4 weeks. The jet fuel exposure was a 1000 mg/m^3 dose of JP-8 for 6 hours/day, 5 days/week for 4 weeks. Errors bars indicate standard errors of the means. Note that there is no significant difference between the groups.

4.6 Cochleograms – LE rats

The cochleogram is a graph of the number of missing or present auditory receptor cells (outer hair cells, see Figure 5). If the noise exposure, fuel exposure or noise plus fuel exposure induced cell death in the cochlea, then the cochleogram would show a reduction in the number of cells that are present when compared to that from the control (non-exposed) group. Cochleae were formalin fixed and micro-dissected from each animal within each group. Cochleae were then processed so that flat whole-mount preparations could be mounted onto microscopic slides and each cell counted. As both male and female LE rats were included in the study, the cochleograms were separated by gender.

Figure 6 shows cochleogram data for the LE male rats. The results demonstrate that the exposures (noise, fuel or combination of noise and fuel) did not reduce the number of outer hair cells. For instance, virtually all the cells (approximating 100 percent) are present in all the groups. This supports the conclusion that the cochlea were not affected by the exposures.

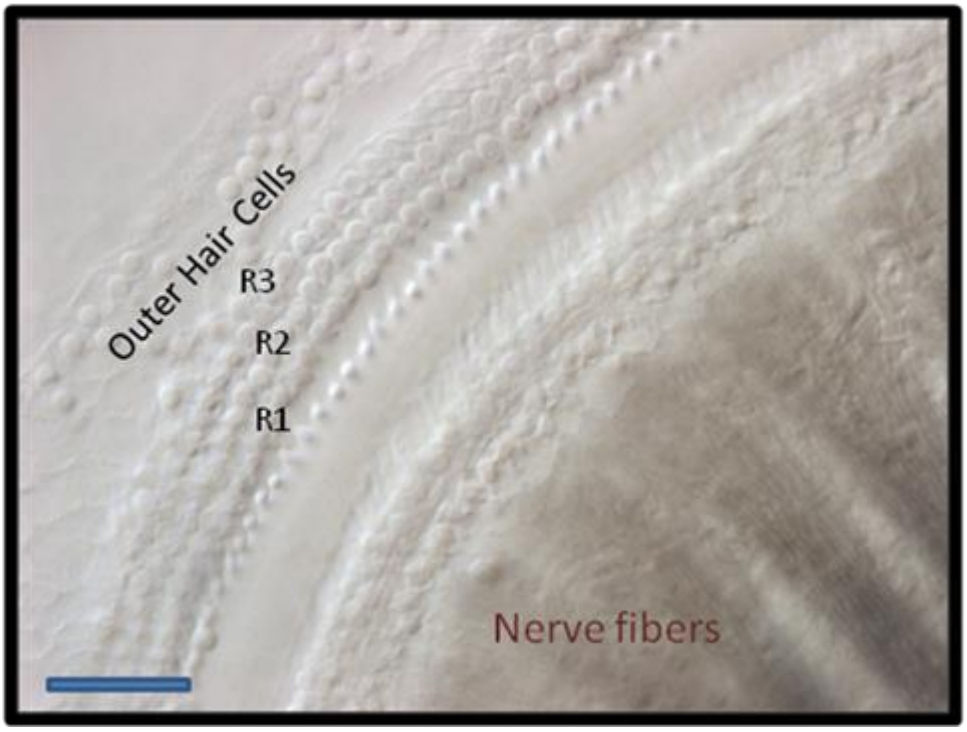

Figure 5. Representative Nomarski Micrograph of the Neurosensory Epithelium used to Construct a Cochleogram for an LE Rat. Outer hair cells form three parallel rows called row 1 (R1), row 2 (R2) and row 3 (R3). These rows run the length of the cochlea and missing (or present cells) can be counted as a function of cochlear length. Scale bar is 50 µm.

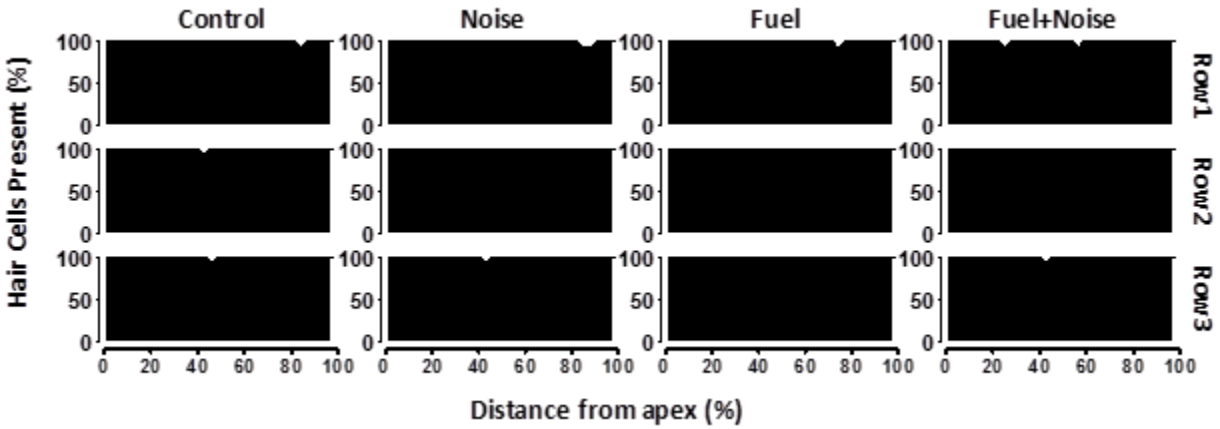

Figure 6. Cochleogram Results for Male LE Rats. The percent of cells present as a function of percent distance along the cochlea are shown for each treatment group: Control (air + background sound), Noise (air + noise), Fuel (fuel + background noise) and Fuel + Noise. The noise exposure was an 8-kHz octave band at 85 dB SPL for 6 hours/day, 5 days/week for 4 weeks. The jet fuel exposure was a 1000 mg/m^3 dose of JP-8 for 6 hours/day for 4 weeks. Black areas represent cells present and white areas (sections showing less than 100%) represent regions of missing cells. For each group, almost 100 percent of the cells are present indicating that the exposures did not affect outer hair cells.

The cochleogram data for the LE female rats (Figure 7) also demonstrate that the exposures (noise, fuel or combination of noise and fuel) did not reduce the number of outer hair cells in these female rats. Again, nearly 100 percent of cells are present in all the groups. The cochlea were not affected by the exposures.

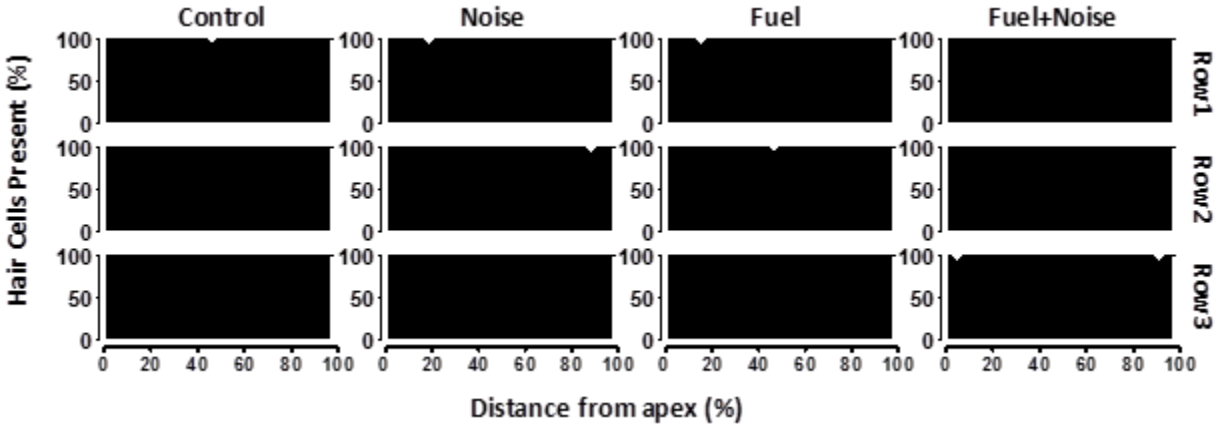

Figure 7. Cochleogram Results for Female LE Rats. The percent of cells present as a function of percent distance along the cochlea are shown for each treatment group: Control (air + background sound), Noise (air + noise), Fuel (fuel + background noise) and Fuel + Noise. The noise exposure was an 8-kHz octave band at 85 dB SPL for 6 hours/day, 5 days/week for 4 weeks. The jet fuel exposure was a 1000 mg/m^3 dose of JP-8 for 6 hours/day for 4 weeks. Black areas represent cells that are present and white areas (sections showing less than 100%) represent regions of missing cells. For each group, almost 100 percent of the cells are present indicating that the exposures did not affect outer hair cells.

5.1 RESULTS OF STUDY 2 WITH FISCHER 344 RATS

5.2 Jet Fuel Exposure – F344 rats

The target concentration for both JP-8 exposure chambers was 1000 mg/m^3. The average nominal concentrations, the percent nominal and the actual measured concentration from the FTIR for the JP-8 alone chamber and the combined JP-8 and noise chamber are shown in Table 3. The table also shows the aerosol concentration from the filters, the MMAD and the GSD for both chambers.

Table 3. Data for JP-8 in the Chambers with Jet Fuel Exposure for Study 2

		JP-8 Alone*	JP-8 plus 85 dB*
Vapor + Aerosol	**Nominal (mg/m^3)**	1006.9 ± 51.2 (23)*	1150.4 ± 95.1 (22)
	Percent Nominal	102.4 ± 4.3 (23)	89.2 ± 7.0 (22)
	FTIR (mg/m^3)	1030.6 ± 24.5 (23)	1026.2 ± 28.3 (22)
Aerosol	**MMAD (µm)**	2.29 ± 0.03 (5)	2.31 ± 0.16 (4)
	GSD	1.19 ± 0.10 (5)	1.85 ± 0.17 (4)
	Filter Concentration (mg/m^3)	74.1 ± 9.2 (5)	100.7 ± 34.4 (4)

* mean ± standard deviation (n)

5.3 Noise Exposure – F344 rats

The two "with sound" chambers had a target concentration of 85 dB while the two "no sound" chambers had background level of noise only. The average decibels for daily readings in each chamber are shown in Table 4.

Table 4. Noise Data in all Four Chambers with and without Jet Fuel Exposure for Study 2

	No sound 1000 mg/m^3	**85 dB alone**	**85 dB + 1000 mg/m^3**	**No sound (air control)**
Mean ± Standard Deviation	45.90 ± 1.87	85.53 ± 0.77	85.64 ± 0.56	46.07 ± 0.81
n	20	20	20	20

5.4 DPOAE – F344 rats

This testing measures the function of auditory receptor cells in the cochlea. If the noise exposure, fuel exposure or noise plus fuel exposure altered cochlear function, then DPOAE levels would be reduced when compared to levels from the control group. The DPOAE testing was performed 4 weeks after the exposures to assess permanent changes in cochlear function. Both male and female F344 rats were included in the study. Therefore, the DPOAE data were separated by gender.

Figures 8 and 9 depict the DPOAE data for the male and female rats, respectively. The results demonstrate that the exposures (noise, fuel or combination of noise plus fuel) did not significantly reduce DPOAE levels relative to that of the control group for either sex.

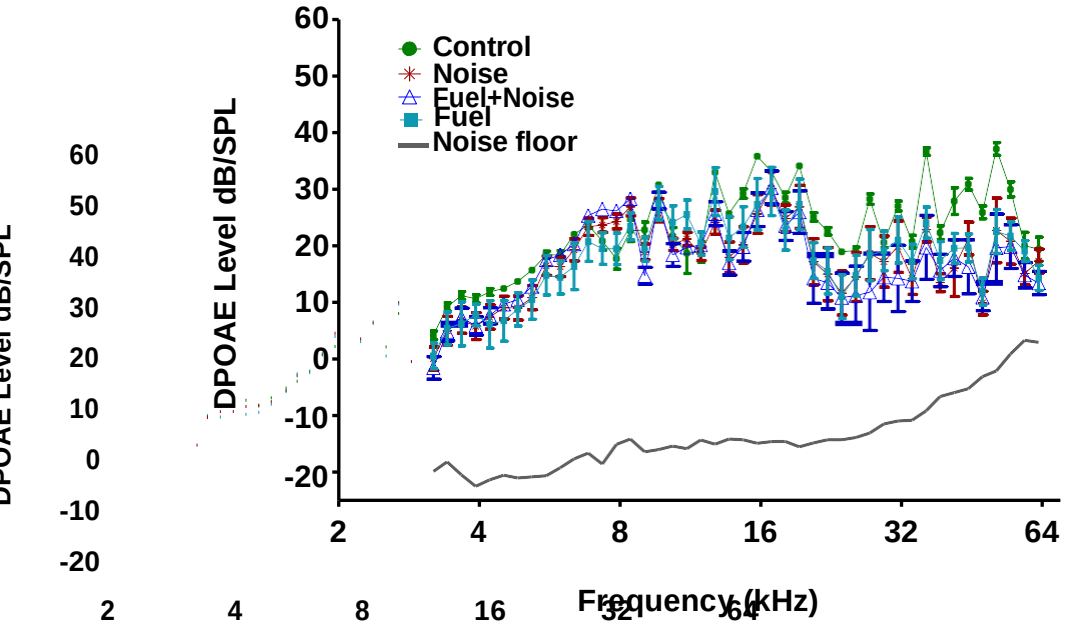

Figure 8. DPOAE Results for Male F344 Rats. DPOAE levels as a function of frequency are shown for each treatment group: Control (air + background sound), Noise (air + noise), Fuel (fuel + background noise) and Fuel + Noise. Primary levels of 55/35 dB SPL were used to stimulate DPOAE responses. The noise exposure was an 8-kHz octave band at 85 dB SPL for 6 hours/day for 4 weeks. The jet fuel exposure was a 1000 mg/m^3 dose of JP-8 for 6 hours/day, 5 days/week for 4 weeks. The noise floor (solid gray line) represents biological and instrumental background activity. Error bars indicate standard errors of the means. Note that there are no significant differences between groups.

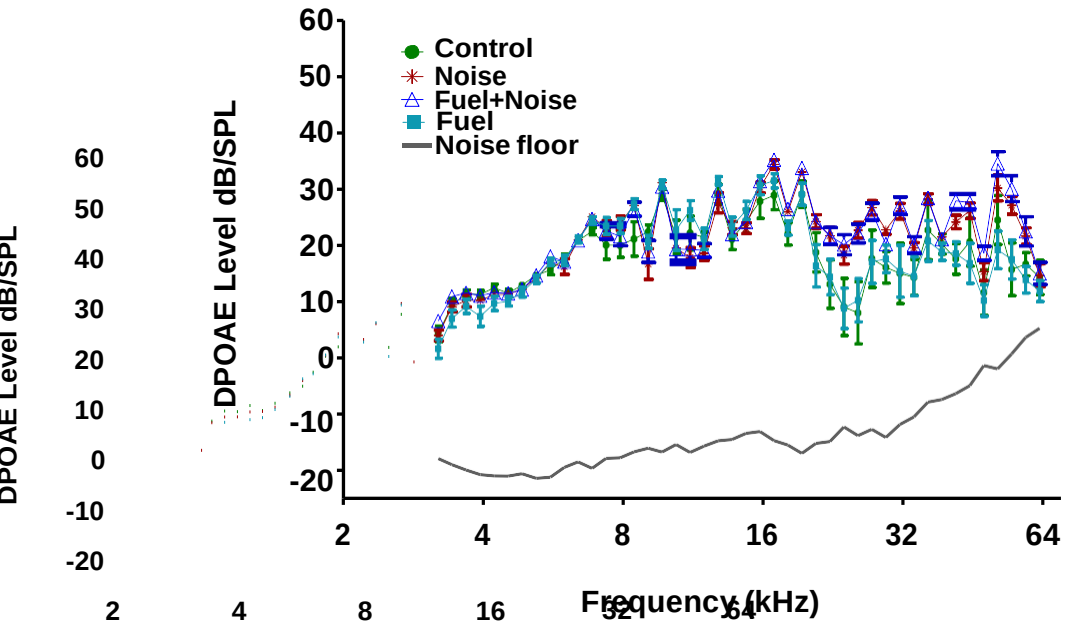

Figure 9. DPOAE Results for Female F344 Rats. DPOAE levels as a function of frequency are shown for each treatment group: Control (air + background sound), Noise (air + noise), Fuel (fuel + background noise) and Fuel + Noise. Primary levels of 55/35 dB SPL were used to stimulate DPOAE responses. The noise exposure was an 8-kHz octave band at 85 dB SPL for 6 hours/day for 4 weeks. The jet fuel exposure was a 1000 mg/m^3 dose of JP-8 for 6 hours/day, 5 days/week for 4 weeks. The noise floor (solid gray line) represents biological and instrumental background activity. Error bars indicate standard errors of the means. Note that there are no significant differences between groups.

5.5 Electrophysiology – F344 rats

The sensitivity of the auditory nerve (Wave I) was monitored in a series of sound evoked waves representing neural action potentials (CAPs) in the cochlea and brainstem. If the noise exposure, fuel exposure or noise plus fuel exposure altered the sensitivity of the auditory nerve, then neural thresholds would increase when compared to thresholds from the control (non-exposed) group. The electrophysiologic recordings were performed 4 weeks after the exposures to assess permanent changes in neural sensitivity. Both male and female F344 rats were included in the study; therefore, the electrophysiologic data were separated by gender.

Figures 10 and 11 show thresholds (CAPs) for male and female F344 rats, respectively. The results demonstrate that the exposures (noise, fuel or combination of noise plus fuel) did not significantly increase thresholds relative to that of the control group in either sex.

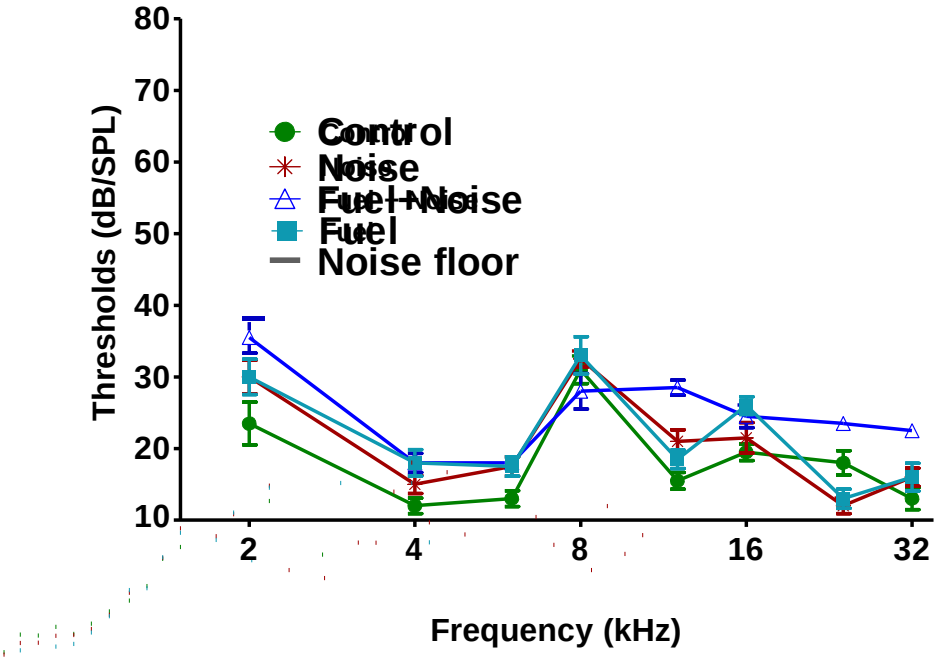

Figure 10. Threshold (CAP) Results for Male F344 Rats. Threshold in dB SPL as a function of frequency are shown for each treatment group: Control (air + background sound), Noise (air + noise), Fuel (fuel + background noise) and Fuel + Noise. The noise exposure was an 8-kHz octave band at 85 dB SPL for 6 hours/day, 5 days/week for 4 weeks. The jet fuel exposure was a 1000 mg/m^3 dose of JP-8 for 6 hours/day, 5 days/week for 4 weeks. Errors bars indicate standard errors of the means. Note that there is no significant difference between the groups.

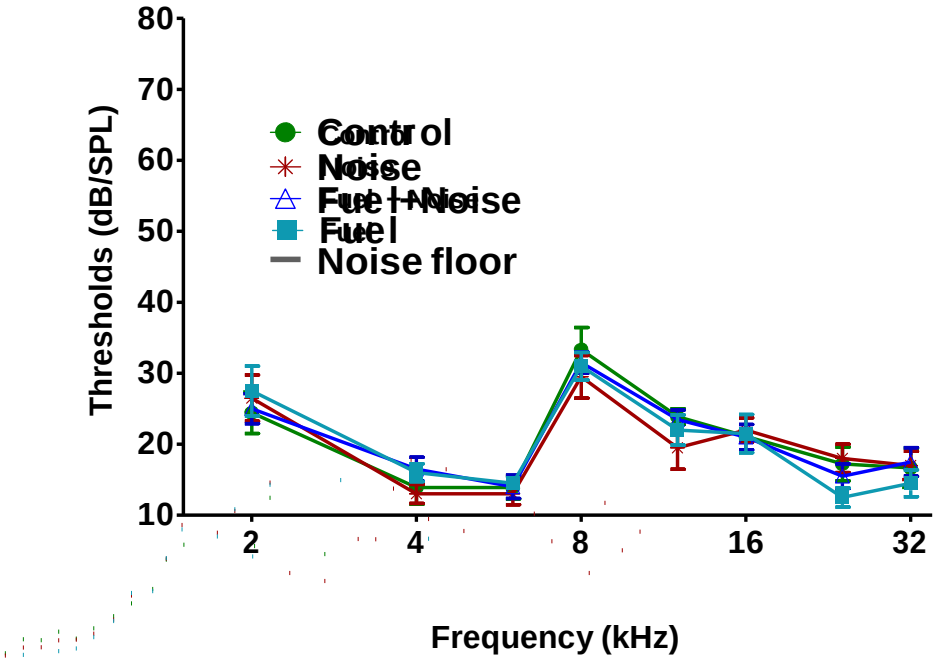

Figure 11. Threshold (CAP) Results for Female F344 Rats. Threshold in dB SPL as a function of frequency are shown for each treatment group: Control (air + background sound), Noise (air + noise), Fuel (fuel + background noise) and Fuel + Noise. The noise exposure was an 8-kHz octave band at 85 dB SPL for 6 hours/day, 5 days/week for 4 weeks. The jet fuel exposure was a 1000 mg/m^3 dose of JP-8 for 6 hours/day, 5 days/week for 4 weeks. Errors bars indicate standard errors of the means. Note that there is no significant difference between the groups.

5.6 Cochleograms – F344 rats

The cochleogram is a graph of the number of missing or present auditory receptor cells (outer hair cells). If the noise exposure, fuel exposure or noise plus fuel exposures induced cell death in the cochlea, then the cochleogram would show a reduction in the number of cells that are present when compared to that from the control (non-exposed) group. Cochleae were formalin fixed and micro-dissected from each animal within each group. They were then processed so that flat whole-mount preparations could be mounted onto microscopic slides and each cell counted. Since both male and female F344 rats were included in the study, the cochleograms were separated by gender. Figure 12 is a representative Nomarski micrograph of a micro-dissected cochlear specimen that is used in constructing cochleograms.

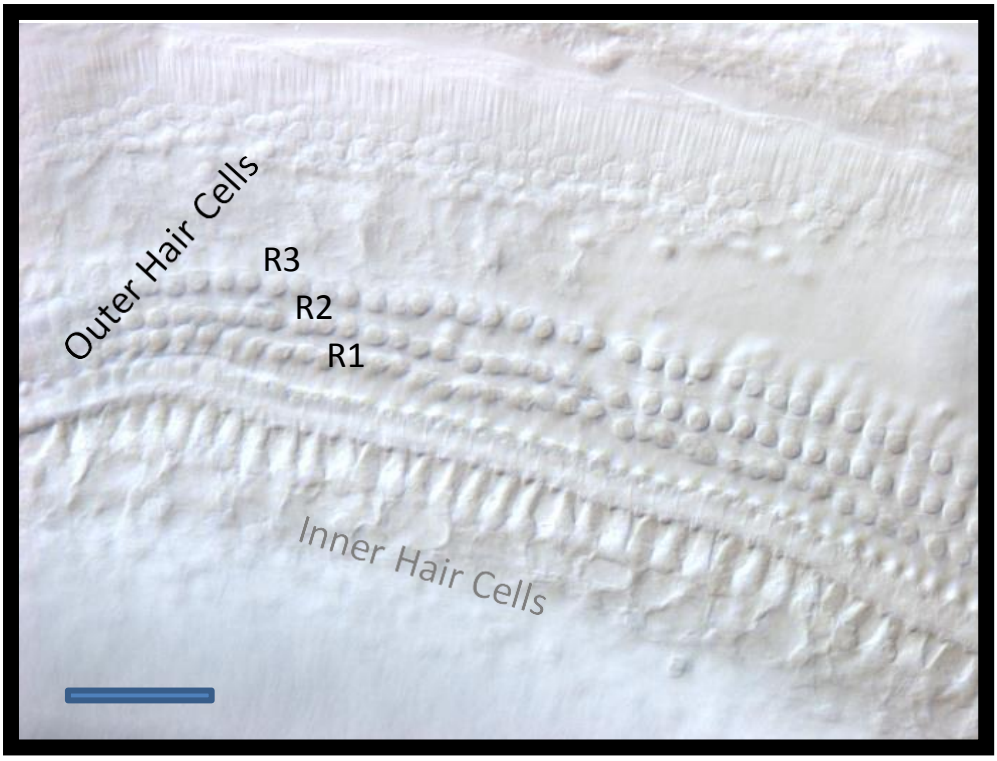

Figure 12. Representative Nomarski Micrograph of the Neurosensory Epithelium used to Construct a Cochleogram for an F344 Rat. Outer hair cells form three parallel rows called row 1 (R1), row 2 (R2) and row 3 (R3). These rows run the length of the cochlea and missing (or present cells) can be counted as a function of cochlear length. Scale bar is 50 µm.

Figures 13 and 14 depict cochleogram data for the male and female F344 rats, respectively. The results demonstrate that the exposures (noise, fuel or combination of noise plus fuel) did not reduce the number of outer hair cells. Almost all the cells (approximately 100 percent) are present in all the groups. These data support the conclusion that the cochlea was not affected by the exposures.

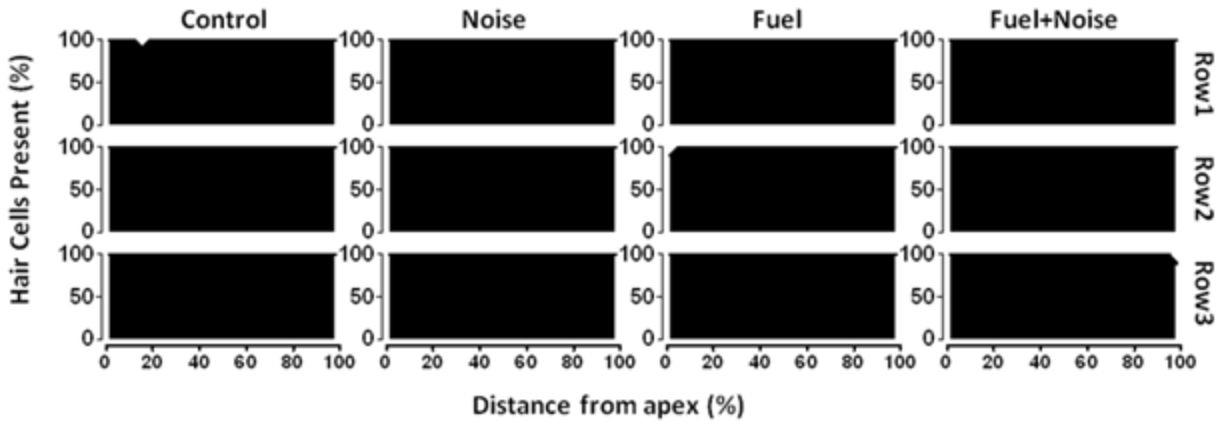

Figure 13. Cochleogram Results for Male F344 Rats. The percent of cells present as a function of percent distance along the cochlea are shown for each treatment group: Control (air + background sound), Noise (air + noise), Fuel (fuel + background noise) and Fuel + Noise. The noise exposure was an 8-kHz octave band at 85 dB SPL for 6 hours/day, 5 days/week for 4 weeks. The jet fuel exposure was a 1000 mg/m^3 dose of JP-8 for 6 hours/day for 4 weeks. Black areas represent cells that are present and white areas (sections showing less than 100%) represent regions of missing cells. For each group, almost 100 percent of the cells are present indicating that the exposures did not affect outer hair cells.

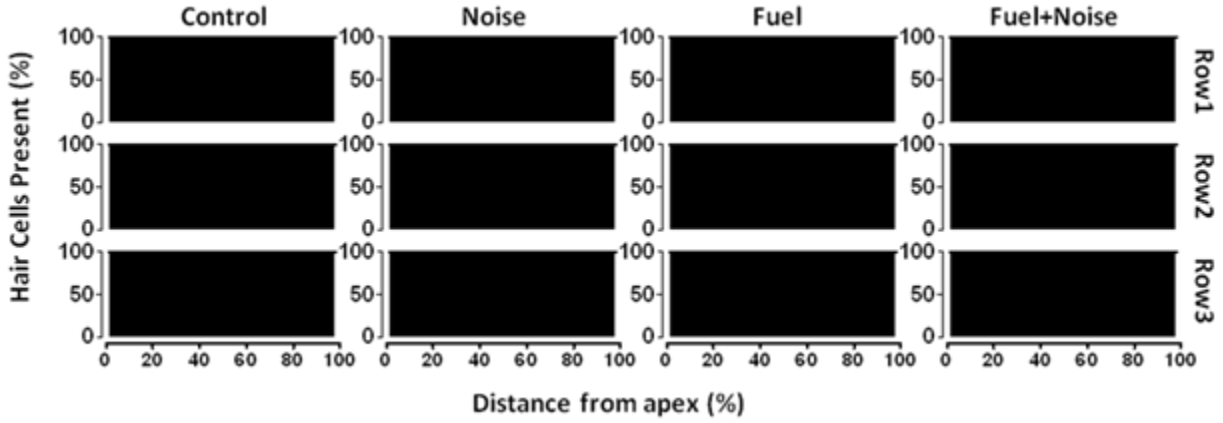

Figure 14. Cochleogram Results for Female F344 Rats. The percent of cells present as a function of percent distance along the cochlea are shown for each treatment group: Control (air + background sound), Noise (air + noise), Fuel (fuel + background noise) and Fuel + Noise. The noise exposure was an 8-kHz octave band at 85 dB SPL for 6 hours/day, 5 days/week for 4 weeks. The jet fuel exposure was a 1000 mg/m^3 dose of JP-8 for 6 hours/day for 4 weeks. Black areas represent cells that are present and white areas (sections showing less than 100%) represent regions of missing cells. For each group, almost 100 percent of the cells are present indicating that the exposures did not affect outer hair cells.

6.1 DISCUSSION

The goal of this project was to determine whether or not combined exposure to low levels of jet fuel and non-damaging noise leads to permanent peripheral auditory dysfunction. The individual noise and jet fuel exposure levels used in the experiments were deliberately chosen to have a subtoxic auditory effect. The hypothesis was that subtoxic levels of each exposure could become toxic/damaging when they are combined (noise plus jet fuel). For both the LE and F344 rat studies, the concentration of jet fuel as measured by FTIR was essentially the same, even though the nominal and percent nominal values varied between each chamber. The concentration of aerosol varied between chambers for both strains, which may have contributed to the difference in nominal values. The MMAD values were very close among the F344 JP-8 exposed study groups (with and without noise); however, in the LE rat study, the MMAD for the JP-8 plus noise exposure was much lower than the MMAD in the JP-8 only exposure. The GSD values were essentially the same for both exposures in the LE rat study, but in the F344 rat study, aerosol variation in the JP-8 plus noise chamber was much higher than in the JP-8 only chamber. Despite these differences in JP-8 exposure values, the same hearing outcomes were observed in both strains. Noise levels were extremely consistent in all chambers for both strains of rats.

The results revealed that Long-Evans and F344 rats exhibited normal pre-neural function as evidenced by robust DPOAE levels, when exposed to jet fuel (1000 mg/m^3, 6 hours/day, 5 days/week for 4 weeks; 20 total exposures), noise (85 dB, 6 hours/day, 5 days/week for 4 weeks; 20 total exposures) or jet fuel combined with noise. This was confirmed by cytocochleograms that revealed that almost 100 percent of hair cells were present from the exposed groups. Additionally, the compound cochlear nerve response (CAP) generated as Wave I in the ABR revealed normal click and puretone thresholds for each experimental group. Therefore, the combined results suggest that the exposures did not induce a detectable peripheral impairment in hearing.

The method used to produce the noise in the chamber had a pathway for mechanical vibration to the rats in their cages. There is an unknown and immeasurable mechanical vibration that could reach the rats' auditory system via bone and tissue conduction and potentially contribute to the noise exposure they received in these studies. The pathway of conduction is from the metal plate at the bottom of the chamber, up the side/mainframe of the chamber, out through the support struts for the cages, down and through the wire mesh cages and into the feet and body of the rats. The intent of the project was to look at the potential for jet fuel combined with noise exposure to produce an increased effect on hearing loss. The experimental conditions did permit co-exposure and did not show a potentiation of hearing loss by the jet fuel at the levels and length of exposure tested. Although we cannot identify how much the mechanical vibration pathway may contribute to the noise exposure of the rats, in these studies it did not appear to affect the overall results.

An interesting observation from the CAPs threshold data is that the animals from all groups (and both genders) had an elevation in threshold at around 8 kHz, relative to laboratory historic controls. DPOAE levels in all groups at 8 kHz are normal (not depressed). A discrepancy between normal DPOAE and elevated threshold has been noted previously in the literature and has been shown to be due to mild noise exposure (Kujawa and Liberman, 2009). It is possible

that the animals may have experienced a noise event that increased their thresholds within a limited range (~8 kHz). Such an event may, for example, occur from exposure to spectrally shaped background sound emitted from the exposure chambers at WPAFB. The sound may also be from an event during shipping from WPAFB to Loma Linda, CA. Since controls were also affected, the increase in threshold does not appear to be due to a vibration effect from the noise generating system. To further assess the basis of this 8 kHz threshold elevation, one recommendation would be to assess the thresholds of animals that did not enter the exposure chambers (an untreated control group). Another recommendation would be to use a sound level meter to measure the frequency response of the background sound generated by the exposure chamber. These measurements would allow us to correlate the bandwidth of maximum energy to the 8 kHz threshold elevation. Studies with other Long-Evans rats (not associated with this study, Fechter *et al.*, 2007 and 2010) do not show evidence of threshold elevations at 8 kHz. This further indicates that the animals in this project had a unique experience that increased their threshold. Since the event occurred in both studies, it is likely due to background noise in the exposure facility. However, all rats were flown to California from Ohio, so there may also have been a common effect due to shipping.

6.2 Central Auditory Processing Dysfunction

In addition to the peripheral auditory system, the central auditory system was also assessed by continuing to measure ABR Waves II and III as part of a pilot study. Central auditory nervous system (CANS) function assessments were conducted on five rats from each group. Only five Long Evans and five F344 rats from each group were tested due to the extensive time needed for these assessments, the fact that it was uncertain if any central impairment would be found, and because this was an additional effort not originally planned for this project. The assessments were centered on brainstem evoked potential recordings. The results are not a part of this report but are summarized below and have been reported separately (Guthrie *et al.*, 2014).

Electrophysiologic assessments of the CANS revealed the presence of a central auditory processing dysfunction (CAPD) that manifested as impaired brainstem encoding of stimulus intensity. This assessment of central brain function allowed for the discovery of three potentially important results. First, low level jet fuel exposure induced a central auditory dysfunction that manifested as impaired brainstem encoding of stimulus intensity. Second, this central auditory dysfunction was exacerbated by low level noise exposure. Third, the brainstem impairment was dominant among neurons that are responsive to high levels of acoustic stimulation. These findings could represent important and major shifts in the theoretical framework that governs current understanding of jet fuel and/or noise induced ototoxicity. From a clinical perspective, the results indicate that jet fuel plus noise exposures have consequences to brainstem function that may be more wide-spread and insidious than what was previously known. Therefore, it is possible that a large population of military personnel who are suffering with the effects of jet fuel plus noise exposure may be misidentified because they would exhibit normal auditory sensitivity (normal hearing thresholds) but harbor a "hidden" brainstem auditory dysfunction. Such brainstem dysfunctions may be associated with a large variety of clinical conditions such as depression, anxiety, sleep disorders, post-traumatic stress, tinnitus (ringing in the ear),

hyperacusis (hypersensitivity to sounds), diploacusis (misperception of pitch) and impaired speech perception (inability to understand speech sounds) (Guthrie *et al.*, 2014).

7.0 CONCLUSIONS

The current data suggest that a noise dose of 85 dB combined with JP-8 exposure of 1000 mg/m^3 for 6 hours/day, 5 days/week for 4 weeks does not significantly affect auditory function in Long-Evans rats. Compared to other strains of rats, such as the albino Fischer 344 rat, the pigmented Long-Evans rat is generally more resistant to ototoxicity. Therefore, higher levels of exposure are often needed to observe ototoxicity. Future experiments may benefit from conducting dose-response studies to determine a noise exposure level that does yield a slight auditory impairment in Long-Evans rats and to use that noise exposure level in combination with jet fuel to determine whether the fuel does increase the adverse effects of the noise.

The current data suggest that the same noise and jet fuel exposure also does not significantly affect auditory function in Fischer 344 rats. This is in contrast to previous studies that showed ototoxicity when F344 rats were exposed to a noise dose of 102 dB for 15 minutes each hour (90 minutes total exposure) combined with JP-8 exposure of 1000 mg/m^3 for 6 hours/day, 5 days/week for 4 weeks (Fechter *et al.*, 2012). Therefore, jet fuel induced ototoxicity may depend on the level of noise exposure. For instance, there may be a critical level for the noise where jet fuel induces ototoxicity when the noise is at or above this critical level (e.g., 102 dB) but when the noise level (e.g., 85 dB) is below the critical level, ototoxicity is avoided. This will be important for establishing military and civilian guidelines for limiting ototoxicity as a result of combined exposures to jet fuel and noise.

The additional data suggest that a noise dose of 85 dB combined with JP-8 exposure of 1000 mg/m^3 for 6 hours/day for 4 weeks induced a significant central auditory dysfunction which manifested as impaired brainstem encoding of stimulus levels in LE rats. It is possible that this central auditory dysfunction may appear as an early sign of combined fuel plus noise ototoxicity. If this is the case, then current approaches and efforts to identify military personnel at risk may not be applying the most sensitive techniques for early detection of ototoxicity since current efforts are based solely on threshold testing. Furthermore, the development of peripheral threshold impairment may represent a later stage in the pathobiology of the toxic exposure. Experimental confirmation of these issues awaits further research.

8.0 REFERENCES

AVMA. 2007. AVMA Guidelines on Euthanasia (Formerly Report of the AVMA Panel on Euthanasia). Schaumburg IL: American Veterinary Medical Association. June 2007.

AVMA. 2013. AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. Schaumburg IL: American Veterinary Medical Association. https://www.avma.org/KB/Policies/Documents/euthanasia.pdf.

Backoff, P.M., and Caspary, D.M. 1994. Age-related changes in auditory brainstem responses in Fischer 344 rats: effects of rate and intensity. Hear. Res. 73(2):163-72.

Fechter, L.D., Fisher, J.W., Chapman, G.D., Mokashi, V.P., Ortiz, P.A., Reboulet, J.E., Stubbs, J.E., Lear, A.M., McInturf, S.M., Prues, S.L., Gearhart, C.A., Fulton, S. and Mattie, D.R. 2012. Subchronic JP-8 jet fuel exposure enhances vulnerability to noise-induced hearing loss in rats. J. Toxicol. Environ. Health A 75(5): 299-317.

Fechter, L.D., Gearhart, C., Fulton, S., Campbell, J., Fisher, J., Na, K., Cocker, D., Nelson-Miller, A., Moon, P. and Pouyatos, B. 2007. JP-8 jet fuel can promote auditory impairment resulting from subsequent noise exposure in rats. Toxicol. Sci. 98(2): 510-525.

Fechter, L.D., Gearhart, C.A. and Fulton, S. 2010. Ototoxic potential of JP-8 and a Fischer-Tropsch synthetic jet fuel following subacute inhalation exposure in rats. Toxicol Sci. 116(1): 239-248.Fitzpatrick DT. 1988. An analysis of noise-induced hearing loss in army helicopter pilots. Aviation, space, and environmental medicine 59:937-941.

Gopal, K.V. and Kowalski, J. 1999. Slope analysis of Auditory Brainstem Responses in children at risk of central auditory processing disorders. Scand. Audiol. 28(2):85-90.

Guest, M., Boggess, M., Attia, J., D'Este, C., Brown, A., Gibson, R., Tavener, M., Gardner, I., Harrex, W., Horsley, K. and Ross, J. 2010. Hearing impairment in F-111 maintenance workers: the study of health outcomes in aircraft maintenance personnel (SHOAMP) general health and medical study. Am J Ind. Med. 53(11): 1159-1169.

Guthrie, O.W. 2008. Preincision complex-I from the excision nuclease reaction among cochlear spiral limbus and outer hair cells. J. Mol. Histol. 39(6): 617-25.

Guthrie, O.W., Gearhart, C.A., Fulton, S., and Fechter, L.D. 2011. Carboxy alkyl esters of Uncaria tomentosa augment recovery of sensorineural functions following noise injury. Brain Res. 1407: 97-106.

Guthrie, O.W., Xu, H., Wong, B.A., McInturf, S.M., Reboulet, J.E., Ortiz, P.A. and Mattie, D.R. 2014. Exposure to low levels of jet-propulsion fuel impairs brainstem encoding of stimulus intensity. J Toxicol Environ Health A 77(5): 261-280.

Hunter, K.P., and Willott, J.F. 1987. Aging and the auditory brainstem response in mice with severe or minimal presbycusis. Hear. Res. 30(2-3): 207-18.

Jaruchinda, P., Thongdeetae, T., Panichkul, S., and Hanchumpol, P. 2005. Prevalence and an analysis of noise--induced hearing loss in army helicopter pilots and aircraft mechanics. J. Med. Assoc. Thai. 88 Suppl. 3: S232-9.

Jerger, J.F., Carhart, R., Tillman, T.W., and Peterson, J.L. 1959. Some relations between normal hearing for pure tones and for speech. J. Speech Hear. Res. 2(2): 126-40.

Kaufman, L.R., LeMasters, G.K., Olsen, D.M. and Succop, P. 2005. Effects of concurrent noise and jet fuel exposure on hearing loss. J. Occup. Environ. Med. 47(3): 212-218.

Kujawa, S. G. and Liberman, M. C. 2009. Adding insult to injury: cochlear nerve degeneration after "temporary" noise-induced hearing loss. J. Neurosci. 29(45): 14077-14085.

Martin, G.K., Stagner, B.B., and Lonsbury-Martin, B.L. 2006. Assessment of cochlear function in mice: distortion-product otoacoustic emissions. Curr. Protoc. Neurosci. Chapter 8: Unit 8 21C.

Ngan, E.M. and May, B.J. 2001. Relationship between the auditory brainstem response and auditory nerve thresholds in cats with hearing loss. Hear. Res. 156(1-2): 44-52.

NRC. 2011. Guide for the Care and Use of Laboratory Animals. Eighth Edition. National Academy Press, Washington, D.C.: Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council.

Raynal, M., Kossowski, M., and Job, A. 2006. Hearing in military pilots: one-time audiometry in pilots of fighters, transports, and helicopters. Aviat. Space Environ. Med. 77(1): 57-61.

Rowe, M.J. 1981. The brainstem auditory evoked response in neurological disease: a review. Ear Hear. 2(1): 41-51.

Smedje, G., Lunden, M., Gartner, L., Lundgren, H., and Lindgren, T. 2011. Hearing status among aircraft maintenance personnel in a commercial airline company. Noise Health 13(54): 364-70.

Stubbs, J.E. 2010.Development of a novel noise delivery system for JP-8 ototoxicity studies. Thesis, Air Force Institute of Technology, Wright-Patterson AFB, OH. AFIT/GIH/ENV/10-M04.

Whitehead, M.L., McCoy, M.J., Lonsbury-Martin, B.L., and Martin, G.K. 1995a. Dependence of distortion-product otoacoustic emissions on primary levels in normal and impaired ears. I. Effects of decreasing L2 below L1. J. Acoust. Soc. Am. 97(4): 2346-58.

Whitehead, M.L., Stagner, B.B., Lonsbury-Martin, B.L., and Martin, G.K. 1995b. Effects of ear-canal standing waves on measurements of distortion-product otoacoustic emissions. J. Acoust. Soc. Am. 98(6): 3200-14.

Whitehead, M.L., Stagner, B.B., McCoy, M.J., Lonsbury-Martin, B.L., and Martin, G.K. 1995c. Dependence of distortion-product otoacoustic emissions on primary levels in normal and impaired ears. II. Asymmetry in L1,L2 space. J. Acoust. Soc. Am. 97(4): 2359-77.

Wible, B., Nicol, T., and Kraus, N. 2004. Atypical brainstem representation of onset and formant structure of speech sounds in children with language-based learning problems. Biol. Psychol. 67(3): 299-317.

Zhou, X., Jen, P.H., Seburn, K.L., Frankel, W.N., and Zheng, Q.Y. 2006. Auditory brainstem responses in 10 inbred strains of mice. Brain Res. 1091(1): 16-26.

LIST OF ABBREVIATIONS

ABR	auditory brainstem response
AFMAN	Air Force Manual
AFMSA	Air Force Medical Support Agency
ANOVA	analysis of variance
CANS	central auditory nervous system
CAP	compound action potential
CAPD	central auditory processing dysfunction
dB	decibel
DHP	Defense Health Program
DoD	Department of Defense
DPOAE	distortion product otoacoustic emissions
DTIC	Defense Technical Information Center
F344	Fischer 344
FTIR	Fourier-transform infrared spectrophotometer
GSD	geometric standard deviation
HEPA	high efficiency particulate air
i.m.	intramuscular
IACUC	Institutional Animal Care and Use Committee
JP-8	jet-propulsion fuel-8
LE	Long Evans
MMAD	mass median aerosol diameter
NAMRU-D	Navy Medical Research Unit – Dayton
NIOSH	National Institute for Occupational Safety and Health
OBN	octave band noise
OSHA	Occupational Safety and Health Administration
OSPL	output sound pressure level
PBS	phosphate-buffered saline
PEL	permissible exposure limit
REL	recommended exposure limit
SG	Surgeon General
SPL	sound pressure level
THRU	Toxic Hazard Research Unit
WPAFB	Wright-Patterson Air Force Base