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## BIOMARKERS OF FATIGUE: Metabolomics Profiles Predictive of Cognitive Performance

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**AFRL-RH-WP-TR-2013-0092**

**BIOMARKERS OF FATIGUE:  
Metabolomics Profiles Predictive of  
Cognitive Performance**

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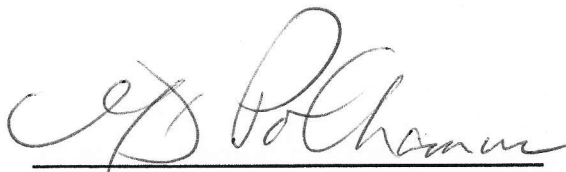
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BIOMARKERS OF FATIGUE: Metabolomics Profiles Predictive of Cognitive Performance

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## **PREFACE**

This research was conducted by the Bioeffects Division, Molecular Bioeffects Branch (711 HPW/RHDJ), Human Effectiveness Directorate of the 711th Human Performance Wing of the Air Force Research Laboratory, Wright-Patterson AFB, OH, under Dr. John J. Schlager, Branch Chief. This technical report was written as the Final Report for 711 HPW/RHDJ Work Unit 7184D420.

Research performed with Dr. Nicholas Reo, Wright State University, under Henry M. Jackson Foundation sub-award HJF 132633, contract FA8650-05-2.

The protocol involving human research subjects (F-WR-2010-0029-H) was approved by the Wright-Patterson Institutional Review Board (IRB), and was conducted at Brooks City-Base Texas under the direction of Dr. Donald Harville, Biosciences and Performance Division, Biobehavioral, Bioassessment, and Biosurveillance Branch.

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## 1.0 SUMMARY

The sleep deprivation-induced fatigue susceptibility ranking portion of the cognitive fatigue study seeking to determine metabolic biomarkers for resistance to sleep deprivation-induced cognitive fatigue was completed at Brooks City-Base, TX in 2010. A total of 23 subjects completed a sleep-deprivation induced fatigue protocol involving informed consent, medical screening, two-day evening training on cognitive tasks, providing five urine samples (beginning with a morning 12 h pre-study baseline), refresher training, and an experimental session (with six, four hour long blocks) starting in the evening. Subjects demonstrated the expected declines in performance during the 36-hour, 15-minute period of sleep deprivation without caffeine. The simple change from baseline results on the Psychomotor Vigilance Task (PVT) was recommended as the primary classifier for cognitive fatigue susceptibility. The Automated Neuropsychological Assessment Metrics-Math, Continuous Performance Test (CPT), and Grammatical Reasoning (ANAM-core), developed by the US Army, was also implemented with the math component recommended as the secondary classifier for cognitive fatigue susceptibility (Harville et al., 2010). The details of the first phase of the cognitive fatigue study involving psychological computer testing in which study participants were ranked according to their cognitive fatigue susceptibility, based on their behavior observed during sleep deprivation, was described in technical report AFRL-RH-WP-TR-2010-0150 entitled: “*Biomarkers of Fatigue: Ranking Mental Fatigue Susceptibility* (Harville et al, 2010).” The present report describes the second phase of the study involving nuclear magnetic resonance (NMR) spectroscopy-based metabolomics analysis of urine samples collected from these same study subjects to determine if metabolite profiles, or biomarkers, could be used as a predictor of cognitive fatigue susceptibility in military personnel prior to selection for demanding missions.

Urine is a noninvasively-collected, information-rich biofluid that can provide insight into the metabolic state of an organism. As a result, urine is often a focus in metabolomics investigations using NMR and liquid chromatography/mass spectroscopy (LC/MS) analyses. Targeted profiling is a powerful tool that can drive such studies, providing direct identification and quantification of a variety of potential metabolite biomarkers. This approach has been demonstrated previously in animal toxicity studies. Statistically significant group classification based on NMR spectral analysis has been previously shown in a metabolomics study investigating the susceptibility of rats to acetaminophen toxicity (Clayton et al., 2006). This work described an alternative and conceptually new 'pharmaco-metabonomic' approach to personalizing drug treatment that used a combination of pre-treatment metabolite profiling and chemometrics to model and predict the responses of individual subjects. This study was able to predict sensitivity/resistance to acetaminophen toxicity in rats prior to exposure to the compound.

Preliminary results from a previous sleep deprivation study indicated that urinary metabolite profiles (biosignatures) identified using NMR and LC/MS analyses were able to classify subjects with respect to cognitive performance during sleep-deprivation induced fatigue. Results

obtained from this previous cognitive fatigue study also indicated that pre-study NMR spectral profiling of human urine, along with chemometric analysis, was capable of predicting cognitive fatigue susceptibility prior to testing (data not reported).

The purpose of the second phase of the current cognitive fatigue research is to identify degree of fatigue resistance/sensitivity and to anonymously identify urinary biomarkers (metabolites) associated with fatigue resistance/sensitivity. Recent advances in our laboratory's bioinformatics tools development for multivariate statistical analysis of NMR-based metabolomics data has resulted in more accurate biosignature and biomarker identification (Anderson et al. 2011). In the present study, bioinformatic analysis of NMR data indicated that urinary metabolite profiles could discern differences in cognitive performance of study subjects 12 h prior to cognitive testing, and of subjects that were sleep-deprived for 28 h. Furthermore, psychological cognitive testing scores (i.e. PVT and ANAM-core), with regards to classification as cognitively resistant or sensitive to sleep deprivation-induced fatigue, were found to correlate with urinary metabolite profiles determined at 28 h of sleep deprivation. Although significant urinary metabolite profile differences were observed by NMR analysis 12 h prior to the start of the psychological testing phase of the study between two cohort subset group classifications, with respect to cognitive performance, no differences in cognitive performance were noted via psychological testing at this time point.

Using a Chenomx database analysis of processed NMR data, we identified twenty three metabolite spectral peaks that were significant in separating two subsets of 23 human subjects; one that was cognitively resistant ( $n = 6$ ) and one cognitively sensitive ( $n = 6$ ) to sleep deprivation. Seven of the 23 NMR spectral peaks could not be assigned a metabolite identification using the Chenomx database. Fourteen of the 23 peaks were statistically significant ( $p \leq 0.05$ ) in distinguishing between the two fatigue classification groups. Five of these 14 metabolites could not be identified. The other nine metabolites were identified and a number of these were noteworthy. These included: tyrosine, homovallinate, 3-hydroxyisovalerate, alanine, 2,2-dimethylsuccinate and taurine. Tyrosine and homovanillate are metabolites associated with the dopaminergic pathway and were elevated in the urine of the fatigue-resistant subjects. This pathway has been shown to be associated with a more awake state. Alanine, 2,2-dimethylsuccinate and taurine were elevated in the urine of fatigue-sensitive subjects and can be linked to high percentage dietary carbohydrate intake that has been found to be associated with inducing sleepiness. Self-recorded food logs from the subjects 8 h prior to the cognitive testing phase indicated that those who were more resistant to sleep deprivation-induced fatigue ingested significantly (2-fold) higher amounts of protein prior to testing. It is possible that using this profile of 14 metabolite peaks that the risk of cognitive decline in demanding missions may be predicted and may be associated with nutritional status.

Identification of urinary biosignatures (spectral profile) of cognitive fatigue may provide for a real-time predictive screen of cognitive performance ability in defined and undefined mission environments for preventing or minimizing mission degradation due to cognitive impairment, as well as to aid in personnel selection for demanding missions. Furthermore, nutritional intervention may yield a non-pharmacological solution to countering the effects of sleep-deprivation induced fatigue on cognitive performance during demanding military missions. Future studies determining the effects of various dietary components and composition, as well as determining optimal time following ingestion, using larger cohorts of human subjects will be needed to validate the initial results of the present study.

The results of this study lead to a working hypothesis and may be useful for developing new methods to rapidly assess and alter an individual's cognitive status. We hypothesize that the nutritional status and subsequent metabolite profile is directly correlated with cognitive performance and fatigue resistance. Further, we suggest that NMR-based urinary metabolomics is a powerful tool to assess the nutritional status of individuals for optimal cognitive performance (i.e. mapping coordinates of an individual's urinary metabolite profile can be visualized graphically in reference to coordinates indicative of a formulated fatigue index). This information can then be used to make appropriate adjustments to the diet, while monitoring the trajectory plot of the metabolite profile until a desired outcome is reached.

## **2.1 INTRODUCTION**

### **2.2 Military Fatigue**

Most humans require approximately 8 h of sleep to maintain optimal cognitive function when awake (Anch et al., 1988). It is also well established that fatigue and cognitive performance are linked. Vigilance tasks have been shown to be very sensitive to fatigue induced by sleep deprivation, and result in cognitive performance degradation (Belenky et al., 2003; Driskell et al., 1991; Driskell and Salas, 1996; Hursh and Bell, 2001; Van Dongen et al., 2003). Research has also shown that the effects of fatigue directly impact vigilance, mood and cognitive performance (Krueger, 1991). Persistent sleep deprivation also contributes to both central and peripheral fatigue. Sleep deprivation has also been shown to have an impact on physical performance (Belmont et al., 2009). When compared to well-rested individuals, sleep deprived individuals perform cognitive and physical tasks more slowly and have problems with memory.

Because cognition is more significantly affected than psychomotor proficiency, decision making in flight operations can be problematic and lead to increases in errors and accidents (Caldwell et al., 2001; Billings et al., 1968; Pereli, 1980; Krueger, et al., 1985). Although the impact of fatigue on flight operations and our scientific understanding of fatigue and the factors involved are well known, fatigue related problems in flight operations continue to persist in both military and commercial aviation (Dinges et al., 1996). Consequently, the problems of pilot fatigue, and fatigue-related concerns regarding aviation safety, have been increasing over time. Even though there is staggering evidence that sleep deprivation has significant detrimental effects on performance, military culture evokes the warfighter mentality that it can be overcome by proper motivation. This belief is contrary to the reality that the warfighter needs adequate sleep to perform optimally.

Continuous and sustained actions in operational environments typically leads to reduction in the amount of sleep required to perform optimally. These operational environments subject the war fighter to intense physical and mental exertion. Other factors present in these environments that impact war fighter performance include, reduced caloric intake, dietary changes, and decision-making induced stress. Aircrews are particularly susceptible to the effects of fatigue due to mission-related extended duty hours and irregular sleeping patterns. Rotating shifts, maintenance delays and mission rescheduling can lead to disruptions in normal sleep cycles in pilots and ground crews causing performance decrements that can have dire consequences on mission effectiveness (Australian Government Civil Aviation Safety Authority, 2004). The Air Force (Luna, 2003; Nicholson et al., 1986), the Army (Caldwell and Gilreath, 2002; Caldwell and Gilreath, 2001), and the Navy (Belland and Bissell, 1994; Ramsey et al., 1997) have all reported that sleep deprivation-induced fatigue is a significant problem that has resulted in performance degradation. This creates a situation where a routine task can suddenly evolve into an event with lethal consequences.



Because fatigue has been shown to be an occupational hazard, leading to cognitive defects in performance, there has been a recognized need for real-time detection technologies that minimize fatigue-induced mishaps (Brown, 1997). Efforts have been made to detect real-time fatigue in pilots using prudent technologies in an attempt to develop and implement effective countermeasures. Although it has been established that changes in electroencephalography (EEG) brain waves are a valid means of assessing fatigue, such assessments are not practical. Furthermore, the validity of an automated technology, such as EEG, may not be legitimate in another operational environment. Therefore, their usefulness is dependent on the requirements and conditions of the operational environment (i.e. flight line or cockpit). Currently, none of the fatigue detection technologies developed has been adequately verified in an operational environment.

## **2.3 Nutrition**

Maintaining good nutrition is an important factor for both physical and cognitive performance. It has been demonstrated that normal variations in nutrition have little effect on sleep anatomy (Lucero and Hicks, 1990; Neumann and Jacobs, 1992); unusual variations in diet may produce mild effects. Severe reduction in dietary intake can lead to sleep disruption and fatigue. Failure to maintain sufficient glucose levels in the blood will result in a situation that is not promotive of restful sleep and can lead to poor cognitive performance. In fact, diets high in carbohydrates prior to bedtime have been shown to be linked to inducing sleepiness (Porter and Horne, 1981).

The central nervous system (CNS) requires a number of amino acids found in food to function adequately (Betz et al., 1994). The importance of various amino acids as precursors for key brain neurotransmitters is well established, and transport mechanisms exist to provide these to the brain (Lieberman 1999). The functional implication of this unique characteristic of the blood-brain barrier (BBB) is that the amino acid composition of food is of greater consequence to the brain than perhaps any other organ system. Results from several lines of related research suggest that the peripheral concentration of particular amino acids can be a factor in the regulation of central neurotransmission, cognitive performance, and mood state; two of these amino acids, tryptophan and tyrosine, are of particular interest.

Tryptophan is an essential amino acid found in food that is a precursor of the neurotransmitter serotonin. The highest levels of serotonin are typically observed in the hypothalamus and cortex following a pure carbohydrate meal. Serotonin is involved in the regulation of sleeping and waking (Portas et al., 2000), and ingestion of a mixture lacking tryptophan has been shown to shorten the sleep cycle (Moja et al., 1984; Voderholzer et al., 1998). Furthermore, it has been shown that severely undernourished soldiers perform poorly on cognitive tasks (Lieberman et al., 1997). However, as the concentration of protein in a meal increases the brain concentration of

serotonin falls. Studies suggested that increasing the amount of tyrosine in the diet causes an increase in brain catecholaminergic neurotransmission leading to a more awake state (Lieberman 1994).

Tyrosine is not considered an essential amino acid because it can be synthesized from phenylalanine in humans. Furthermore, protein foods typically contain higher levels of tyrosine compared to tryptophan. Tyrosine, a precursor of the neurotransmitters norepinephrine, epinephrine and dopamine, enhances cognitive performance in animals (Ahlers, et al., 1994; Rauch and Lieberman, 1990; Shukitt-Hale et al., 1996; Shurtleff et al., 1993) and in humans under both physical and psychological stress (Banderet and Lieberman, 1989; Shurtleff et al., 1994; Deijen and Orlebeke, 1994).

## **2.4 Metabolomics**

Metabonomics is defined as “the quantitative measurement of the time-related multiparametric metabolic response of living organisms to pathophysiological stimuli or genetic modification” (Nicholson et al., 1999). The field of metabonomics is concerned with the study of fixed cellular and biofluid concentrations of endogenous metabolites, as well as dynamic metabolite fluctuations, exogenous species, and molecules that arise from chemical rather than enzymatic processing (Lindon et al., 2003). Metabonomics is an approach used to characterize the metabolic profile of a specific tissue or biofluid. Because many biofluids can be easily obtained either non-invasively (urine) or minimally invasively (blood), they are typically used in metabonomic studies. Metabolic alterations in various biofluids are expressed as “fingerprints” of biochemical perturbations that are characteristic of the type of stressor (biological, psychological, chemical, etc.) and target tissue insult (brain, liver, kidney, etc.). These metabolic alterations are often seen in the urine as changes in metabolic profile in response to the types of stressors or insults mentioned above.

Metabolomics is a powerful tool for assessing the overall physiology of an organism. Because metabolomics measures the results of a multitude of biochemical and environmental interactions within an organism, it serves as a good indicator of an organism’s phenotype. Therefore, metabolomics allows for quantitative assessment of molecular phenotype in research studies.

The most common analytical platforms used today in metabonomics are proton NMR, and mass spectroscopy (MS) coupled to liquid chromatography (LC) and gas chromatography. The advantages of NMR-based metabonomics include being a nondestructive analysis, applicable to intact biomaterial, and information-rich with respect to determinations of molecular structure, especially in complex mixtures. The non-selectivity, lack of sample bias and reproducibility of NMR (Keun et al. 2002) is of critical importance when considering screening applications.

Changes in NMR-derived urinary metabolite levels have proven to be a sensitive indicator of chemical-induced toxicity (Robertson et al., 2000; Holmes and Shockcor, 2000; Waters et al., 2002; Nicholson, et al., 2002). MS offers the ability to detect chemical classes not detected by NMR (i.e. sulfates), and the capability to detect lower abundance metabolites with little sample processing. This is of critical importance if one is searching for novel biosignatures (or biomarkers) of physiological, psychological or chemical insult. Therefore, MS is complementary to NMR data and facilitates metabolite identification.

The mammalian metabolome is comprised of both endogenous and exogenous metabolites. The latest version of the Human Metabolome Database (v. 2.5; released August, 2009) includes approximately 8,000 identified mammalian metabolites, with over 1,000 of these metabolites in the Human Metabolome Library ([www.hmdb.ca](http://www.hmdb.ca)). Endogenous metabolites are small molecules that are produced by the host genome, whereas exogenous metabolites, also known as xenometabolites, are "foreign" substances derived from food, drugs or host-specific microbes. Endogenous metabolites vary little between species (i.e. rats, mice, humans, etc.). However, compared to exogenous metabolites, the number of endogenous metabolites is relatively small. The large number of exogenous metabolites is reflected by the highly varied diet of mammals that includes ingestion of a wide spectrum of animal, plant and microbe products. Further adding to the complexity of the exogenous metabolite pool is the inclusion of drugs, nutraceuticals, and other xenobiotics. In addition, metabolites from gut microflora offer another important source of exogenous metabolites.

Urine is the only biofluid where the entire accumulation of water-soluble metabolites can be found. This makes urine the ideal biofluid for metabolite analysis. Although urine cannot be sampled continuously, such as blood, it can be obtained in copious amounts and by non-invasive means. However, urine analysis only reflects metabolic events that occurred at an earlier time during host genome processing (i.e. gene and protein expression). While it is possible to maintain experimental control over diet and environment in animal studies, it is essentially impossible in the case of human studies. However, utilizing specialized diets and dietary restrictions (i.e. eliminating specific confounding food items, drugs, smoking) one can partially control for the variations intrinsically associated with human subjects. Furthermore, utilization of food/beverage intake logs can also further reduce this variability. These dietary issues can also be minimized by incorporating over-night fasting into the experimental design.

The rationale for performing metabolomics analysis in the current cognitive fatigue study is based on preliminary analysis of NMR data of human urine collected in a previous cognitive fatigue study in 2005 (data not reported). Preliminary NMR-based metabolomics results from this study indicated that distinctive urinary profiles (or biosignatures) were identified that could distinguish fatigue-susceptibility prior to being placed on-study. This finding was supported by previous metabolomics work investigating susceptibility to acetaminophen toxicity in rats (Clayton et al., 2006). This work described an alternative and conceptually new 'pharmaco-metabonomic'

approach that used a combination of pre-dose metabolite profiling and chemometrics to model and predict the responses of individual subjects. Results obtained from the previous cognitive fatigue study also indicated that pre-study NMR profiling of human urine, along with chemometric analysis, was capable of predicting cognitive performance outcome under sleep deprivation-induced fatigue prior to testing. Identification of urinary biosignatures predictive of fatigue susceptibility may provide for a real-time screen of cognitive/physical performance ability in defined and undefined mission environments for preventing or minimizing mission degradation due to cognitive/physical impairment, as well as to aid in personnel selection for demanding missions.

### **3.1 MATERIALS AND METHODS**

In the present study, a total of 23 human subjects were recruited. The human-use protocol (F-WR-2010-0029-H) for the cognitive fatigue research project was approved by the Wright-Patterson Institutional Review Board, and was conducted at Brooks City-Base, San Antonio, Texas under the direction of Dr. Donald Harville, Biosciences and Performance Division, Biobehavioral, Bioassessment, and Biosurveillance Branch. Human subjects were subjected to pre-study dietary restrictions as outlined in the “Informed Consent Document” (ICD) and maintained food and fluid intake logs over the course of the 36 h and 15 min sleep-deprivation period of this study. All research subjects were male (19 to 39 years old), were informed concerning all study experimental procedures, were informed concerning risks and benefits and were made aware of their entitlements and confidentiality rights. All research subjects read and signed an ICD prior to participating in any aspects of this research project. The psychological testing conducted during the first phase of cognitive fatigue study included the PVT and ANAM-core tests. During the psychological testing phase of the study, beginning 12 h after awaking, subjects were provided standard meals (Table 1). Descriptions of these psychological task classifier tests are provided in the previously published technical report for this study (Harville et al., 2010). Study subjects were required to record sleep/wake cycles for 3 days prior to the start of the study using wrist-worn actigraphs to ensure all subjects received approximately the same amount of sleep within the time window of 2300 to 0700 h. After two evenings of training, study participants were exposed to 36 hours-15 minutes of sustained wakefulness, during which, urine samples were collected at various time-points. During the last 24 hours-15 minutes, operational work was simulated using repeated cognitive tests (six 4 h blocks). Urine samples were collected cold from all subjects beginning with the first void urine following an eight hour overnight fast (0 h) and then at 12 h, 20 h, 28 h and 36 h. Urine samples were then immediately frozen and stored at -20 °C prior to being shipped on dry ice to Air Force Research Laboratory (AFRL), Molecular Bioeffects Branch, for NMR analysis. A schematic of the experimental design and time-line for urine collection is shown in Figure 1.

**Table 1: Standard 2400 calorie meal plan (male) provided during 36 h sleep deprivation study**

**Friday 7pm, Dinner (750 calories)**

1 cup chicken noodle soup  
6 oz sirloin  
1 medium baked white potato  
1 cup broccoli  
3 tsp margarine  
1 fat free sour cream  
1 pkg Teddy Grahams Cinnamon  
Salt & pepper

**Friday 11pm, Snack (200 calories)**

1 Quaker Cereal Bar-Apple Crisp  
1 cup orange juice

**Saturday 3am, Snack (200 calories)**

1 Quaker Cereal Bar-Apple Crisp  
1 cup fat-free milk

**Saturday 7am, Breakfast (450 calories)**

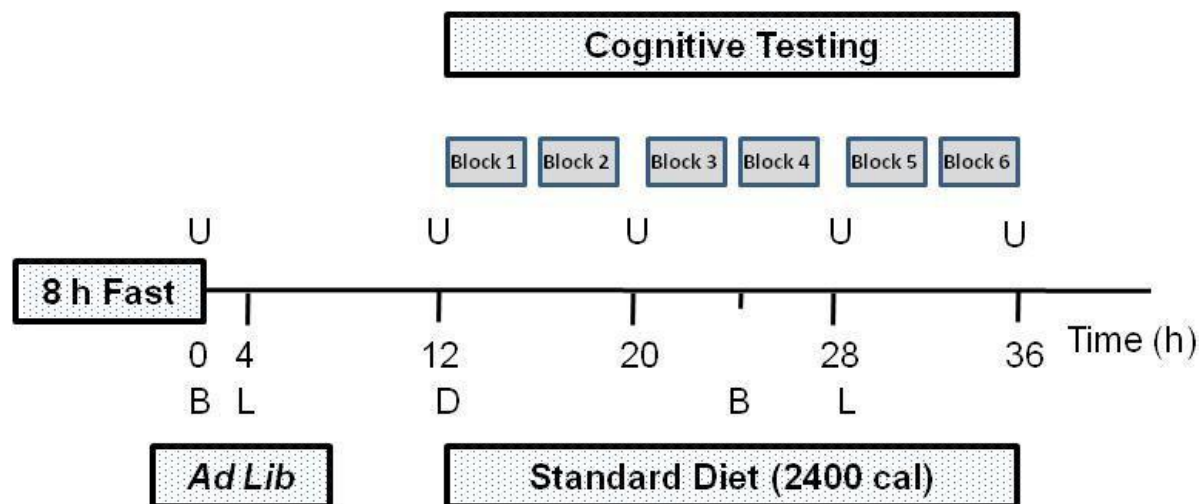
1 container Wheaties cereal  
1 banana  
1 cup fat-free milk  
1 cup 100% orange juice  
3 pkgs sugar

**Saturday 11am, Lunch (600 calories)**

2 slices whole-wheat bread  
4 oz turkey lunch meat  
1 slice cheese  
2 tsp mayonnaise  
1 tsp mustard  
½ cup baby carrots  
1 apple

**Saturday 3pm, Snack (200 calories)**

1 Quaker Cereal Bar-Apple Crisp  
1 cup fat-free milk



**Figure 1. Cognitive fatigue study experimental design.**

### 3.2 Pre-Study Restrictions

Participants were not allowed any naps or caffeine during the 1-hour, 30-minutes refresher training or during the 24-hour, 15-minutes protocol. Research subjects were instructed at the first of two training sessions for personal computer (PC)-based cognitive testing tasks (i.e. PVT and ANAM) to reduce their daily caffeine consumption 1/3 each day and to consume no caffeine on the study start day. Subjects were also instructed 72 h prior to the start of the study to get a minimum of seven hours sleep each night. Study participants were not allowed to take medications of any kind (including over-the-counter), use tobacco, or ingest alcohol from 24 h prior to providing their first urine sample until the end of the study. Additionally, they were told not to consume fish, cheese, garlic, onion, beetroot, asparagus, cherries, grapefruit, or liquorice over this same time period. Furthermore, subjects were instructed not to perform any strenuous exercise during this period of time. Strenuous exercise was defined as anything that increased the heart rate above 140 beats per minute. Study participants were also subjected to an overnight fast (8 h) prior to providing their first urine sample 12 h prior to the start of the study.

### 3.3 Caloric Intake

Food logs were used to record dietary intake (food and beverage) over the total 36 h sleep deprivation period and cognitive testing phases of the study. Following the 8 h overnight fast,

subjects were allowed to eat *ad libitum* (except those food items noted above as pre-study restricted) for breakfast (~ 0700 h) and lunch (~1130 h), and recorded their food intake prior to the start of the study PC-based cognitive testing phase at 1900 h. Identical standard meals of 2400 calories/day (dinner at 1900 h, breakfast at 0700 h, and lunch at 1100 h) were provided during the 24 h and 15 minute repeated cognitive testing phase of the study for all subjects beginning at 1900 on the day of testing (Table 1). Subjects also recorded their standard meal food intake during this phase of the study. There was free access to permitted fluids throughout the 24 h PC-based cognitive testing phase. Nutritional information of *ad libitum* diets of subjects was derived using a web-based nutritional database called Nutrition Data ([www.NutritionData.com](http://www.NutritionData.com)) developed by Nutrition Data. The information in Nutrition Data's database is derived from the United States Department of Agriculture's "National Nutrient Database for Standard Reference" and is supplemented by listings provided by restaurants and food manufacturers. The Nutrition Data analysis of type and amount of food ingested was conducted by a registered dietitian at Wright-Patterson Air Force Base, Dayton (WPAFB), OH and yielded nutritional information regarding levels of calories, carbohydrates, protein and fat ingested. Food logs were provided to the dietitian without any personal identifiers or information on cognitive performance testing outcomes. Nutritional analysis was not performed on standard meals because all subjects ingested the complete standard meals. Statistical analysis of macronutrient amounts was performed using a two-tailed Student's t-test ( $p < 0.05$ ).

### 3.4 Urine Sample Collection

Subjects were given instructions, a cooler, ice packs, and supplies for their first urine sample during training. Urine was collected in a chilled graduated container. A total of five urine samples were collected over the course of the 36 h sleep-deprivation study. Subjects provided a urine sample as soon as they woke at the start of the 36 h sleep-deprivation period and prior to the start of the 24 h psychological testing phase of the study at 0700 h and 1900 h, respectively. The remaining three urine samples were collected on the second day of the study over the 24 h PC-based psychological testing phase during the 1 hour study breaks at 8 hour intervals (0300 h, 1100 h, and 1900 h). The volume of the spot urine sample was recorded and then a 20-30 mL portion was transferred to a plastic tube on ice prior to freezing and storage at -20 °C according to the provided instruction. All urine collections occurred prior to ingestion of any food or fluids. Furthermore, volunteers were asked to keep a detailed food log on day-1 from 0700 h to 1900 h and during their scheduled 1 hour breaks throughout the 24-hours and 15-minutes psychological testing period. The urine samples were then transported from Brooks City Base, TX to WPAFB on dry-ice in an insulated pack. Upon arrival at WPAFB, all urine samples were thawed at 4 °C and then centrifuged at 2500 x g for 10 min at 4 °C to remove any solid debris from the urine. Portions (1.0 mL) of the supernatant fluid were then stored at -80 °C prior to analysis using NMR spectroscopy.

### 3.5 Urine Sample Preparation

Urine samples for NMR analyses were prepared as described by Robertson et al. (2000) and modified as follows. Frozen (-80 °C) urine samples were thawed at 4 °C overnight, and then equilibrated to room temperature just prior to NMR sample preparation. A 600 µL aliquot of urine was transferred to a 1.5 mL Eppendorf tube, mixed with 300 µL of phosphate buffer (0.2 M monosodium phosphate and 0.2 M disodium phosphate, pH 7.4), and allowed to equilibrate for ten minutes. Samples were then centrifuged at 5000 rpm for ten minutes to remove any particulate matter, and a 550 µL aliquot of supernatant was transferred to a 5 mm NMR tube. An internal standard consisting of 150 µL of trimethylsilylpropionic (2, 2, 3, 3 d<sub>4</sub>) acid (TSP) dissolved in deuterium oxide was then added at a final concentration of 2 mM. Study urine samples were stored at -80 °C until all samples had been satisfactorily analyzed by NMR and all data collection results had been validated. Urine samples were then placed in biohazards bags and disposed of as biohazardous waste.

### 3.6 NMR Data Acquisition and Processing

Proton NMR spectra were acquired at 25 °C on a Varian INOVA NMR instrument operating at 600 MHz. Water suppression was achieved using the first increment of a Nuclear Overhauser Effect Spectroscopy (NOESY) pulse sequence, which incorporates saturating irradiation (on resonance for water) during the relaxation delay (7.0 s total; 2 s with water pre-saturation) and the mixing time (50 ms total; 42 ms with water irradiation). Data was signal averaged over 128 transients using a 4.0 s acquisition time and interpulse delay of 11.05 s.

NMR spectral data were processed using Varian software (VNMR 6.1c) and employing exponential multiplication (0.3 Hz line-broadening), Fourier transformation, and baseline flattening (fifth-order polynomial and spline fitting routines). Spectra were then baseline corrected (flattened) in MATLAB (The Mathworks, Inc. Natick, MA; v. R2010b) using the Whittaker Smoother algorithm (with lambda value of 200) on selected spectral noise regions (Eilers 2003; Whittaker 1923). Spectral resonances in urine from TSP (0.0 ppm), residual water (4.72-5.00 ppm) and urea (5.54-6.01 ppm) were excluded from the analyses. Spectra were then preprocessed using a probabilistic quotient normalization (PQN) method. This method is based on the calculation of a most probable dilution factor by looking at the distribution of the quotients of the amplitudes of a test spectrum by those of a reference spectrum (Dieterle et al., 2006). To reduce the dimensionality and mitigate peak misalignment, a dynamic programming-based adaptive binning technique was employed (Anderson et al. 2011) using a minimum and maximum distance between peaks in a single bin of 0.001 and 0.04 ppm, respectively. Bin boundaries were then manually adjusted to further mitigate peak misalignment, and to keep known J-coupled multiplets within that same bin (e.g., doublets, triplets, etc). Integrated bin



areas were transferred to an Excel file and normalized to the TSP signal intensity. Data were then autoscaled using various datasets as reference.

### 3.7 NMR Data Acquisition and Processing

Multivariate data analyses were conducted on binned, scaled spectral data using MATLAB software. Binned NMR data were scaled to a chosen reference dataset by subtracting each bin value from the mean value for the corresponding bin in the reference data, then dividing this value by the standard deviation of the reference data (auto-scaling).

Principal Component Analysis (PCA) provided a first-approach, unsupervised technique, for data visualization. As previously described (Mahle et al. 2010), PCA model constructs were based on specific experimental groups to explore any systematic differences between groups that may exist. Once the model is constructed, other groups can then be superimposed into the visualization, by applying the model-specific bin coefficients (PCA loadings), to show how they compare. Thus PCA models were constructed to maximize visualization of specific responses based upon the nature of the effects being assessed, and PCA scores plots were used to help identify the time points of maximum effects for treatments.

Orthogonal Projection onto Latent Structures - Discriminant Analysis (OPLS-DA) was used as a supervised technique to classify data and identify salient features that allow class separation (Wold et al. 2001). In order to apply OPLS-DA, spectral data were collected into a matrix of variables or bins (X) and a vector of categorical labels (Y), representing the effects. These data were then analyzed and modeled as follows: (1) determine a specific time point of interest; (2) encode each treatment and corresponding control group as a two-group problem and analyze with OPLS; (3) using the model created for this specific two-group problem, project the remaining samples from other groups into the OPLS model. Therefore, OPLS enabled classification into specific groups. The OPLS model was evaluated on its predictive ability, using the  $Q^2$  (coefficient of prediction) metric.  $Q^2$  was calculated as follows:

$$Q^2 = 1 - \frac{PRESS}{SSY} = 1 - \frac{\sum_{i=1}^n e_i^2}{\sum_{i=1}^n (y_i - \bar{y})^2} = 1 - \frac{\sum_{i=1}^n (y_i - \hat{y}_i)^2}{\sum_{i=1}^n (y_i - \bar{y})^2}$$

where PRESS is the Predicted Residual Sum of Squares calculated as the residual  $e_i$  between the predicted and actual Y (class labels) during leave-one-out cross-validation, SSY is the Sum of Squares for y,  $\bar{y}$  is the y mean across all samples, and  $\hat{y}_i$  is the y value for  $i^{th}$  sample. As  $Q^2$  approaches 1, the more predictive capability the model exhibits. A  $Q^2$  value less than zero

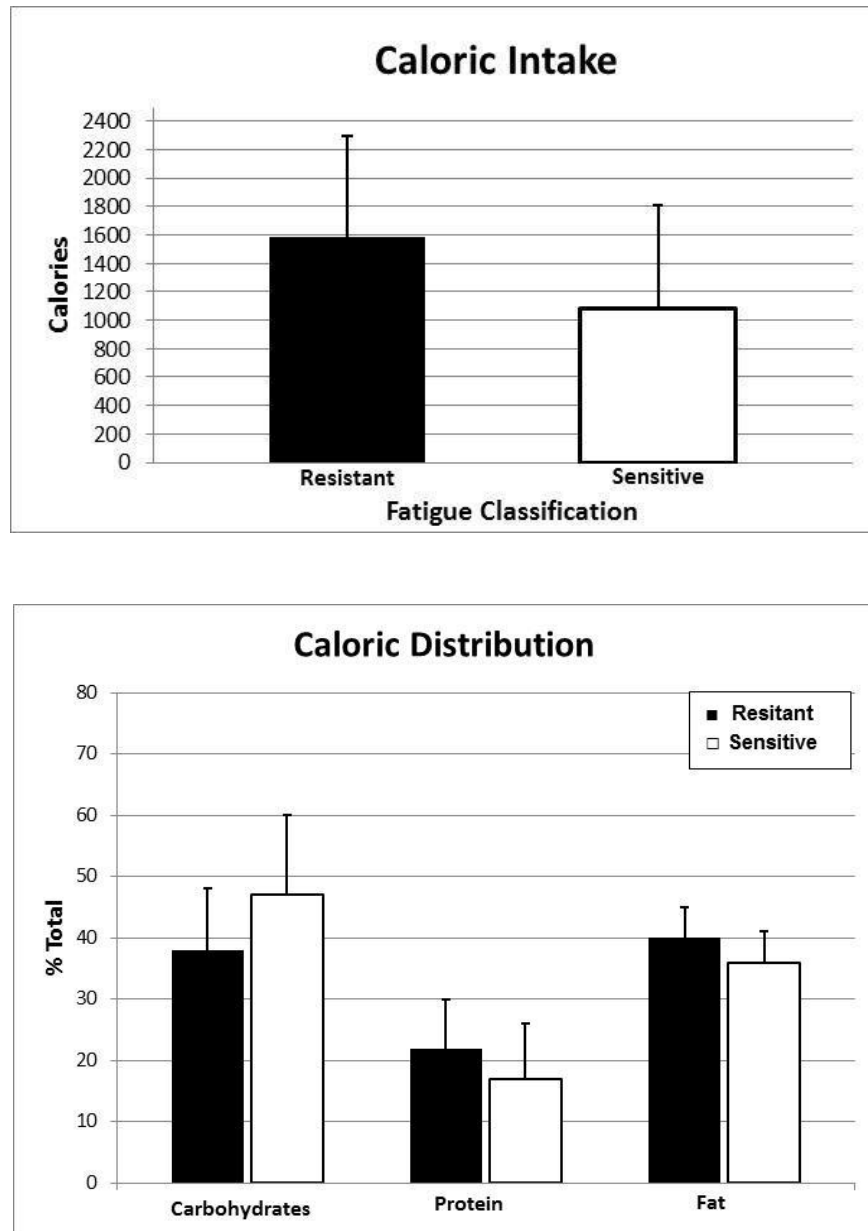
indicates that the model has no predictive power. A permutation test was performed to evaluate the significance of the  $Q^2$  metric. The test involved repeatedly permuting the data labels and re-running the discrimination analysis, resulting in a distribution of the  $Q^2$  scores (Westerhuis et al. 2008). The  $Q^2$  from the correctly labeled data is then compared to the distribution to determine the significance of the model at a specified alpha (set herein at  $\alpha = 0.01$ ).

Variable selection (salient bins) from OPLS-DA was also statistically evaluated. The bin loadings, commonly referred to as coefficients, were compared to calculated null distributions in order to select for significance. The null distribution for each bin was determined by refitting the OPLS model to datasets in which each bin was independently and randomly permuted to remove any correlation between it and the control/treatment groups. The true OPLS model loading was then compared to the resulting null distribution of loadings, and values in the tail (greater than 99.5% or less than 0.5% of the null distribution; corresponding to  $\alpha = 0.01$ ) were assumed to contribute significantly to the model. The permutation was initially repeated 500 times for each bin and those near-significant loadings (greater than 92.5% or less than 7.5% of the null distribution; corresponding to  $\alpha = 0.15$ ) were selected for 500 additional permutations (total 1000). Comparisons between individuals were used to help identify metabolite profiles or markers of fatigue susceptibility. The salient spectral resonances were assigned to metabolites using Chenomx 5.1 software, on-line NMR databases (i.e., [mmcd.nmr.fam.wisc.edu](http://mmcd.nmr.fam.wisc.edu); U Wisc, etc), and by "spiking" samples with known compounds, if necessary.

## 4.1 RESULTS

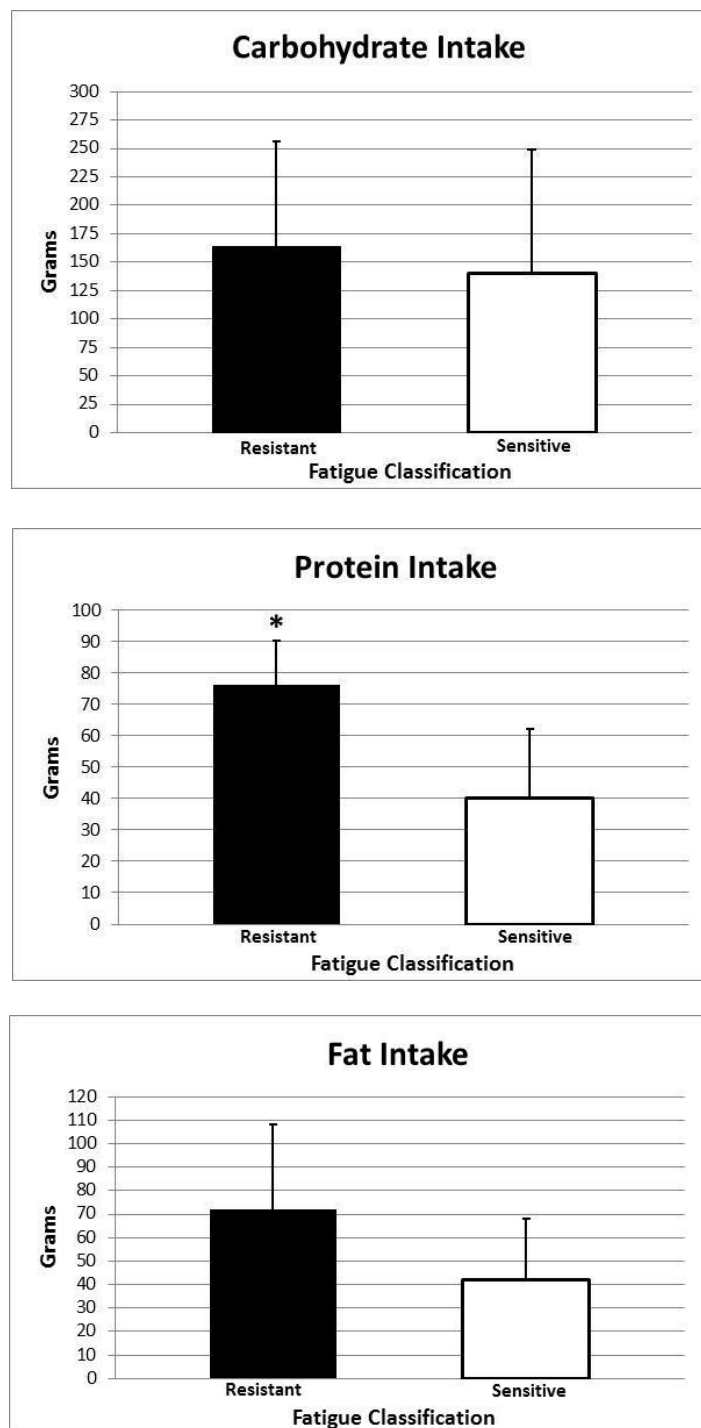
### 4.2 Pre-Study Caloric Intake

Following an overnight fast (7 h), study subjects were allowed to consume breakfast and lunch food items of their choosing, except those identified as dietary restricted in the ICD document, and the amount and type of food eaten was recorded. All subjects ate breakfast at approximately 0630 h and lunch at approximately 1130 h. No additional food-intake occurred over the next 7 h until the start of the cognitive testing phase of the study at 1700 h. Following the study, food logs were sent to an Air Force dietitian, Wright-Patterson AFB, OH without any personal identifiers or cognitive performance rankings. Food logs were analyzed using Nutrition Data. Pre-study caloric intake determinations indicated that study subjects that were identified as more resistant to the effects of sleep deprivation-induced fatigue had an average higher caloric intake (1590 calories) when compared to those subjects classified as sensitive to sleep deprivation-induced fatigue (1087 calories; Figure 2A). Furthermore, those subjects classified as sensitive to the effects of sleep deprivation-induced fatigue derived a higher percentage of their total caloric intake from carbohydrates, and demonstrated a lower percentage of their total caloric intake from protein and fat, when compared to the fatigue resistant group (Figure 2B). Due to the higher caloric intake of the fatigue resistant group, these subjects did demonstrate higher levels of



**Figure 2 Average caloric intake for subjects classified via psychological testing as fatigue-resistant (n = 6) and fatigue-sensitive (n = 6).** Bars represent mean + SD. Fatigue-resistant subjects generally derived a higher percentage of their dietary intake from protein, while fatigue-sensitive subjects derived a higher percentage of their dietary intake from carbohydrates. Caloric intake and macronutrient distribution determined from subject food logs 12 h prior to psychological testing using Nutrition Data analysis.

carbohydrate, fat and protein intake than the fatigue sensitive group. However, only the amount of protein ingested was found to be significant (2-fold higher) when compared to the fatigue sensitive group (Figure 3).



**Figure 3. Quantitation of macronutrients ingested by fatigue-resistant (n = 6) and fatigue-sensitive (n = 6) subjects 12 h prior to cognitive testing using Nutrition Data analysis. Bars represent mean + SD. Although fatigue-resistant subjects ingested higher amounts of macronutrients, only protein amounts were found to be significant. (\* p ≤ 0.05)**

### 4.3 Psychological Testing

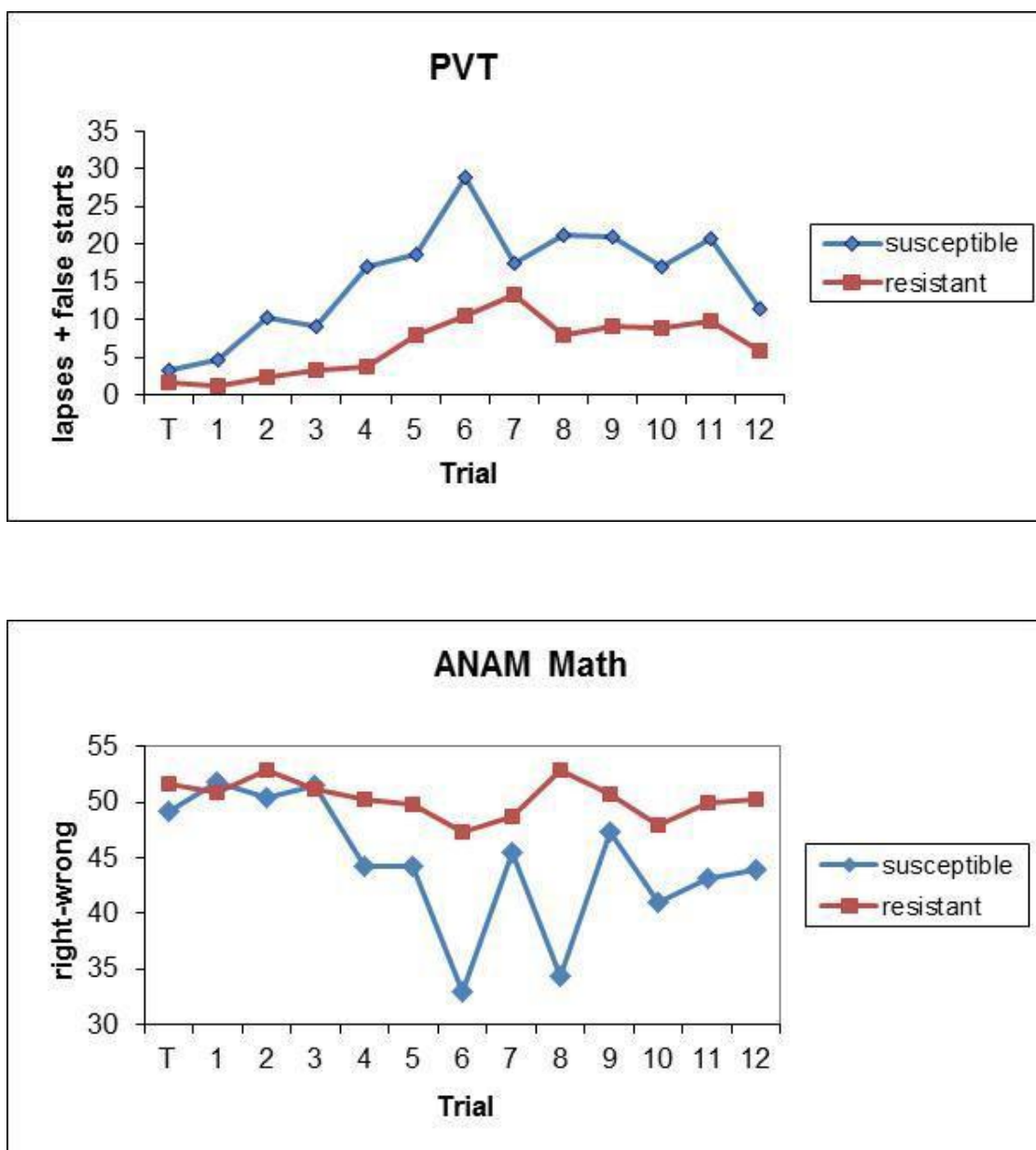
Psychological testing consisting of the PVT and ANAM-core tests were performed in six 4 h blocks over 24 h and the results were reported in the technical report by Harville et al. (2010). The findings reported in this technical report indicated that the PVT and the ANAM-math tests were the primary and secondary classifiers for cognitive fatigue susceptibility, respectively (Figure 4). Psychological testing results from all cognitive tests given indicated that two subsets of subjects were identified from the median subject fatigue classification split (i.e. 11 resistant and 12 sensitive). One of the cohort subsets ( $n = 6$ ) was consistently classified as “resistant” to the effects of sleep deprivation-induced fatigue (i.e. scored high in all test modules given). The other cohort subset ( $n = 6$ ) was consistently classified as “sensitive” to the effects of sleep deprivation-induced fatigue (i.e. scored low in all test modules given). The remaining 11 subjects demonstrated mixed results on all psychological testing, scoring highly on one test and poorly on another, and were classified as variable to the effects of fatigue.

### 4.4 NMR Spectroscopy of Human Urine

The representative NMR spectra of human urine shown in Figure 5 show the changes in endogenous components of urinary metabolites observed at 0 h hours (12 h prior to start of psychological testing) and at 28 h of sleep deprivation between two subset populations ( $n = 6/\text{group}$ ) of the cohort that were identified as being sensitive (Figure 5A) or resistant (Figure 5B) to sleep deprivation-induced fatigue. The NMR instrument phasing and baseline correction of the data was not used to generate the spectra in Figure 5. Instead, the automatic data processing procedure of the Chenomx (Chenomx Inc., Edmonton, Canada) NMR Processor application was implemented. In order to visualize the data, the NMR traces were binned with bin sizes of 0.0005 ppm (i.e. full resolution), and the data were scaled using the area of the peak associated with the internal standard (i.e. TSP). Water and urea resonances were removed from the NMR traces. Visual inspection of the spectral resonance intensity peaks from a number of metabolites clearly shows that they have been altered when compared either between group classification (i.e. resistant vs. sensitive), or time (i.e. 0 h vs. 28 h). Psychological testing involving PVT and ANAM testing indicated that 28 h of sleep deprivation resulted in peak fatigue in all subjects (Harville et al., 2010) and correlated with changes observed in urinary metabolite profiles between the two extreme cohort groups at that time.

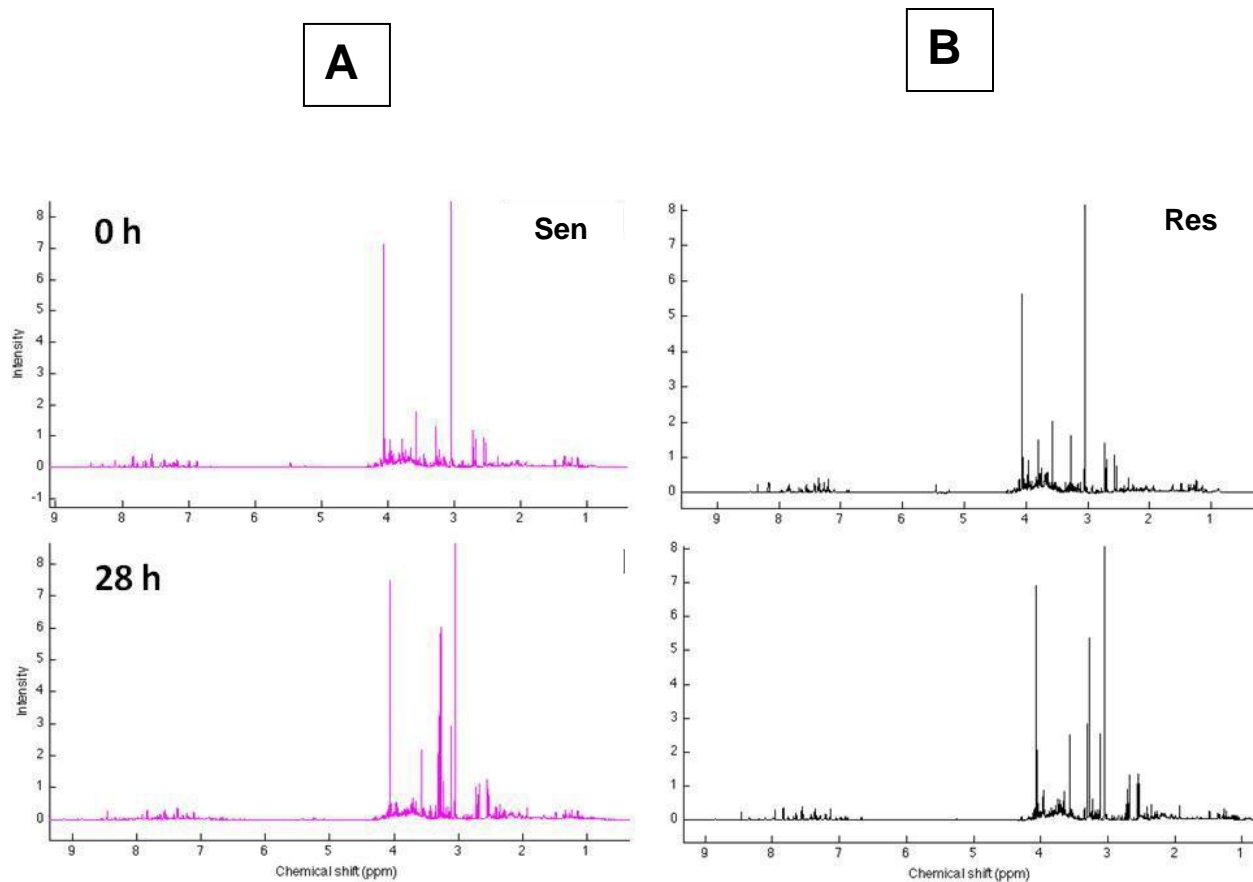
### 4.5 Unsupervised Principal Component Analysis (PCA) of NMR Data

Unsupervised PCA analysis assumes no knowledge *a priori* and is the predominant linear dimensionality reduction technique applied to a wide variety of scientific datasets. PCA attempts to map data points from a high dimensional space to a low dimensional space while keeping all relevant linear structure intact. The only input parameters are the coordinates of the data points and the number of dimensions that will be retained in the mapping. The NMR spectral intensities



**Figure 4. Plots of the primary (PVT) and secondary (ANAM-Math) psychological classifiers for fatigue susceptibility reported by Harville et al. (2010) for fatigue resistant and fatigue susceptible subjects for average training (T) and testing trials (1-12). Testing trials were performed two times within six 4 h time-blocks over a 24 h period as indicated in Figure 1.**

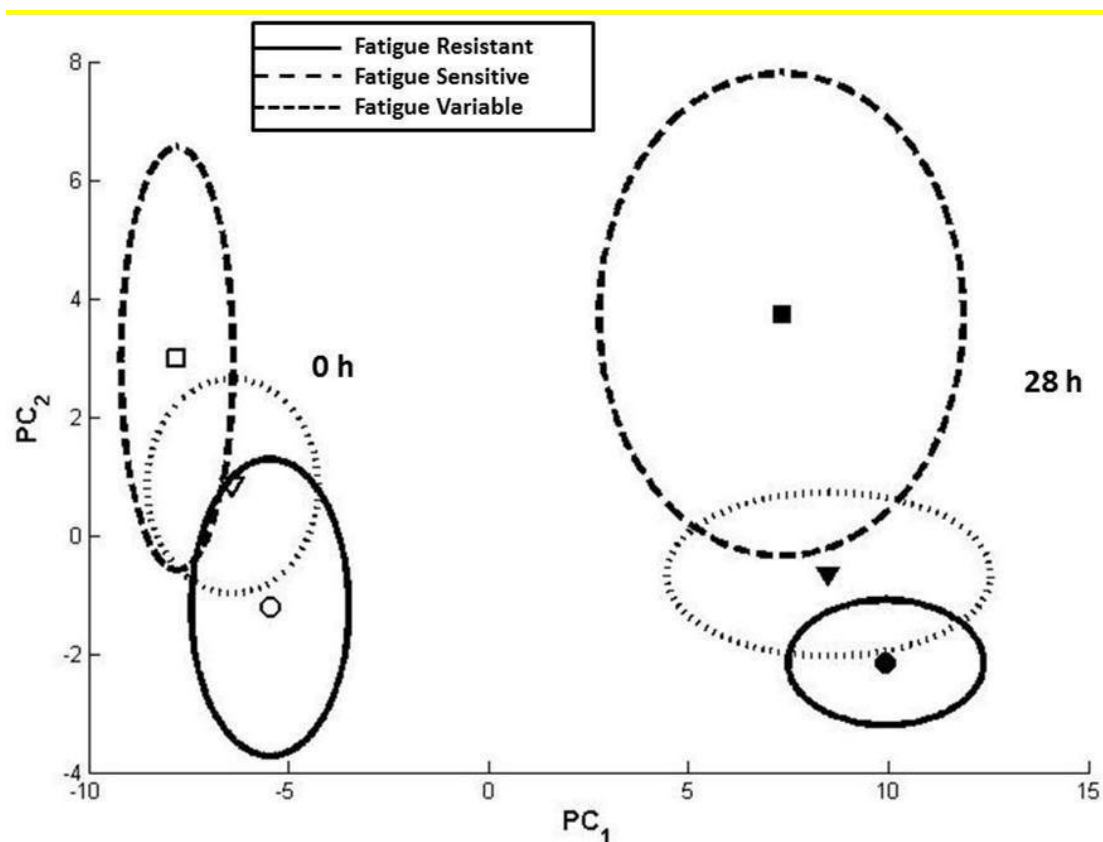
from each dynamically adaptively binned urine spectrum provided multivariate data input to PCA. The model parameters used to generate PCA scores plots (PC1 vs. PC2; representing 33% data variance) for the three classification groups (resistant, sensitive and variable) were based on



**Figure 5. Representative 600 MHz NMR spectrum of urine from a fatigue-sensitive (A) and fatigue-resistant (B) subject 12 h prior to cognitive testing (0 h) and after 28 h of sleep deprivation.**

NMR spectra from all 23 subjects at 0 h and 28 h sleep deprivation time points; maximal differences in cognitive performance were noted between these two time points via psychological testing in a previous study (Harville et al., 2010). Although all time points from all subjects were included in the data analysis, only data for the 0 h and 28 h sleep deprivation time points were plotted for clarity (Figure 6). ). It should be noted that ideal model parameters for the analysis work best when differences exist between time points or treatment groups. PCA parameter models generated using similar NMR spectral profiles between time points or treatment groups will not result in distinguishing treatment group classification (i.e. no treatment effect). The data shown in Figure 6 indicate the centric mean  $\pm$  2SEM (or 95 % confidence interval [CI]) for the various fatigue classification groups at 0 and 28 h of sleep deprivation. Differences in mapping position reflect changes in biochemical composition of the urine. This scores plot clearly shows separation between the 95% CIs at 0 h and 28 h for each of the fatigue classification groups. Furthermore, the 95% CIs do not overlap in space between the fatigue resistant and fatigue sensitive subjects at both 0 h and 28 h. Interestingly, the fatigue variable

subjects' 95% CI at 28 h was located in space between that of the fatigue resistant and fatigue sensitive groups 95% CIs (Figure 6). The 95% CIs for the fatigue variable group was found to overlap both the fatigue resistant and fatigue sensitive group 95% CIs at both 0 and 28 h of sleep deprivation (Figure 6).

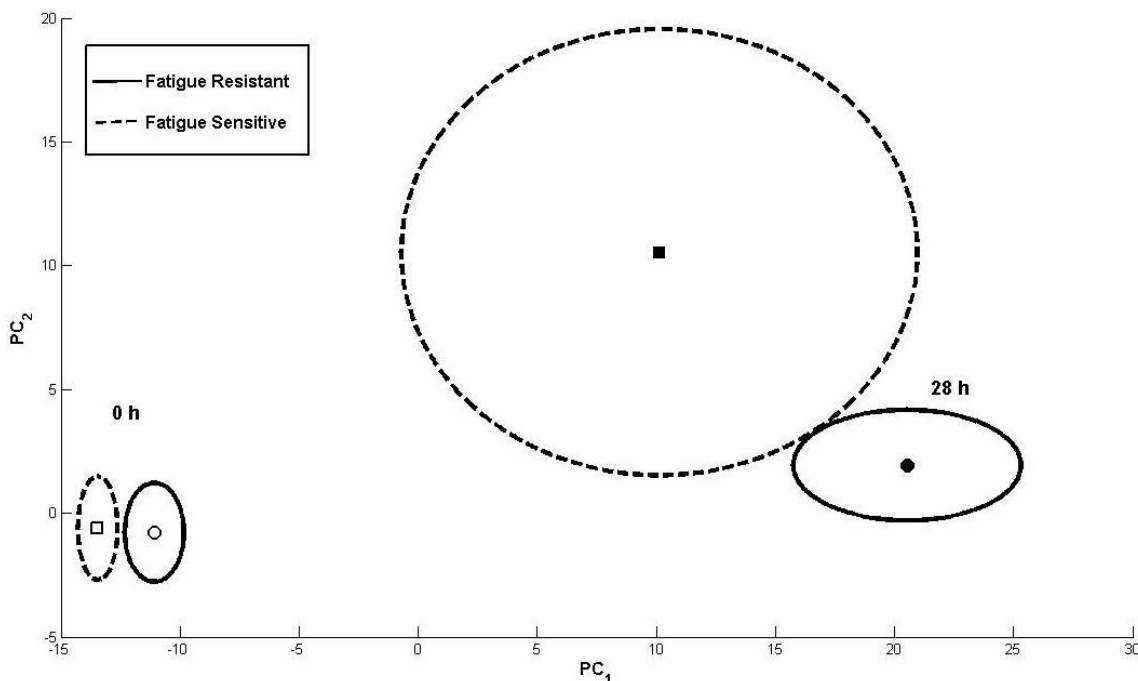


**Figure 6. PCA scores plot (PC1 vs. PC2) of all study subjects as a function of sleep deprivation time.** Fatigue resistant (n =6; circles), fatigue sensitive (n =6; squares) and fatigue variable (n = 11; triangles) data are plotted at 0 h (open symbols) and 28 h (filled symbols) of sleep deprivation as the centroid mean value for the first two principal components (PC1 vs. PC2)  $\pm$  2SEM (95% CI) in two dimensions (ellipse). PCA model was generated from urine NMR spectra from all subjects at 0 h (12 h prior to cognitive testing) and 28 h of sleep deprivation. Other time points were included in the analysis, but were omitted from this plot.

A PCA scores plot (PC1 vs. PC2; representing 47 % data variance) was then generated from the subject urinary metabolite profiles using the same model parameters as described above, but derived from only the fatigue-resistant and fatigue-sensitive classification groups at all time points. Although the 95% CIs derived from the subjects of these two fatigue classification groups clearly shows separation between classification group (resistant vs. sensitive) at 0 h (12 h prior to the start of cognitive testing) and 28 h of sleep deprivation (Figure 7), the CIs were larger than that observed in Figure 6. The larger CIs observed when PCAs were performed using

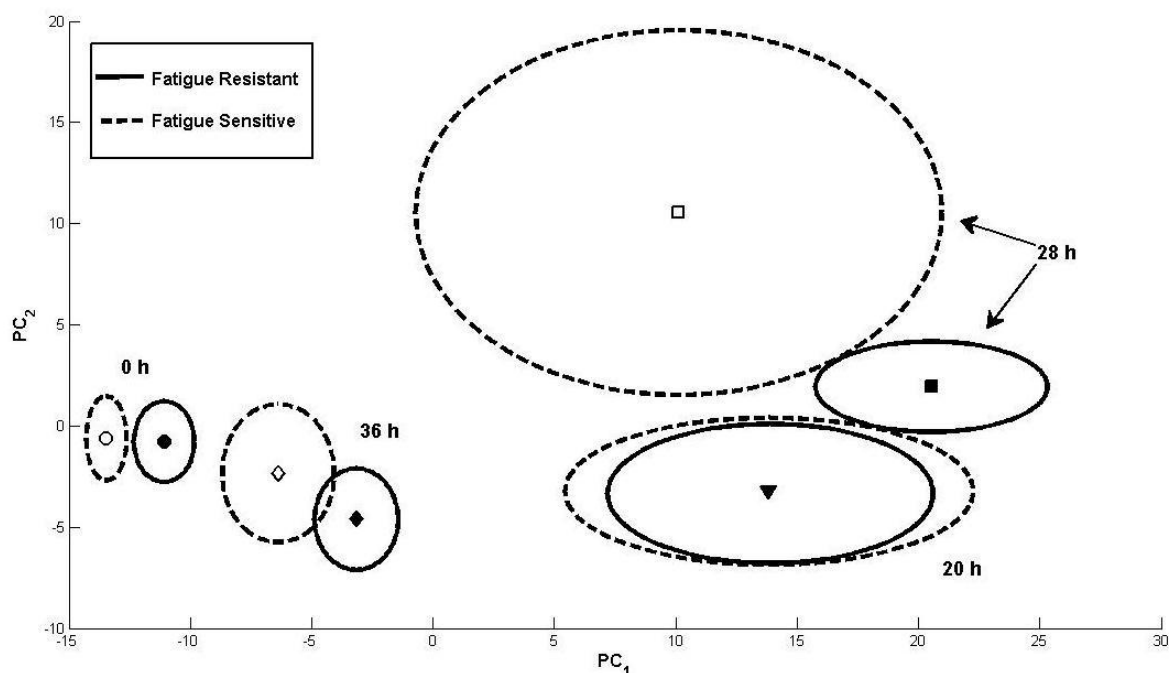


only two classification groups (fatigue resistant and fatigue sensitive) were due to the greater variability (or SE) resulting from the decrease in “N” value from 23 to 12.



**Figure 7. PCA scores plot (PC1 vs. PC2) of fatigue-resistant and fatigue-sensitive subjects as a function of sleep deprivation time.** Fatigue resistant (n =6; circles) and fatigue-sensitive (n =6; squares) data are plotted at 0 h (open symbols) and 28 h (filled symbols) of sleep deprivation as the mean value for the first two principal components (PC1 vs. PC2)  $\pm$  2SEM (95% CI) in two dimensions (ellipse). PCA model was generated from urine NMR spectra as described in Figure 6 legend. Other time points were included in the analysis, but omitted from this plot.

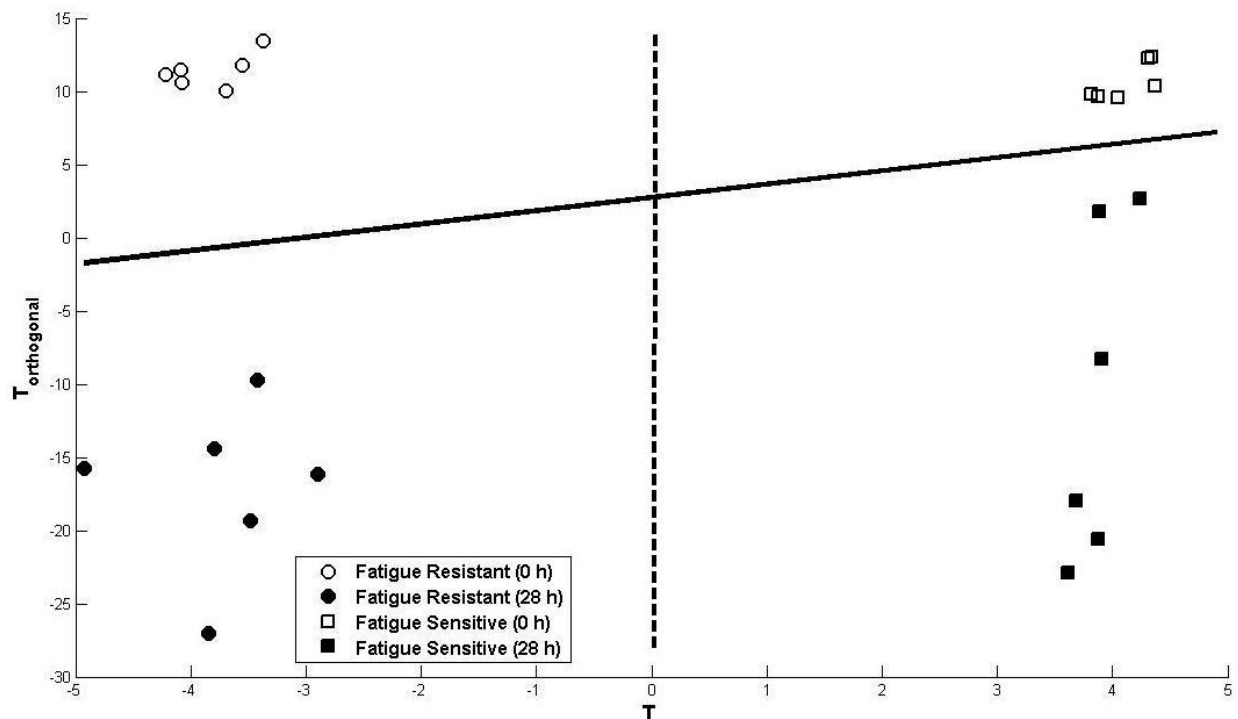
A PCA scores plot (PC1 vs. PC2) using the same model parameters as above and plotted over time, indicated that maximal group separation, based on urine analysis, occurred at 28 h of sleep deprivation (Figure 8). The urine collected at this time was 4 h following the maximal fatigue impact as determined by PVT and ANAM psychological testing performance reported in the previous technical report (Harville et al., 2010). This PCA plot also indicated that at 36 h of sleep deprivation the urinary metabolite profiles between the two fatigue classification groups appeared to be returning to that observed at the beginning of the study (Figure 8). This result also correlated with psychological testing results reported in the previous technical report that described improved performance related to what was termed a “going home” effect (Harville et al., 2010).



**Figure 8. PCA plot of fatigue-resistant and fatigue-sensitive subjects over 36 h of sleep deprivation.** Fatigue resistant (n =6; filled symbols) and fatigue-sensitive (n =6; open symbols) data are plotted at 0 h (circle), 20 h (triangle), 28 h (square) and 36 h (diamond) of sleep deprivation as the mean value for the first two principal components (PC1 vs. PC2)  $\pm$  2SE (95% CI) in two dimensions (ellipse). The 12 h sleep deprivation time point data overlapped with the 0 h data and is not shown for purposes of clarity.

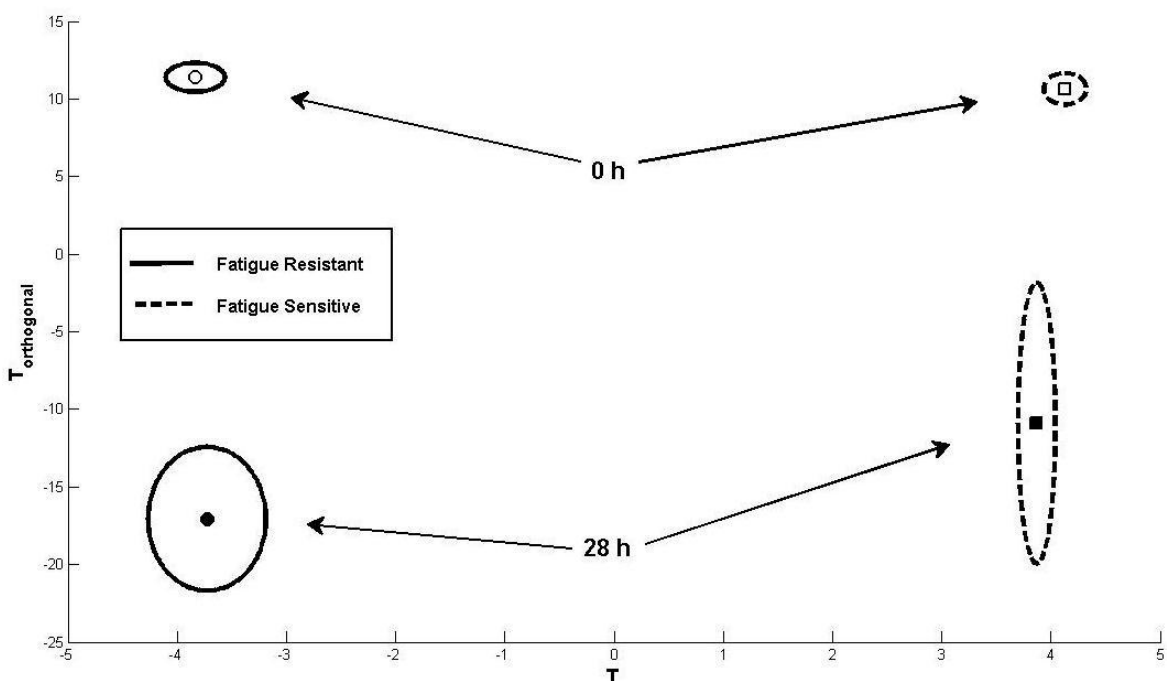
#### 4.6 Supervised Orthogonal Projection onto Latent Structures (OPLS) Discriminant Analysis of NMR Data

Supervised discriminant analysis is a statistical technique to classify objects into mutually exclusive and exhaustive groups based on a set of measurable object's features. The purpose of OPLS-DA is to classify objects (people, things, etc.) into one of two or more groups based on a set of features that describe the objects (e.g. gender, age, fatigue state, diet, etc.). Therefore, supervised analysis attempts to reduce variability within a classification group while maximizing separation between classification groups. An OPLS-DA was conducted modeling NMR spectra from Resistant (all times) + Sensitive (all times) subjects. Results from this analysis ( $Q^2 = 0.68$ ; threshold = -0.044; leave-one-out predictive accuracy = 97%) indicated that these data are significant at  $p < 0.01$ . The T-score scatter plot of study subjects clearly indicated that fatigue-resistant and fatigue-sensitive subjects clustered together and were separated from each other in graphical space at both 0 h and 28 h (Figure 9). Furthermore, within-group fatigue classification separation was also noted between 0 h and 28 h. It was also observed that clustering of subjects in both fatigue-resistant and fatigue-sensitive groups at 0 h was less variable than at 28 h;



**Figure 9.** T-scores scatter plot derived from an OPLS-DA of fatigue-resistant (n = 6; circle) and fatigue-sensitive (n = 6; square) data modeled by fatigue classification group at 0 h (open symbols) and 28 h (filled symbols). Fatigue resistant and fatigued-sensitive group subjects are observed to cluster together and be separated by group classification (dashed line) and time point (solid line).

presumably, due to autoscaling the NMR data to this time point. However, it is also possible that overnight fasting prior to sample collection contributed to this observation. Variability in urinary metabolite profiles was observed to be greatest in the fatigue-sensitive group at 28 h when compared to the fatigue-resistant group at this time (Figure 9). When the subject clusters were plotted as the centric mean  $\pm$  2SEM (95% CI), clear separation between fatigue classification groups and time was clearly evident (Figure 10).



**Figure 10. T-scores plot showing 95% CI derived from OPLS-DA of fatigue-resistant (n = 6; circle) and fatigue-sensitive (n = 6; square) data modeled by fatigue classification group at 0 h (open symbols) and 28 h (filled symbols).** Data are plotted as the centric mean  $\pm$  2SEM (95% CI ellipse). OPLS-DA was performed using a -1 cross validation, an alpha of 0.01 and 500 permutation runs. Significant differences between fatigue classification groups was indicated with a  $Q^2$  value of 0.685, threshold value of -0.03 and accuracy value of 97%. OPLS-DA identified 23 significant NMR spectral peaks that were responsible for classification group separation.

Using the significant variables, or spectral features, derived from this OPLS analysis that were responsible for the fatigue classification separation, 43 significant bins (spectral features) were identified that were found to be associated with 23 independent metabolite signals (Table 2). Of these 23 signals, we were able to identify 16 metabolites using the Chenomx commercial software database; 7 signals are unidentified as of yet (Table 2). Statistical analysis (t-test and Bonferroni multiple testing) indicated that 14 of the 23 metabolites were significantly altered when fatigue-resistant and fatigue-sensitive groups were compared; five of these metabolites could not be identified.

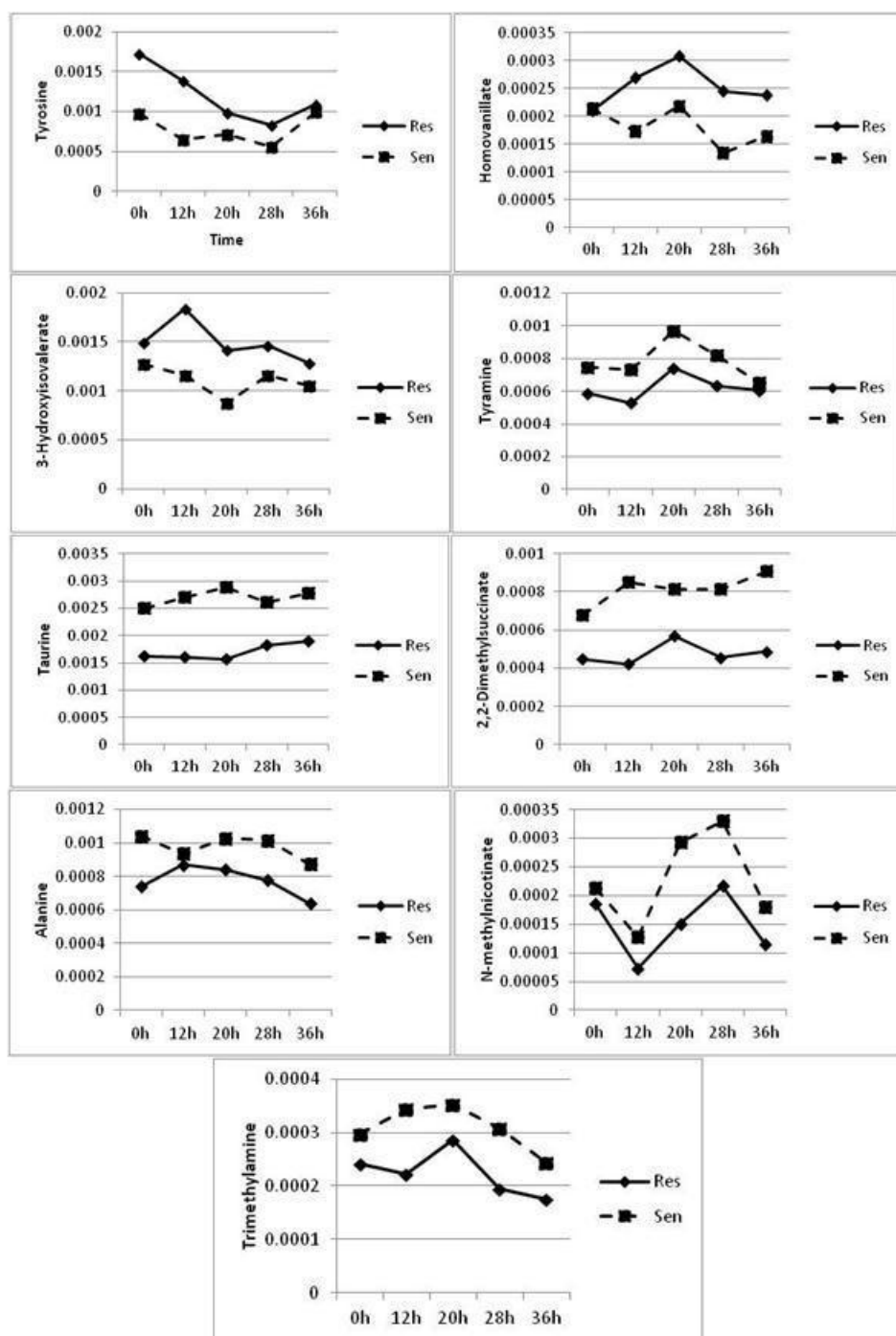
**Table 2. List of 23 urinary metabolites determined to be significant by OPLS-DA modeling Resistant (all times) versus Sensitive (all times).** NMR spectral data were normalized using Probabilistic Quotient Normalization (PQN). Data represent the Mean  $\pm$  SEM relative signal intensity values. Statistical analyses tested whether the means were different between Resistant vs. Sensitive groups. Statistically significant metabolites are highlighted.

Metabolite	PPM	All times combined Mean $\pm$ SEM ( $\times 10^{-4}$ ); N = 30	
		Resistant	Sensitive
Methylmalonic acid	1.23	12.18 $\pm$ 0.77	13.15 $\pm$ 0.61
2,2-Dimethylsuccinate	1.28	4.76 $\pm$ 0.27 **	8.13 $\pm$ 1.04
Alanine	1.49	7.73 $\pm$ 0.50 *	9.77 $\pm$ 0.59
3-Hydroxyisovalerate	2.35	14.99 $\pm$ 1.61 *	11.06 $\pm$ 1.11
Pyruvate	2.37	2.11 $\pm$ 0.12	2.78 $\pm$ 0.34
Alpha-ketoglutarate	2.45	8.70 $\pm$ 0.43	10.14 $\pm$ 0.62
Trimethylamine	2.88	2.24 $\pm$ 0.17 *	3.09 $\pm$ 0.27
Tyramine	2.91	6.19 $\pm$ 0.32 ‡	7.84 $\pm$ 0.33
Creatinine	3.05	412.34 $\pm$ 13.33	411.41 $\pm$ 18.43
Phosphorylcholine	3.23	19.57 $\pm$ 2.61	23.27 $\pm$ 2.35
Betaine	3.27	12.97 $\pm$ 1.24	17.10 $\pm$ 1.91
Taurine	3.43	17.01 $\pm$ 1.69 ‡	26.97 $\pm$ 2.27
N-methylnicotinate	4.48	1.48 $\pm$ 0.14 *	2.29 $\pm$ 0.28
Homovanillate	6.99	2.54 $\pm$ 0.28 *	1.81 $\pm$ 0.18
Tyrosine	7.21	12.01 $\pm$ 1.26 **	7.75 $\pm$ 0.85
Hippurate	7.84	21.03 $\pm$ 2.31	18.32 $\pm$ 1.43
Unidentified # 1	1.128	0.80 $\pm$ 0.05 ‡	0.54 $\pm$ 0.03
Unidentified # 2	2.15	3.52 $\pm$ 0.34	4.43 $\pm$ 0.43
Unidentified # 3	2.86	0.70 $\pm$ 0.08 *	1.06 $\pm$ 0.11
Unidentified # 4	3.36	5.81 $\pm$ 0.31 ‡	4.21 $\pm$ 0.26
Unidentified # 5	4.14	7.30 $\pm$ 0.35 *	17.38 $\pm$ 4.16
Unidentified # 6	7.015	0.47 $\pm$ 0.07	0.35 $\pm$ 0.04
Unidentified # 7	7.31	1.02 $\pm$ 0.06 ‡	0.62 $\pm$ 0.04

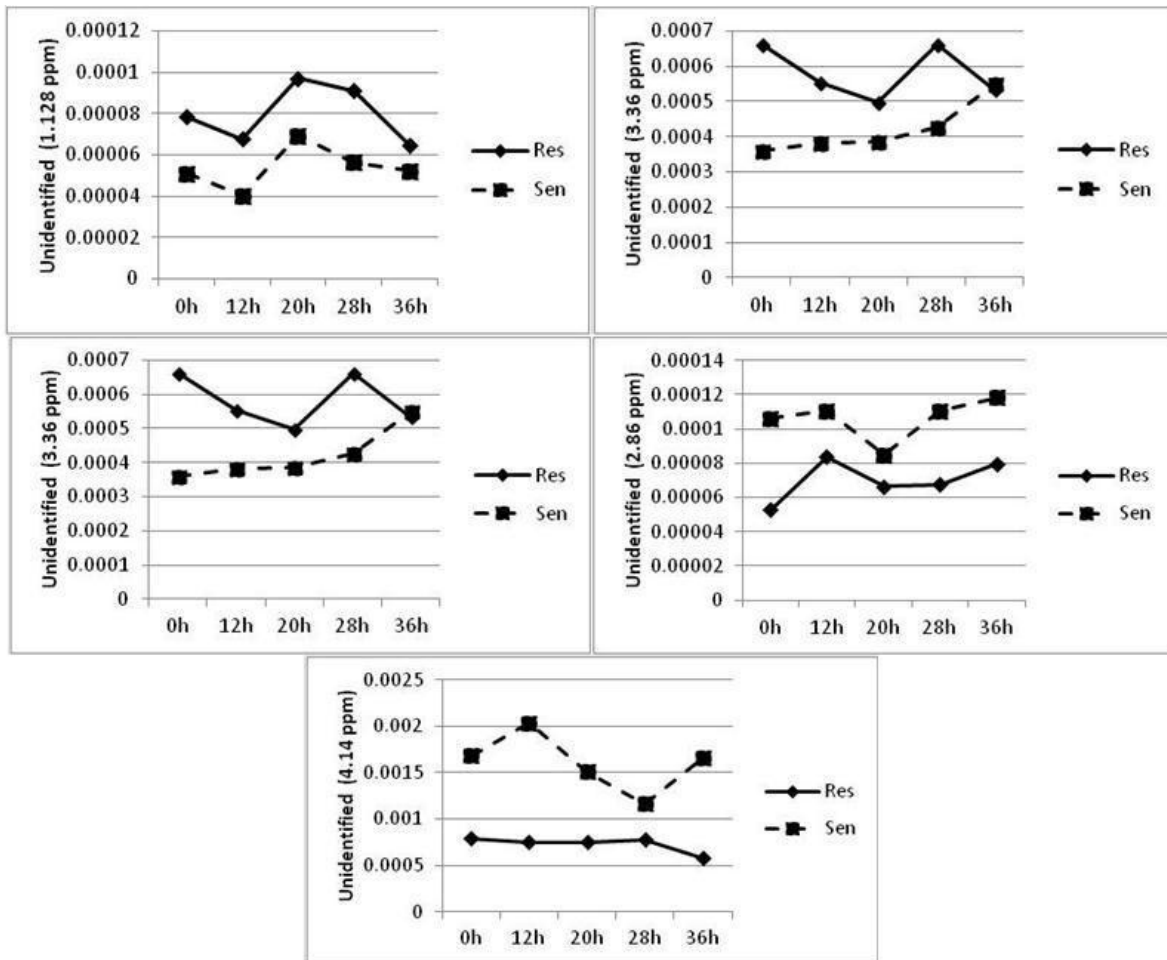
Asterisk(s) indicates means are significantly different by t-test (\*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ), and the dagger (‡) indicates means are significantly different by t-test and Bonferroni correction for multiple testing ( $p \leq 0.05$ ).

#### **4.6 Urinary Levels of Significant Metabolites**

Urinary levels of the statistically significant 14 metabolites highlighted in Table 2 were plotted over the 36 h sleep deprivation-induced fatigue study. Metabolite levels in urine were based upon relative signal intensities from the PQN-adjusted spectra, which accounts for differences in urine sample concentrations. Urinary metabolite levels at various time points over the 36 h of sleep deprivation indicated that tyrosine, homovalinate and 3-hydroxyisovalerate were observed to be at higher levels in fatigue-resistant subjects when compared to fatigue-sensitive subjects (Figure 11). In contrast, metabolite levels of tyramine, N-methylnicotinate, taurine, alanine, 2,2-dimethylsuccinate and trimethylamine were found to be elevated in fatigue-sensitive subjects over 36 h compared to fatigue-resistant subjects (Figure 11). Three of the five significant unidentified urinary metabolites with peaks at 1.128 ppm, 3.36 ppm and 7.31 ppm were found to be elevated in fatigue-resistant subjects over 36 h, when compared to fatigue-sensitive subjects, while the remaining two unidentified metabolite NMR peaks at 2.86 ppm and 4.14 ppm were found to be elevated in fatigue-sensitive subjects, when compared to fatigue resistant subjects (Figure 12).



**Figure 11. Levels of significantly identified urinary metabolites, using Chenomx database, responsible for fatigue classification and monitored over 36 h of sleep deprivation using an interactive spectral de-convolution algorithm in MATLAB.**



**Figure 12. Levels of significant unidentified urinary metabolites responsible for fatigue classification, and monitored over 36 h of sleep deprivation using an interactive spectral deconvolution algorithm in MATLAB.**

## 5.0 SUMMARY AND DISCUSSION

High operational tempos, sustained operations, disruptive sleeping environments and insufficient staffing make fatigue a growing problem in military operations. The impact of military fatigue on operational performance has been long known and dates back to the 1920s (Caldwell et al., 09). Fatigue in the military affects both soldiers and aviators and is a significant safety and operational issue resulting in diminished alertness and performance (Kruger, 1991; Miller et al., 2007). Pilot fatigue is well documented and is a significant problem in modern aviation operations, largely due to long duty periods, circadian disruptions, and insufficient sleep that can turn a routine operation into a potentially fatal situation (Weeks et al. 2010). With regards to the soldier, as a decision-maker, they interface with increasingly sophisticated technology for



communications, targeting, and weapons systems. Therefore, the human operator in both air and ground operations becomes the weakest element of these operations where sustained optimal performance is required. Scheduling, sleep deprivation, circadian disruptions, and extended duty periods continue to challenge the alertness and performance levels of both pilots and ground crews. At present, none of the real-time fatigue detection technologies (i.e. predictive modeling algorithms, EEG, biomathematical models of alertness) are adequately proven in an aviation environment to permit extensive utilization (Caldwell et al., 2009).

Although there has been much research into sleep and sleep disorders, discovery of biomarkers that accurately characterize sleep deprivation, or predict sleepiness, has been difficult to realize. Currently, objective determinations of sleepiness, such as psychomotor vigilance and multiple sleep latency tests are not inclusive of all types of sleep deficiencies and are not conducive to use in environmental settings outside of the laboratory. Development of accurate and easy to use biomarkers is important because current subjective assessment of sleepiness and sleep disorders are not reliable. To date, there have been relatively few articles published concerning identification of biomarkers related to sleep deprivation or sleepiness.

The current study investigated whether NMR-based metabolomics could be used to identify early metabolic markers of sleep deprivation-induced fatigue that was predictive of cognitive performance degradation. There is precedence for this approach in animal studies for predicting drug sensitivity using rats. Clayton et al., (2006) demonstrated that NMR analysis of rat urine prior to exposure to acetaminophen could predict animal sensitivity to this compound prior to being exposed. The urine is ideally suited for metabolomics analysis because it contains and concentrates essentially all endogenous and exogenous metabolites found in the body. These metabolites represent the end-products of countless interdependent macromolecular interactions.

Therefore, the power of metabolomics is derived from the fact that these small molecules effectively reside at the top of the genomic pyramid. Because metabolomics measures the "end result" of multiple protein, gene, and environmental interactions, it is a particularly good indicator of an organism's phenotype or physiology (Wishart 07). Furthermore, it allows researchers the capacity to quantitate these molecular phenotypes. However, as with any technology, one must be aware of the limitations of the technology being applied to address a research hypothesis.

Although NMR-based metabolomics requires minimal sample preparation, is less destructive and is quantitative, it is not as sensitive as other methods, such as mass spectrometry, and metabolites that are found in low concentration ( $< 0.1$  mM) may not be detected. Metabolite variability, especially in humans, is another concern. It has been shown in rodents that species, strain, genetics, sex, age, hormone concentrations, diurnal cycles, diet, temperature, stress, and gut microflora all contribute to the metabolic composition of the urine (Dai 08). Variations in diet and environment are important confounding factors in human studies. However, these can be

controlled somewhat by monitoring food and drink intake and by restricting ingestion of specific foods and drugs that would compromise data analysis. In addition, collecting samples after fasting can also help eliminate dietary issues. This approach to reducing metabolite variability in human studies has been implemented and suggests that it is possible to collect consistent metabolomics data in clinical studies (Lentz 03). The current study utilized the same dietary and drug restrictions and fasting described previously by Lentz (2003). Visual inspection of representative NMR spectra in the present study (Figure 5) clearly indicated differences in metabolite profiles between subjects who were classified as fatigue-sensitive and fatigue-resistant via psychological testing (Figure 4; Harville et al., 2010).

Cognitive testing conducted during Phase I of this study and reported by Harville et al. (2010), used a median split of the test scores for PVT and ANAM-core (math, grammatical reasoning and continuous performance) tests to classify subjects as fatigue-sensitive or fatigue-resistant. Findings published in this technical report indicated that PVT and ANAM-math tests were found to be the primary and secondary classifiers for fatigue, respectively. In the current technical report concerning classifying subject fatigue status based on urinary metabolite profiles, we further refined the subject classification groups. Use of the median split of cognitive test scores reported in the previous technical report of the 23 study subjects often led to variable results when different cognitive tests were compared. Therefore, for metabolite analysis we classified fatigue-resistance subjects ( $n = 6$ ) as those subjects who performed well on all cognitive tests given (PVT and ANAM-core) and fatigue-sensitive subjects ( $n = 6$ ) as those subjects who performed poorly on all cognitive tests given. The remaining 11 subjects were classified as fatigue-variable as they were found to perform well on some cognitive tests and poorly on others.

Unsupervised PCA is the first step in the multivariate statistical analysis of NMR spectral data. This unsupervised PCA assumes no knowledge *a priori* and is based on the distribution of eigenvectors, or principal components, which best explains the variance in the NMR spectral data. To reduce the dimensionality of the transformed data, typically only the first 4 or 5 principal components are used and these should capture a high percentage of the variance in the data if distinct patterns exist. PCA of urinary NMR spectra from fatigue-resistant, fatigue-sensitive and fatigue-variable subjects indicated that the urinary profiles for fatigue-resistant and fatigue-sensitive subjects were distinctive, exhibiting group separation occurring at both 0 h and 28 h (Figure 6). Indeed, these analyses showed that 50-58 % of the data variance is captured in the first 4 PCs. It is important to note that the 0 h time point is actually the baseline urine sample obtained after an overnight fast and is 12 h prior to the 24 h cognitive testing portion of the study (see Figure 1). Therefore, the reported time points in all figures reflect subject awake-time. The fatigue-variable group demonstrated urinary metabolite profiles that were similar to the other two classification groups at 0 h and was found to be intermittent between the other two fatigue classification groups at 28 h. Interestingly, this separation was distinct from the fatigue-sensitive group and more similar to the fatigue-resistant group (Figure 6).

When PCA was performed using only the fatigue-resistant and fatigue-sensitive subjects, separation between these two groups is observed at both 0 h and 28 h of sleep deprivation (Figure 7). However, compared to Figure 6 where all 23 subjects were included in the analysis, the separation, although still significant, between the fatigue-resistant and fatigue-sensitive subjects at 28 h was not found to be as great; PCA correlation appeared to be greater when the model was built on the fatigue resistant and fatigue sensitive groups only (i.e. % variance captured in first 4 PCs was greater) compared to the 3-group analysis. Furthermore, the variability within the fatigue-sensitive group was also found to be greater than that observed in Figure 6. This was more likely due to the reduced “N” value from 23 to 12 subjects in the PCA resulting in a greater SE. When all time points were superimposed onto the scores plot from the analysis involving only the fatigue-resistant and fatigue-sensitive subjects, changes in urinary metabolite profiles can be observed over time (Figure 8). The 12 h time point is not shown in this Figure for clarity because it clustered with the 0 h baseline samples. An interesting observation here was that after 36 h of being awake, the urinary profiles between the fatigue-resistant and fatigue sensitive subjects were becoming more similar and were moving back towards their 0-12 h profiles.

Harville et al. (2010) noted that cognitive test scores improved towards the end of their physiological study (36 h time point) and hypothesized this improvement to have been the result of a psychological phenomenon called “the going home effect”; subjects’ cognitive performance increases because of the realization that they would be returning home soon. Interestingly, the amounts of tryrosine, HV, and 3HI, all of which were elevated in the urine of the fatigue-resistant subjects, appeared to become similar in concentration between the two fatigue classification groups at the end of the 36 h sleep-deprivation study (Figure 11). This also was found to be associated with the so called “going home” effect reported in the previous technical report (Harville et al., 2010) to account for improved cognitive performance by all subjects during the last testing session of the study. In contrast, with the exception of tyramine, the significant metabolites elevated in the urine of the fatigue-sensitive subjects appeared to remain elevated throughout the 36 h sleep-deprivation period of the study. The levels of urinary tyramine in the fatigue-sensitive group becoming more like that observed in the urine of the fatigue-resistant group towards the end of the study may be the result of increased protein intake (i.e. tyrosine metabolism) in this group because all subjects were on a standard diet at the onset of the 24 h period of sleep-deprived cognitive testing. Previous work has shown that as little as a 4% increase in protein to a high carbohydrate diet can abolish the effects of tryptophan (induces sleepiness) and improve cognitive performance (Dye et al., 2000).

Once it was shown that PCA of human urine from study subjects demonstrated group separation with regards to fatigue classification, a supervised analysis (OPLS-DA) was conducted to determine which metabolites were responsible for this observed separation in PCA scores plot. In

an OPLS-DA, knowledge is imparted to the data sets (i.e. fatigue classification, time, etc.). The objectives of an OPLS-DA are to maximize the separation between groups, while minimizing the variation within groups, and to identify the salient spectral features (or metabolites) responsible for group differences. When OPLS-DA was performed using NMR data obtained from the fatigue-resistant and fatigue-sensitive groups ( $n = 6/\text{group}$ ), a scatter plot of all subjects involved in the analysis show a clear separation between fatigue-group classification and time (Figure 9). As observed with PCA, the OPLS-DA scatter plot shows that the fatigue-sensitive group demonstrated the greatest variability between subjects. When the data were plotted as the mean  $\pm$  2 SE (95% CI; Figure 10), maximal separation between groups and reduced variability within groups is clearly evident when compared to similar PCA scores plots between these two groups (Figure 7).

In generating the figures for OPLS-DA, NMR spectral peaks are identified that were significant and responsible for group separation. These spectral peaks can then be identified using commercial software database such as Chenomx. OPLS-DA identified 23 significant spectral peaks that were responsible for classifying study subjects as resistant or sensitive to sleep deprivation-induced fatigue, and 16 of these peaks were assigned to specific metabolites identified (Table 2). Although Chenomx is a leading database for identifying metabolites from NMR data, it is not complete. It is noteworthy that three of the significant unidentified metabolites that were elevated in the urine of the fatigue-resistant group (i.e. 1.128 ppm, 3.36 ppm and 7.31 ppm) demonstrated a similar concentration pattern as that observed for the three significantly identified urinary metabolites (i.e. tyrosine, HV and 3HI) of fatigue-resistant group over time (Figure 12). The remaining two unidentified significant metabolites (i.e. 2.86 ppm and 4.14 ppm), as that observed in the fatigue-sensitive group, remained elevated in the urine over the 36 h period of sleep deprivation. Although 7 of the 23 metabolite spectral peaks could not be identified, it may be possible to identify them using 2D NMR in the future (e.g. TOCSY, HMQC).

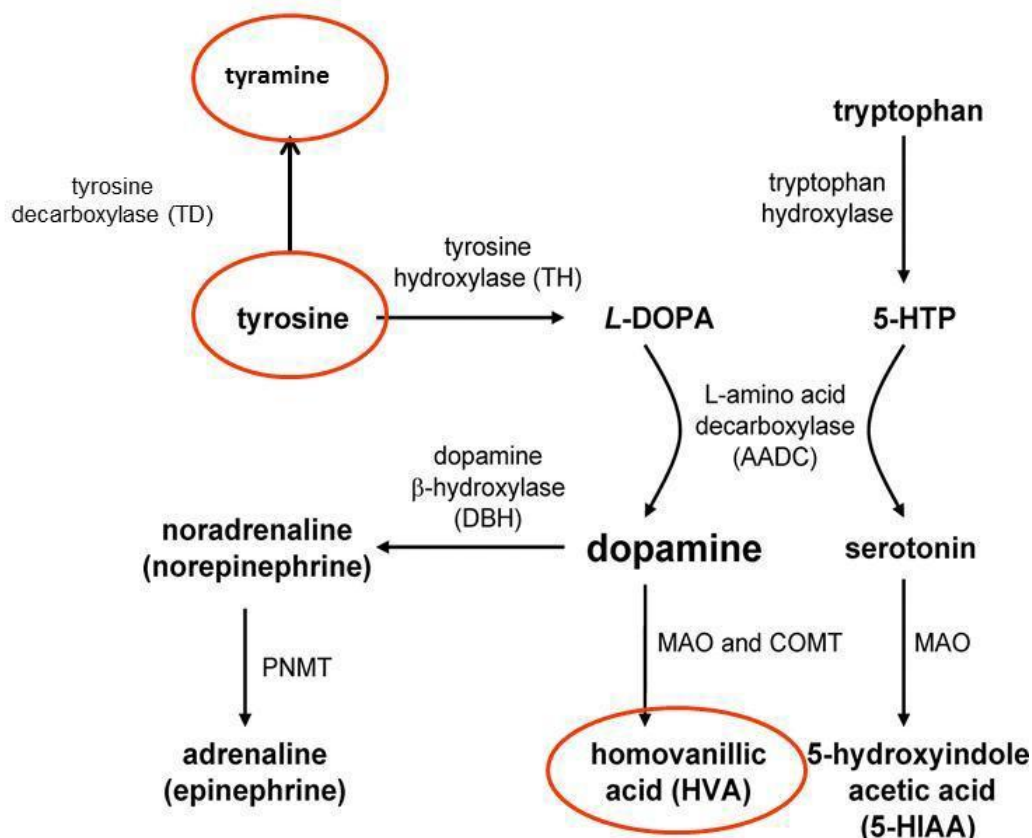
Statistical analysis of the 23 significant spectral peaks identified by OPLS-DA that were responsible for fatigue group classification indicated that 14 were statistically significant. Nine of these spectral peaks could be identified using Chenomx, while 5 could not (Table 2; highlighted metabolites). A number of the identified urinary metabolites in both the fatigue-resistant and fatigue-sensitive subjects were of particular interest. Taken together, our data showed that measured biomolecular endpoints (urinary metabolite profiles) correlated with psychological endpoints (PVT and ANAM) for determining cognitive performance outcome. This was observed not only at the 36 h time point, but also at the 28 h time point where psychological testing indicated peak fatigue occurring between 24-28 h of sleep deprivation in all groups (Harville et al., 2010).

## 5.1 Fatigue Resistant

With regards to the fatigue-resistant group, 6 of the statistically significant urinary metabolite levels were found to be higher when compared to the fatigue-sensitive group. However, only 3 metabolites could be identified using the ChemoMx metabolomics database and these were: 3-hydroxyisovalerate (3-HI), tyrosine and homovanillate (HV). Increased levels of these three metabolites in urine are suggestive of greater protein metabolism in the fatigue-resistant group. Tyrosine is of particular interest because it is the precursor to three neurotransmitters: dopamine, norepinephrine and epinephrine (see Figure 13).

Although tyrosine can be synthesized from phenylalanine in humans, it is generally derived from the diet where it is found in high quantities in protein-rich foods. The role of tyrosine in countering the effects of fatigue are well known and suggests that supplemental administration of tyrosine increases brain catecholaminergic neurotransmission and has beneficial effects on various behavioral parameter, associated with resistance to stress (Lieberman 1994 and 1999). Other human studies have shown that tyrosine has a positive effect on cognitive performance during exposure to various stresses such as temperature and altitude (Banderet and Lieberman, 1989; Shurtleff et al., 1994). Tyrosine has also been shown to enhance performance in individuals exposed to psychological stress (Deijen and Orlebeke, 1994). These findings in humans are similar to those observed in animals showing improved cognition when under stress.

As indicated above, the dopaminergic pathway has been associated with a more wakeful state. Dopamine synthesis in dopaminergic neurons begins with tyrosine where it is obtained from the circulation. Tyrosine, like other large neutral amino acids (i.e. tryptophan, phenylalanine,



**Figure 13. Schematic pathways for synthesis of dopamine, noradrenaline, adrenaline, and serotonin.** 5-HTP, 5-Hydroxytryptophan; PNMT, phenylethanolamine-N-methyl transferase; MAO, monoamine oxidase; COMT, catechol-O-methyl transferase. (Modified from Rubí and Maechler, 2010). Ovals indicate metabolites found to be significantly elevated in the urine of the fatigue-resistant group by NMR.

leucine, histidine, etc.), cannot cross the BBB and must be actively transported across. Once tyrosine crosses the BBB, it is taken up and concentrated in catecholamine neurons. Once inside the cell, tyrosine is converted to dopamine via dihydroxyphenylalanine (DOPA). Dopamine can then be used as a substrate to generate norepinephrine and epinephrine or be metabolized to HV.

In the kidneys, more than 95% of amino acids are filtered from the plasma and reabsorbed by the proximal tubules and returned back into circulation (Parvy et al., 1988). Therefore, urinary levels of amino acids are typically low. However, in the present study we observed significantly higher levels of tyrosine in the urine of the fatigue-resistant group by NMR analysis than that observed in the urine derived from the fatigue-sensitive group. We believe that this finding was due to greater dietary intake of protein in the fatigue-resistant group than that in the fatigue-sensitive group. This is supported by a blind dietary analysis of subject food logs using the Nutrition Data database that indicated a significant 2-fold greater protein intake by the fatigue-resistant subjects

~ 8 h prior to the 24 h of cognitive testing (Figure 3). Furthermore, a number of previous studies have shown increases in tyrosine, as well as other amino acids, in urine following ingestion of protein-rich foods (Brand et al., 1997; Parvy et al., 1988; Steele et al., 1950). This is believed to occur when the kidney tubular load for amino acids exceeds the rate of reabsorption and urinary excretion of amino acids increases proportionally (Odle et al., 1992).

Homovanillate was another metabolite found to be elevated in the urine of fatigue-resistant subjects when compared to fatigue-sensitive subjects. A review article by Amin et al. (1992) indicated that dopamine is predominantly metabolized to HV and mostly excreted in a free unconjugated form. The unconjugated HV produced in the brain moves directly into the blood via active transport (organic anion transporter) across the BBB. This review also indicated that animal and human studies have shown that a portion of urinary measured HV originated from the brain. Therefore, it is possible that HV may provide an index of central dopamine neuronal activity. Our finding of elevated HV in the urine of the fatigue-resistant group lends further support of increased protein consumption leading to increased tyrosine levels and enhancement of the dopaminergic pathway in the brain. Elevated levels of 3-HI in the urine adds additional evidence that increased protein consumption in the fatigue-resistant group contributed to enhanced cognitive performance. This metabolite can be formed enzymatically utilizing acetyl CoA and is a catabolic intermediate of leucine associated mitochondrial metabolism. Increased dietary protein intake by the fatigue-resistant group would have increased the availability of leucine for mitochondrial metabolism to form 3-HI. Three other metabolites were also found to be elevated in the urine of the fatigue-resistant group, but they could not be identified using the Chenomx database. Future analysis by 2D NMR will shed some light as to the identity of these metabolites. Taken together, our data suggest that the higher levels of protein ingested by the fatigue-resistant group, when compared to the fatigue-sensitive group, ultimately lead to enhanced cognitive performance via stimulation of the dopaminergic pathway (Figure 13) as evidenced by higher levels of tyrosine, HV and 3-HI measured in the urine by NMR.

## 5.2 Fatigue Sensitive

In contrast to the urine profiles of the fatigue-resistant group, a number of urinary metabolites were identified in the fatigue-sensitive group that could be associated with a more sleepiness, or more fatigued, state. Eight metabolites in the urine of the fatigue-sensitive group were found to be significantly elevated when compared to the fatigue-resistant group. Of these eight metabolites, only six could be positively identified using the Chenomx database and these were: alanine, 2,2-dimethylsuccinate, trimethylamine (TMA), tyramine, taurine and N-methylnicotinate (NMN; Table 2). Two additional metabolites were observed to be significantly elevated, but could not be identified using Chenomx database. In addition, blind dietary analysis using the Nutrition Data database indicated that the fatigue-sensitive subjects ingested fewer calories (Figure 2A) and a greater percentage of their total caloric intake was derived from

carbohydrates (47%; Figure 2 B). Interestingly, half of the identified significant urinary metabolites in the fatigue-sensitive group may be linked to this observed higher percentage of dietary intake being derived from carbohydrates.

Alanine is a nonessential amino acid that can be formed in the body from the conversion of carbohydrate pyruvate (generated via the glucose-alanine cycle). Therefore, alanine plays an important role in, and in the regulation of, glucose metabolism. Since alanine levels parallel glucose levels in the blood, significantly increased levels of alanine observed in the urine of the fatigue-sensitive group in the present study may be attributed to their higher dietary carbohydrate intake. This is partially supported by the observation that urinary pyruvate (an intermediate product in the alanine-glucose cycle) was also found to be elevated in the fatigue-sensitive group when compared to the fatigue-resistant group, but was not statistically significant (Table 2). However, pyruvate was one of the original 23 metabolites identified by OPLS-DA to be responsible for group classification (Table 2).

Previous studies have shown that methyl esters of succinate are potent insulin secretagogues in pancreatic  $\beta$ -cells (MacDonald, 1993; MacDonald and Fahein, 1988). Once taken-up by the cell, these esters of succinate are hydrolyzed intracellularly to succinate. Initiation of insulin release by esterified succinate, and not by other esters of TCA intermediates, indicates that mitochondrial metabolism alone is sufficient to initiate insulin release (MacDonald and Fahein, 1988). 2,2-Dimethylsuccinate is a nutrient secretagogue that has been shown to be as effective as glucose in stimulating insulin release from the pancreas (Fahien and MacDonald, 2002). Its significant elevation in the urine of the fatigue-sensitive group is suggestive of greater carbohydrate ingestion and metabolism.

Taurine, a sulfur amino acid, has many diverse biological functions (i.e. brain inhibitory neurotransmitter, cell membrane stabilizer, ion transport facilitator, etc.). One of the important functions of taurine is to reduce blood sugar. Studies involving diabetic rat strains have shown that taurine significantly reduces weight and blood sugar in these animal models (Pop-Busui et al., 2001; Nakaya et al., 2000). Taurine has been shown to act as a glycation inhibitor, resulting in decreased formation of advanced glycation end products, both *in vitro* and *in vivo* (Huang et al., 2008; Verzola et al., 2002). Therefore, it is possible that the significantly elevated level of taurine found in the urine of the fatigue-sensitive group is a response to increased blood glucose levels due to high carbohydrate ingestion. Although taurine can be obtained from the diet by ingestion of protein, it can also be synthesized in adult humans. Therefore it is also possible that the elevated taurine levels observed in the fatigue-sensitive group may have been due to synthesis in response to high carbohydrate intake. Taurine has been shown to be synthesized and stored in adipocytes by two cellular pathways (Ueki and Stipanuk, 2009).



The role of the remaining three significantly elevated urinary metabolites, with regards to fatigue sensitivity, is not clear. Tyramine (metabolite of tyrosine), trimethylamine (TMA) and N-methylnicotinate (NMN) are metabolites more likely to have been obtained from the diet and, with the exception of tyramine, are plant-based metabolites. Tyramine, a monoamine, is found in foods as the result of tyrosine decarboxylation during fermentation. NMN (or trigonelline) is an alkaloid found in a wide variety of plants and in coffee. However, NMN is an important dietary betaine. Trimethylamine is a product of decomposition and is derived from gut microflora. Although these metabolites do not appear to have a link to a specific biochemical pathway of mammalian metabolism linked to fatigue, significant changes in methylamine (TMA) and nucleotide (NMN) metabolism have been observed in type 2 diabetes in both rodent and human models (Salek et al., 2007).

## 6.0 CONCLUSIONS

The results of the present study demonstrated that NMR-based metabolomics could be used as a noninvasive approach to predict and characterize sleep deprivation-induced fatigue impact on cognitive performance. Furthermore, our results indicated that NMR-based metabolomics analysis of human urine identified changes in urinary metabolite profiles that corresponded to psychological testing scores of cognitive performance under sleep deprivation-induced fatigue. More importantly, NMR-based metabolomics was also capable of predicting susceptibility to sleep-deprivation-induced fatigue 12 hours prior to the 24 hour cognitive testing phase of the study. Elevated levels of tyrosine, HV and 3-HI in the urine of the fatigue-resistant subjects suggest that enhanced cognitive performance under sleep deprivation-induced fatigue may have been due to increased protein intake prior to the start of the cognitive testing phase of the study (see Figure 1). A protein-rich diet can lead to activation of the dopaminergic pathway via increased plasma tyrosine levels following protein digestion. This was partially supported in the present study by the observation that nutritional analysis of subject food logs 8 h prior to the start of the cognitive testing phase of the study indicated that the fatigue-resistant subjects ingested a significant 2-fold higher amount of protein than the fatigue-sensitive subjects. A number of studies have shown the benefits of dietary protein and tyrosine supplementation on improving cognitive function (Fischer et al., 2001 and 2002; Dye et al., 2000; Lieberman, 2003). Military studies from all three US services, and one European military study, have all reported positive findings for tyrosine with regards to enhanced cognitive performance. In addition to the above findings, three of the significant metabolites that were found to be elevated in the urine of the fatigue-sensitive subjects (taurine, alanine and 2,2-dimethylsuccinate) were strongly associated with a high carbohydrate diet.

Taken together, the collection of six identified urinary metabolites (tyrosine, HV, 3-HI, taurine, alanine and 2,2-dimethylsuccinate) may provide a useful subset of urinary metabolite markers to screen, or select, personnel for demanding missions where fatigue is expected to play a role. It is

possible that future identification of the five significant unidentified metabolites using 2D NMR may result in an expansion of the number of metabolites used for personnel mission readiness screening.

The results of this study also raise the issue of adequate nutrition for optimal performance. Based on analysis of the study food logs, it was apparent from visual inspection of the food logs that a number of service members were making poor choices with regards to food selection. This was later verified by the study dietitian using the Nutrition Data analysis of the food logs. The study results also indicated that nutritional education of young service men and women may be necessary as part of their training. Particular attention should be paid to assuring that proper nutritional requirements are being met prior to, during and following missions to maintaining optimal physical and cognitive performance readiness.

Although the previous technical report attributed increased cognitive performance at the end of the sleep-deprivation study due to a psychological “going home” effect, our data suggest that this phenomenon may have resulted due to changes in nutritional status. There has been a growing interest in the potential effects of food on various aspects of psychological state, and mental and physical performance (Dye et al., 2000). All of the subjects ate the standard diet provided (Table 1) during the 24 h cognitive testing portion of the study. Thus, any nutritional deficiencies prior to the cognitive testing phase of the study would progressively be reversed during the 24 h course of cognitive testing, and may explain the increase in cognitive performance observed at the end of the 36 h sleep deprivation-induced fatigue testing. This was partially supported by the finding that NMR urinary profiles obtained from the fatigue-resistant and fatigue-sensitive study subjects began to overlap at this time point and appeared to be more representative of their pre-testing profiles at 0 h (Figure 8).

It is important to note that this study was not designed as a nutritional investigation into the effects of macronutrients on cognitive performance, but that NMR data analysis of urine from sleep-deprived subjects indicated that nutrient metabolites seemed to play a role in the classification of subject cognitive performance outcome under this condition of induced fatigue. Therefore, care should be taken when considering the interpretation of the current study results due to the low sample size (N-value) commonly seen with these types of studies. We attempted to control as many confounding factors as possible, such as dietary, pharmacological, alcohol and smoking restrictions, overnight fasting, maintenance of food logs, gender, and timing of all meals. However, other numerous factors exist (e.g. age, ethnicity, underlying medical issues, susceptibility to stress, etc.). Additional work with macronutrients, especially carbohydrate, and diets that vary in protein to carbohydrate ratio, is also necessary. In general, little work on the effects of dietary constituents in mixed diets has been conducted and their chronic effects on behavior have not been investigated. Such studies would be of great value, not only to address military requirements, but for the civilian population as well. Further research is required to

elucidate the specificity of the mechanisms involved in the effects of macronutrients on mental performance.

Currently, objective biomarkers of fatigue are based on difficult to interpret and frequently burdensome electrophysiologic or behavioral tests (e.g. multiple sleep latency test, PVT and pupillometry). Although these tests have been shown to be practical for scientific investigations, they are difficult to use in field studies and would have little useful application in military operational environments. To date, no reliable biomarkers of fatigue have been developed. However, there is an important need for this technology. Given the magnitude of the problem of sleep deprivation-induced fatigue to the public and the military, this is not surprising. Therefore, more engaged basic research is required to address this problem. A general theme that has been emerging as a result these types of research efforts is the importance of identifying simple and quantifiable biomarkers of fatigue (Jones et al., 2005; Van Dongen et al., 2005; Dawson and McCulloch, 2005). The results from this study demonstrated that changes in urinary metabolite profiles, using NMR-based metabolomics, were found to correlate with changes in psychological testing performance (PVT and ANAM) under conditions of sleep deprivation. The present study also provided evidence that metabolomics may be a useful tool for identifying potential molecular biomarkers (urinary metabolites) predictive of cognitive performance ability when sleep deprived.

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## LIST OF ACRONYMS

AFRL	Air Force Research Laboratory
ANAM	Automated Neuropsychological Assessment Metrics
BBB	Blood Brain Barrier
CI	Confidence Interval
CNS	Central Nervous System
CoA	Coenzyme A
CPT	Continuous Performance Test
DOPA	Dihydroxyphenylalanine
EEG	Electroencephalography
3-HI	3-Hydroxyisovalerate
HMQC	Heteronuclear Multiple-Quantum Correlation
HV	Homovanillate
ICD	Informed Consent Document
LC/MS	Liquid Chromatography/Mass Spectroscopy
NMN	N-Methylnicotinate
NMR	Nuclear Magnetic Resonance
NOES	Nuclear Overhauser Effect Spectroscopy
OPLS-DA	Orthogonal Projections onto Latent Structures-Discriminant Analysis
PC	Personal Computer
PCA	Principal Component Analysis
ppm	Parts Per Megahertz
PQN	Probabilistic Quotient Normalization
PRESS	Predicted Residual Sum of Squares
PVT	Psychomotor Vigilance Task
SEM	Standard Error of the Mean
SSY	Sum of Squares “Y”
TMA	Trimethylamine
TOCSY	Total Correlation Spectroscopy
TSP	Trimethylsilylpropionic (2, 2, 3, 3 d <sub>4</sub> ) acid
WPAFB	Wright-Patterson Air Force Base