

## A COMPARATIVE BEAR MODEL FOR IMMOBILITY-INDUCED OSTEOPENIA

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**Abstract:** The National Institutes of Health (NIH) and the National Aeronautics and Space Administration (NASA) are seeking solutions to the human problem of osteopenia, or immobility-induced bone loss. Bears, during winter dormancy, appear uniquely exempted from the debilitating effects of immobility osteopenia. NIH and ESA, Inc. are creating a large database of metabolic information on human ambulatory and bedrest plasma samples for comparison with metabolic data obtained from bear plasma samples collected in different seasons. The database generated from NASA's HR113 human bedrest study showed a clear difference between plasma samples of ambulatory and immobile subjects through cluster analysis using compounds determined by high performance liquid chromatography with coulometric electrochemical array detection (HPLC-EC). We collected plasma samples from black bears (*Ursus americanus*) across 4 seasons and from 3 areas and subjected them to similar analysis, with particular attention to compounds that changed significantly in the NASA human study. We found seasonal differences in 28 known compounds and 33 unknown compounds. A final database contained 40 known and 120 unknown peaks that were reliably assayed in all bear and human samples; these were the primary data set for interspecies comparison. Six unidentified compounds changed significantly but differentially in wintering bears and immobile humans. The data are discussed in light of current theories regarding dormancy, starvation, and anabolic metabolism. Work is in progress by ESA Laboratories on a larger database to confirm these findings prior to a chemical isolation and identification effort. This research could lead to new pharmaceuticals or dietary interventions for the treatment of immobility osteopenia.

*Ursus* 10:507-520

**Key words:** amino acids, bear, bone, comparative study, fasting, hibernation, human, immobility, metabolic patterns, metabolism, osteopenia, protein metabolism, Ursidae.

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This project was designed to demonstrate the use of large multi-parameter databases in identifying biomolecules involved in degenerative disorders. Immobility osteopenia is a degenerative disorder that affects patients who, by injury or illness, are bedridden (Raisz 1988). Astronauts in microgravity environments also lose bone in a process closely resembling immobility osteopenia (NASA 1991). Human immobility osteopenia is a degenerative disorder categorized as a metabolic bone disease, which is manifest by imbalanced osteoclastic and osteoblastic activity. Metabolic bone diseases, including osteoporosis, thyroid-related bone disease, Paget's disease, primary hyperparathyroidism, renal osteodystrophy, hypercalcemia, and osteomalacia, have been characterized by histomorphometric analyses of iliac crest biopsies. While this technique precisely measures bone loss, it is invasive and impractical for repeated measurements and prolonged monitoring. In recent years, efforts have been made to identify specific and sensitive markers of bone turnover (Tamburino and Fiore 1990, Delmas 1991, Riis 1991, Ebling et al. 1992, McGowan 1993, Demers and Kleerekoper 1994).

Bears present a unique opportunity for the study of immobility osteopenia. During winter dormancy, bears do not experience the debilitating effects of immobility

osteopenia (Lundberg et al. 1976; Haller and Zimny 1977; Azizi et al. 1979; Nelson et al. 1983, 1984; Floyd et al. 1990). Unlike other true hibernators, the ability of bears to cope with long periods of food deprivation includes strategies for controlling anabolic metabolism at levels sufficient to maintain bone and lean body mass despite anorexia. Bears accumulate sufficient stored fat to assure survival during winter dormancy. The stored fat is used as energy and metabolic water to maintain essential functions during dormancy. In contrast, humans can not fast for 6 months without risking starvation or remain relatively immobile without suffering bone loss. Studying seasonal metabolic changes in bears may lead to understanding the mechanism by which they avoid bone loss.

Study of the complex biochemistry and etiology of immobility osteopenia, and metabolic bone disorders in general, can benefit from development of new and more powerful analytical tools. Traditionally, investigators choose analytical methods that offer sensitivity and specificity, ultimately fitting results into an overall global picture or evolving theory of a disorder. This approach is effective in studying disorders resulting from a single cause or a disorder where prior knowledge indicates a clear or probable mechanism. This approach is less satisfactory when applied to multifocal degenerative disorder.

ders, where extreme vigilance is necessary to avoid overlooking facets of a disorder and where there may be multiple unknown or seemingly unrelated influences.

An emerging approach to biochemical investigation of degenerative disorders focuses on the network of metabolites and their differences between the well and pathological states. Once biochemical differences are determined, further investigation focuses on these differences. This approach is radical in that there is little *a priori* hypothesis-testing. For example, in the case of immobility osteopenia, we expect measurable changes in the overall biochemistry of low-molecular-weight metabolites between ambulatory and bedrest subjects. The use of individuals as their own controls and the analysis of many individuals in bedrest experiments should minimize noise in identifying changes in both known and unidentified compounds. After identifying compounds that changed during bedrest, we asked the question, "How does the bear, the exception to the immobility osteopenia rule, handle those particular compounds?" Compounds that change in both species with immobility and that change differentially between species are more likely to be involved in immobility-induced metabolic changes and in immobility-induced osteopenia in humans. These compounds may implicate unsuspected pathways in the process and they may yield new markers. With this approach, all low-molecular-weight oxidizable plasma compounds can be compared. This approach is different from that used in locating species-specific or conditionally (i.e., summer vs. winter) unique compounds, although identification of such biochemicals can be easily and concurrently accomplished using archived chromatographic data.

## MATERIALS AND METHODS

Plasma samples from black bears taken in different seasons were provided by Natural Resource Sciences Department at Washington State University (WSU), Maine Department of Inland Fisheries and Wildlife, and the Virginia Cooperative Fish and Wildlife Unit at Virginia Polytechnic Institute and State University (VPISU). Although we received several hundred samples, many were too old for HPLC analysis. Other samples, such as those from VPISU were from black bears taken into captivity in the fall and released in the spring. Plasma samples were collected predominantly from female black bears. For this study, 31 summer samples and 63 winter samples were available. Human plasma samples were generously provided by NASA from bedrest studies conducted at Ames Research Facility, Moffet Field, California. These samples included initial and bedrest times up to 30 days,

with a limited number of post-experiment recovery samples ( $n = 21$ ). All samples were analyzed using HPLC and coulometric electrode array detectors (CEAS). Prototypic instrumentation and software were assembled for this study.

Three ml of blood was collected from bears or humans into vacutainer tubes containing 7.2 mg EDTA (ethylenediamine tetraacetic acid) and chilled on ice prior to centrifugation at 2,000G. Plasma was frozen and shipped on dry ice to ESA, Inc. for storage at -80 C until analysis. A plasma aliquot of 250  $\mu$ l was extracted with 1 ml of acetonitrile (An) in a 4% acetic acid (HAc) solution by volume at 0 C. The centrifuged supernatant was removed, dried by evaporation, and reconstituted in the initial mobile phase for injection onto the HPLC column. This protocol conserved easily oxidizable species better than extraction with cold-buffered 1% perchlorate or methanol.

Chromatograms were analyzed for compounds (Table 1) by comparison with authenticated standards. Currently, standards (Sigma Chemical Co., St. Louis, Mo.) are available for compounds describing tyrosine and tryptophan pathway metabolites, purines, vitamins, phenolics, and bone and muscle metabolites (e.g., carnosine, osteocalcin, and 3-methylhistidine). All standards were stored at -80 C. Stock solutions were made by diluting 100 mg of standard in 100 ml of 0.9% saline containing 1% phosphoric acid. Subsequent working solutions were made into 0.9% saline containing 0.001 molar ascorbic acid as an antioxidant.

Chromatographic solvents were obtained as follows: isopropyl alcohol from Chempure, C.M.S., Houston, Tex.; methanol and acetonitrile from EM Science, Gibbstown, N.J.; glacial acetic acid and pentane sulfonic acid from J.T. Baker, Phillipsburg, N.J.; and lithium hydroxide from Sigma Chemical Co., St. Louis, Mo. Gradient mobile phase A consisted of 11 g/L of pentane sulfonic acid in water adjusted to 3.00 pH with acetic acid; mobile phase B was 0.1 molar lithium acetate (LiAc) in 80–10–10 (volume basis) methanol–acetonitrile–isopropanol adjusted to 3.00 pH with acetic acid. A 120-minute complex gradient from 0% B (aqueous) to 100% B (organic), was used with flow rate adjusted to compensate for azeotropic viscosity effects. Gradient operation was provided by 2 Shimadzu LC-10AD HPLC pumps from ESA, Inc., Chelmsford, Mass.

We developed an electrochemically activated porous carbon and precolumn 18 carbon chain (C18) bed device to eliminate spurious mobile phase contaminant peaks that occurred late in the gradient. The device incorporated a C18 precolumn to trap and spread mobile phase contami-

**Table 1. Compounds quantified with authenticated standards in comparative NIH-funded study of plasma samples from a NASA study of immobile humans and from black bear samples collected in 4 seasons.**

a-methylhistidine	ferulic acid
1-methylhistidine	glutathione
2-hydroxyphenylacetic acid	glutathione disulfide
3,4-dihydroxymandelic acid	guanine
3,3,5-triiodothyronine	guanosine
3,4-dihydroxyphenylacetic acid	homocarnosine
3-O-methyl dopa	homogentisic acid
3-hydroxy-4-methylphenethylamine	homovanillic acid
3-hydroxyanthranilic acid	homovanillyl alcohol
3-hydroxykynurenine	homoveratric acid
3-hydroxymandelic acid	hypoxanthine
3-hydroxyphenylacetic acid	indole-3-lactic acid
3-methoxy-4-hydroxyphenylglycol	indole-3-propionic acid
3-methoxytyramine	indoleacetic acid
3-methylhistidine	isatin
4-hydroxy-3-methylmandelic acid	isoproterenol
4-hydroxybenzoic acid	kynurenine
4-hydroxyphenylacetate	levodopa
4-hydroxyphenyllactic acid	melatonin
4-O-methyl dopamine	metanephrine
5-hydroxyindoleacetic acid	methionine
5-hydroxytryptophan	methoxamine
5-hydroxytryptophol	n-acetylserotonin
5-methoxytryptamine	n-methylserotonin
5-methoxytryptophan	norepinephrine
5-methoxytryptophol	normetanephrine
5-methylcysteine	pyridoxal
6-hydroxymelatonin	serotonin
7-methylguanin	tryptamine
7-methylxanthine	tryptophan
acetylhistidine	tryptophol
anserine	tyramine
anthranillic acid	tyrosine
ascorbic acid	uric acid
carnosine	vanillic acid
cysteine	vanillylmandelic acid
dopamine	xanthine
epinephrine	xanthosine

nants and porous carbon unit bored to mixing flow. This precolumn treatment converted contaminant signals from the mobile phase to waves that were eliminated during data reduction by the software. Analytes occurring in this region of the chromatogram were measured on top of this baseline. Separation was achieved on dual columns containing 3- $\mu$ m octadecylsilyl particles, and each column measured 80 mm long x 4.6 mm inner diameter.

The 16-channel coulometric electrode array incremented in 70 mV units from -100 mV to 990 mV detected both reducible and oxidizable compounds. We confirmed chromatographic peak identities by spiking sample pools with the corresponding standard. Final confirmation was made by comparing the channel response ratios (*R*) for the standard and the sample peaks. A given

compound oxidizes at a specific potential, and every compound can be described by a retention time and a potential. In practice, compounds oxidize on a dominant detector and exhibit a smaller response on the prior and following detector. The ratios (*R*) between detector response on the dominant and adjacent detectors are characteristic of a given compound (Beal et al. 1990).

Information on consistent but unidentified compounds was collected. During data reduction, CEAS software constructs digital time-potential maps of the 16-channel chromatographic data. Peak tables were constructed to represent all potentially detectable compounds in the plasma sample. However, because of chromatographic resolution limitations, the study was restricted to those analytes that could be reliably and automatically analyzed in all samples. Setting the compound table from HR113 cohort pools to 100 resulted in reported values representing percentages. In this study, HR113 "pool standards" reliably found 506 of the 603 potentially detectable analytes in all samples. Once the data report files were generated, they were imported to Lotus 123<sup>®</sup> spreadsheets obtained from Lotus Development Corp., Cambridge, Mass.

We analyzed data with hierarchical cluster analyses and principal components analysis using commercial software (Einsight<sup>®</sup>, Infometrics, Inc., Seattle, Wash.). Data were autoscaled for hierarchical cluster analysis and principal component analysis as described by Harman (1967). Categories were known *a priori*, providing a means for testing validity. The clustering algorithms used in this analysis have been previously described (Hartigan 1975, Hartigan and Wong 1979). Metabolic comparisons between human bedrest and recovery samples were made using the Wilcoxon Signed Rank test. Comparisons between summer and winter samples of bears were made with the Mann-Whitney U-test.

Some NASA samples from Ames Research Facility (study HR62) had been frozen at -70 C since 1987 and were 7 years old at the time of analysis. Absence of many of the most easily oxidized compounds, such as homogentisic acid, strongly suggested sample degradation. Many of the bear samples had also been stored for years in freezers at various temperatures prior to arrival at ESA, Inc. High variability in some samples made them unreliable, and these samples were used only for confirmation. To investigate questions relative to variability resulting from bear blood collection in the field under difficult and variable conditions, a small Eppendorf centrifuge (Millipore Corporation, Bedford, Mass.) was fitted with a small DC-AC power converter and powered by a small 12-volt DC battery. This field centrifuge could be easily carried to

remote den sites in Maine, permitting standardized blood centrifugation and sample preparation within minutes of collecting. In a controlled experiment, blood was carried back in pocket, in thermos, and on ice to a field laboratory for routine preparation, storage, and delivery to ESA, Inc. for analysis. Plasma samples were prepared at each site for storage. Acetonitrile extracts were also prepared at each site for evaporation in the protocol described previously.

## RESULTS

There were no detectable differences between the plasma metabolite profiles or the chromatograms from 2 samples from the same bear, 1 prepared in the field and the other transported in a cold thermos and prepared after several hours delay. Delayed samples appeared pink and showed a small but insignificant elevation in serotonin, indicating possible hemolysis. Repeated analysis over 6-months period of a sample stored at -80 C showed no detectable changes in chromatograms or metabolic profiles.

Chromatographic retention times over 6-month course of the study varied <1%. The absolute qualitative channel ratio responses over the 16-channel detector array varied <20% and was controlled by authentic standards to <5% (Milbury 1992). Typically, about 600 compounds were resolved in plasma at a 20 nanoAmpere gain. Sensitivity limits were defined as the amount of analyte that could be detected while maintaining an SD of half the amount injected and were typically 0.1–100 picograms on the column, depending on the analyte. Precision (coefficient of variation) of individual components in the database varied between  $\pm 3$  and 25%.

Categorical separation was achieved between control samples and the samples from 16-day bedrest and 1-day recovery periods using hierarchical cluster analysis (Fig. 1). The 1-day recovery metabolic samples were not separated from the 16-day bedrest samples, despite NASA's evidence that cardiac function and baroreflex measures in this experiment had recovered. Recovery samples contained both exercised and non-exercised subjects. It was anticipated that metabolic measures might lag behind cardiac functions in recovery; however, samples from later recovery were not available from HR113 to confirm this hypothesis. Trends of those compounds that showed categorical differences indicated that metabolic recovery was underway. Control samples exhibited a greater degree of variability than bedrest samples, as indicated by the tightness of the groupings (representative of eigenvectors).

Scores and loadings were used to determine the relative importance of each variable to categorical separation (Table 2). Compounds located at the extremes of the loading charts represent compounds that had a greater influence in the calculation of the eigenvectors. In the human samples, these compounds included serotonin, ascorbic acid, xanthine, hypoxanthine, unknown compounds 366, 350, 181, and some 34 other compounds (Table 3).

## Comparisons of Human and Bear Metabolic Patterns

Serotonin, guanosine, 5-hydroxyindoleacetic acid, and hypoxanthine decreased in the human subjects ( $P = 0.028$ ,  $0.028$ ,  $0.015$ , and  $0.018$ , respectively) but did not change in the bear. Ascorbic acid and homocarnosine increased in the human bedrest subjects ( $P = 0.018$  and  $0.019$ , respectively) but remained unchanged in the bear.

Twenty-eight identified and 33 unidentified compounds differed in the bear between summer and winter, with  $P$  values of  $4.8 \times 10^{-3}$ – $2.13 \times 10^{-11}$  (Table 3). Compounds associated with energy metabolism were expected to change in the bear, and 3-hydroxykynurenine was increased in dormancy ( $P = 2.13 \times 10^{-11}$ ) while kynurenine and kynurenic acid decreased ( $P = 0.039$  and  $0.001$ , respectively). These compounds were unchanged in plasma samples from ambulatory and bedrest humans. Pyridoxal increased in winter ( $P = 0.03$ ). Prominent among the identified compounds increasing significantly in the bear were metabolites of the tyrosine pathway: homovanillic acid ( $P = 0.046$ ), 3-methoxy-4-hydroxyphenylglycol ( $P = 0.001$ ), and the 2-, and 4-hydroxyphenylacetic acids ( $P = 0.029$  and  $4.65 \times 10^{-8}$ ). Decreases were observed in 3-O-methyl-dopa ( $P = 0.0003$ ), levodopa ( $P = 0.001$ ), tyrosine ( $P = 0.005$ ), and tyramine ( $P = 0.002$ ). Increases were observed in acetyl- and n-methylserotonin ( $P = 5.57 \times 10^{-8}$  and  $8.87 \times 10^{-8}$ , respectively).

We found 31 unidentified compounds that could be involved in bedrest-induced metabolic pattern changes in humans (Table 3). Of these compounds, 6 changed significantly ( $P < 0.043$ ) and diametrically in humans and bears with immobility. Another 10 compounds, with  $P$  ranging from 0.012 to 0.047 in human bedrest subjects, changed differently in wintering bears. Twenty-six unidentified compounds changed ( $P = 4.5^{-2} \times 10^{-1}$ – $1.88 \times 10^{-3}$ ) in the bear, but were unchanged in ambulatory and bedrest humans.

Compound 240 is an example of an unidentified compound that decreased ( $P = 0.012$ ) in human bedrest but was present at very low concentrations and unchanged from summer to winter in the bear. While we concen-

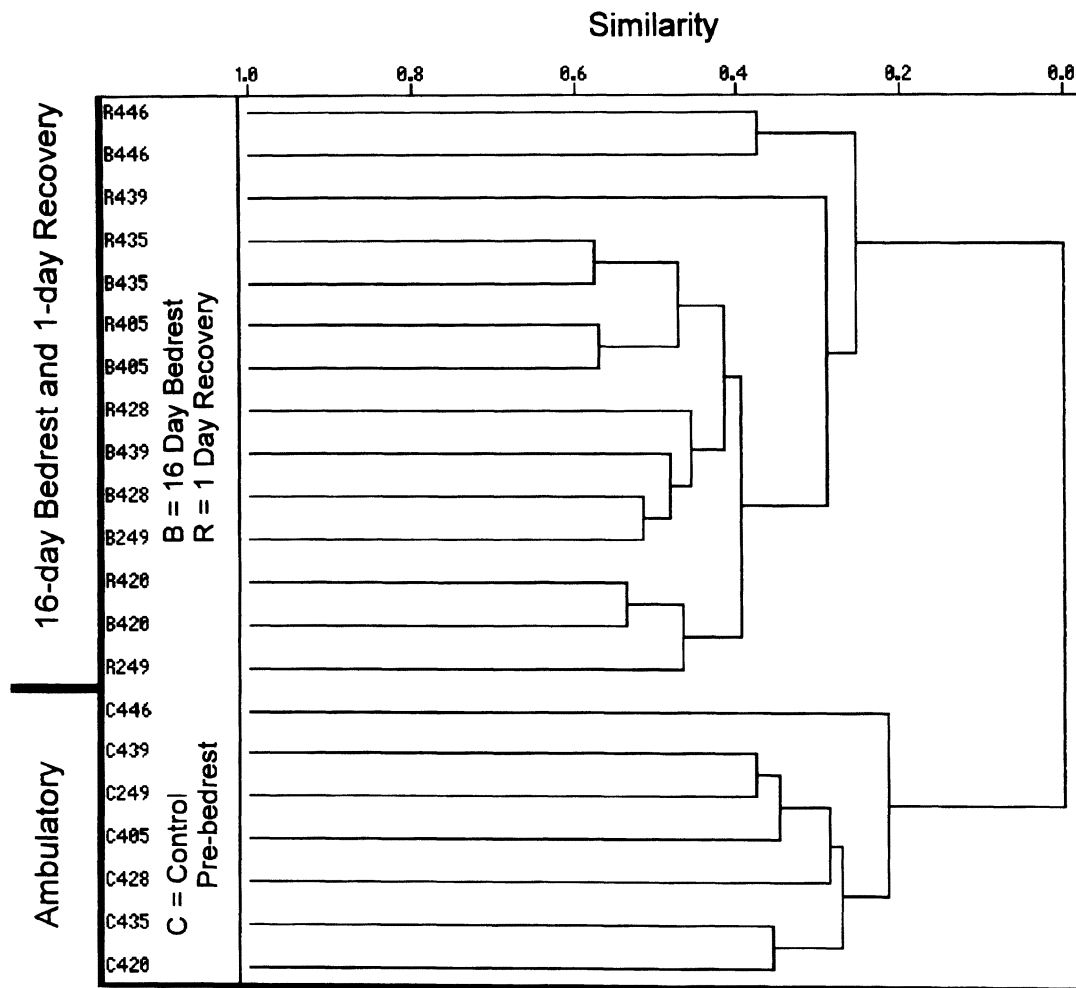


Fig. 1. Dendrogram from hierarchical cluster analysis of NASA HR113 human bedrest experiment (1992–93), demonstrating that the database is capable of categorical separation between ambulatory and bedrest conditions. The dendrogram also shows that 1 day of recovery is insufficient to re-establish ambulatory metabolic profiles.

trated on only summer to winter comparisons, some bears were sampled frequently enough to provide year-long trajectories. In these samples, compound 240 was elevated in black bear during the fall (Fig. 2), thus highlighting the importance of all-season analysis to completely understand compounds that may be involved in osteopenia.

Compound 366 presents a clearer justification for an identification effort. The change from human ambulatory to bedrest conditions ( $P = 0.018$ ) showed a clear recovery (Fig. 3). The concentration of this compound did not vary by season in black bears.

## DISCUSSION

This study suggests fundamental differences in immobility-induced metabolic changes between bears and hu-

mans. In humans, osteopenia resulting from extended bedrest or space flight is believed to result from a mechanical process, such as changes in skeletal loading. Bone homeostasis, however, ultimately depends on the biochemical balance between anabolism and catabolism. Because both osteopenia and osteoporosis result from a change in the rate of bone synthesis while bone degradation appears to be unaffected, mechanistic similarities underlying both disorders have been proposed. In endocrine studies of osteoporosis, emphasis has been placed on anabolic steroids because osteoporosis is observed in so many endocrine-related disorders; for example, osteoporosis occurs in anorexic patients, in estrogen-deficient women, and in men and women with hypothalamic hypothyroidism or hyperprolactinemia (Selby and Francis 1988, Baxter and Sheppard 1991, McGowan 1993,

**Table 2. Loadings of identified and unidentified compound, demonstrating their relative contribution to principal component 1 (PC1) and 2 (PC2).**

Compounds	Loadings		Compounds	Loadings	
	PC1	PC2		PC1	PC2
Serotonin	0.1534	0.0175	002	-0.1393	-0.0908
304	0.1531	-0.0116	Ascorbic acid	-0.1016	-0.1263
Xanthine	0.1479	-0.0476	147	-0.0993	0.0501
Guanosine	0.1463	0.0719	208	-0.0919	-0.0631
Hypoxanthine	0.1438	-0.0213	Carnosine	-0.0918	0.0800
366	0.1406	0.0873	144	-0.0911	0.0987
454	0.1366	-0.0547	430	-0.0879	-0.0198
350	0.1363	-0.0679	059	-0.0861	-0.0753
391	0.1306	-0.0347	311	-0.0858	0.0349
Norepinephrine	0.1306	0.0301	061	-0.0852	-0.0726
181	0.1293	-0.0297	Homocarnosine	-0.0769	0.0392
5-methoxytryptamine	0.1284	-0.0685	352	-0.0757	-0.0144
205	0.1268	-0.0371	067	-0.0722	-0.0312
012	0.1268	-0.0371	2-hydroxyphenylacetic acid	-0.0692	0.1136
Xanthine	0.1242	-0.0507	Anserine	-0.0656	0.0630
505	0.1235	0.0014	265	-0.0619	0.0864
374	0.1228	-0.0338	Methionine	-0.0614	-0.0572
206	0.1222	-0.0484	Vanillylmandelic acid	-0.0614	0.0842
324	0.1222	0.0013	245	-0.0571	0.0937
022	0.1217	0.0242	3-OH-4-ME-phenylethylamine	-0.0552	-0.1225
442	0.1211	0.0361	289	-0.0542	0.0609
523	0.1192	-0.0679	Homovanillic acid	-0.0524	0.1336
050	0.1177	0.0257	255	-0.0501	-0.0045
433	0.1164	-0.0629	421	-0.0480	-0.0211
5-hydroxyindole-acetic a	0.1162	-0.0307	Tryptamine	-0.0463	0.1114
357	0.1148	0.0159	n-methyl dopamine	-0.0462	0.0968
367	0.1147	0.0709	Anthranilic acid	-0.0439	0.0363
388	0.1143	-0.0197	Epinephrine	-0.0437	0.0018
396	0.1141	0.0967	404	-0.0435	-0.1432
188	0.1130	-0.1061	371	-0.0354	0.0325
294	0.1128	-0.0889	365	-0.0347	-0.1159
234	0.1082	0.0865	Tyramine	-0.0318	0.0561
240	0.1024	-0.0395	086	-0.0291	0.0804

Hordon and Wright 1994). Because the bear avoids osteopenia during denning despite immobility, starvation, and hypothyroidism, it appears that osteopenia is more complex than an altered corticosteroid and anabolic steroid balance.

A fundamental argument underlying the approach of evaluating small-molecule CEAS patterns is that changes in both known compounds (tyrosine- and tryptophan-derived neurotransmitters, cofactors, purines and peptides) and unknown compounds can predict and precede or trigger the changes in anabolic or catabolic steroids. A current research direction in degenerative disorders, for instance, is based on the postulate that the patterns of the low-molecular-weight constituents of biological samples represent the operational expression of the genome (Beal et al. 1990, Bird et al. 1990, Matson et al. 1990).

Maintaining experiment-wise error probabilities is problematic with large, multiparameter databases. In establishing significance levels, the investigator must decide arbitrarily what is an acceptable risk of rejecting a true null hypothesis when it should be accepted (type 1 error). Bonferroni recognized that comparing large numbers of variables increases the risk of type 1 error and suggested imposing statistical limits in the form of higher confidence levels (Hassard 1991). The Bonferroni rule of thumb states that given about 500 variables, a corrected  $P = 8.3 \times 10^{-5}$  is required to establish significance. Under these limits, only 1 of the 500 analytes studied in the bear was found to change significantly from summer to winter.

Considering current understanding of bear physiology, Bonferroni's correction may be too stringent and increases the risk of accepting the null hypothesis when it is not

**Table 3. Identified and unidentified oxidizable compounds changing in bedrest humans and denning bears. Data for numbered unknown compounds are expressed as percent of the study control pool value, and data for known compounds are expressed as nanograms/ml of plasma.**

Retention time (min)	Dominant detector channel	Oxidation potential (milli-V)	Direction of change		Human data						Bear data					
			Human	Bear	Ambulatory		Bedrest		Summer		Winter		Mann-Whitney U-test	P		
					Avg	SD	Avg	SD	Avg	SD	Avg	SD				
			Wilcoxon signed/rank test		P		P		P		P					
050	7.79	14	-	+	88.74	19.94	55.48	11.67	0.018	0.018	5.81	4.16	13.92	9.78	3.40 x 10 <sup>-10</sup>	
294	58.54	5	+	-	100.92	27.09	69.36	10.39	0.018	0.018	29.93	12.70	60.34	54.52	1.12 x 10 <sup>-04</sup>	
147	25.82	14	-	+	111.18	55.39	221.56	52.58	0.018	0.018	803.30	169.85	566.83	521.88	0.017	
462	91.40	12	-	+	136.65	29.93	86.79	20.89	0.028	0.028	1001.63	2694	2990.43	6077	0.039	
523	103.13	6	-	+	123.97	29.64	77.96	26.32	0.043	0.043	6.58	5.77	10.60	7.75	0.001	
388	77.32	15	-	+	130.78	17.31	81.86	19.86	0.018	0.018	56.69	28.93	36.77	15.20	0.005	
144	25.14	15	-	+	82.07	18.18	124.09	24.16	0.043	0.043	189.94	248.07	371.68	579.71	0.046	
<b>Unidentified and identified compounds changing in human only<sup>a</sup></b>																
240	45.04	12	-	+	95.25	50.32	23.16	21.02	0.012	0.012	115.89	100.97	122.99	147.12	0.730	
304	60.93	15	-	+	98.83	46.26	20.23	8.84	0.018	0.018	20.95	12.38	16.96	17.37	0.119	
433	86.05	13	-	-	100.92	27.09	62.49	17.12	0.018	0.018	162.39	139.55	114.40	120.84	0.432	
367	72.53	15	-	+	147.84	22.11	87.13	22.87	0.018	0.018	138.38	47.96	149.64	82.47	0.551	
366	72.51	12	-	+	119.80	28.93	48.31	15.53	0.018	0.018	29.23	16.59	32.83	47.24	0.493	
350	70.03	6	-	+	124.82	25.54	75.08	19.50	0.018	0.018	26.71	17.77	30.78	23.17	0.462	
245	45.93	15	-	+	81.60	20.61	124.25	23.91	0.018	0.018	361.59	615.66	1663.42	7084	0.056	
505	98.11	15	-	+	119.86	37.88	58.63	17.88	0.018	0.018	107.75	146.06	61.82	46.97	0.162	
218	39.55	9	-	+	156.71	22.00	98.37	40.61	0.018	0.018	30.95	25.46	45.69	35.10	0.063	
208	37.16	4	-	+	104.71	9.61	126.16	15.56	0.018	0.018	142.55	114.49	273.34	676.76	0.167	
068	10.42	1	-	-	109.46	55.39	50.68	12.86	0.018	0.018	217.59	87.59	200.50	93.86	0.299	
248	46.63	12	-	-	143.01	21.49	113.07	18.12	0.023	0.023	23694.71	73526	11953.21	38499	0.314	
002	3.80	15	-	+	138.60	43.04	370.37	85.60	0.028	0.028	342.16	417.42	388.68	398.24	0.642	
391	78.08	4	-	+	227.09	103.66	108.45	38.96	0.028	0.028	115.44	112.90	156.69	93.31	0.131	
190	33.35	13	-	-	112.39	38.03	56.03	32.25	0.028	0.028	551.68	1086	290.19	261.38	0.117	
427	85.02	12	-	+	165.57	21.32	101.80	45.33	0.028	0.028	302.34	308.45	332.74	451.01	0.635	
181	31.85	9	-	-	111.09	25.09	65.80	11.21	0.028	0.028	140.42	157.08	135.10	121.93	0.840	
396	78.49	15	-	+	131.96	35.55	69.19	26.28	0.028	0.028	9.10	3.94	10.19	4.61	0.298	
314	62.93	8	-	-	108.32	24.15	78.03	21.88	0.042	0.042	145.40	116.78	102.81	63.19	0.359	
357	71.53	11	-	-	123.26	41.82	70.44	16.45	0.043	0.043	307.21	851.54	222.93	453.17	0.638	
324	64.30	16	-	-	132.30	86.92	32.70	17.93	0.043	0.043	1608.51	4811	399.44	1132	0.198	
067	10.19	15	-	+	93.96	27.85	181.28	83.60	0.044	0.044	120.83	139.66	112.78	106.55	0.736	
022	4.79	11	-	-	83.97	25.46	51.47	7.45	0.046	0.046	525.97	757.90	303.58	520.88	0.227	
204	36.04	6	-	-	103.48	53.34	48.92	14.82	0.047	0.047	41.64	40.55	27.09	36.42	0.122	

<sup>a</sup> Change in bears was not significant.  
<sup>b</sup> Change in humans was not significant.

Table 3. Continued.

Direction of change		Human data										Bear data							
		Ambulatory (n = 7)			Bedrest (n = 7)			Wilcoxon signed/rank test				Summer (n = 31)			Winter (n = 63)			Mann-Whitney U-test	
		Human	Bear		Avg	SD	P	Avg	SD	P	Avg	SD	Avg	SD	Avg	SD	Avg	SD	P
-	+		6.84	3.63	2.16	0.398	6.24	2.16	0.398	3.22	2.32	10.07	9.27	10.07	9.27	2.13 x 10 <sup>-11</sup>			
-	+		5.93	1.06	0.77	0.043	5.30	0.77	0.043	10.01	5.31	18.00	11.60	18.00	11.60	3.82 x 10 <sup>-08</sup>			
+	+		139.33	86.36	68.46	0.612	165.02	68.46	0.612	1989.45	1030	3303.52	1770	3303.52	1770	4.65 x 10 <sup>-08</sup>			
-	+		0.91	0.77	0.48	0.866	0.73	0.48	0.866	73.11	84.91	206.91	216.98	206.91	216.98	1.18 x 10 <sup>-07</sup>			
-	+		0.37	0.17	0.35	1.000	0.35	0.21	1.000	0.83	0.07	194.22	291.04	194.22	291.04	5.57 x 10 <sup>-07</sup>			
-	+		4.68	4.68	3.96	1.76	3.96	1.76	1.76	53.82	86.12	398.91	508.09	398.91	508.09	8.87 x 10 <sup>-07</sup>			
-	+		2.29	1.81	1.50	0.866	1.50	0.35	0.866	1.47	0.95	2.48	1.80	2.48	1.80	4.83 x 10 <sup>-05</sup>			
-	+		61.46	15.82	8.59	0.499	57.30	8.59	0.499	708.22	478.75	1105.93	656.57	1105.93	656.57	5.24 x 10 <sup>-05</sup>			
-	-		0.52	0.66	0.10	0.866	0.10	0.05	0.866	1.20	0.87	0.50	0.26	0.50	0.26	1.47 x 10 <sup>-04</sup>			
-	-		17.81	6.60	4.11	0.176	15.54	4.11	0.176	30.68	20.78	18.02	8.64	18.02	8.64	2.84 x 10 <sup>-04</sup>			
-	-		0.07	0.04	0.02	0.465	0.06	0.02	0.465	0.56	0.21	0.34	0.06	0.34	0.06	3.16 x 10 <sup>-04</sup>			
-	-		77.27	29.27	21.76	0.399	64.21	21.76	0.399	32.79	28.20	16.96	8.05	16.96	8.05	0.001			
-	-		0.87	0.37	0.65	0.311	0.65	0.41	0.311	2.18	0.77	1.07	0.66	1.07	0.66	0.001			
+	-		27.28	13.18	6.38	0.612	31.90	6.38	0.612	61.28	25.29	46.70	20.41	46.70	20.41	0.001			
+	+		51.82	24.19	27.09	0.735	51.90	27.09	0.735	4.31	5.85	8.77	10.51	8.77	10.51	0.001			
-	+		4.01	1.39	3.41	0.091	3.41	0.76	0.091	0.80	0.39	1.03	0.35	1.03	0.35	0.001			
+	-		4.22	0.62	4.51	0.116	4.51	0.73	0.116	1.22	0.61	0.48	0.10	0.48	0.10	0.001			
-	-		1.80	0.81	1.51	0.311	1.51	0.32	0.311	0.32	0.16	0.19	0.10	0.19	0.10	0.001			
+	-		9.28	2.42	11.74	0.128	11.74	5.42	0.128	0.36	0.10	0.25	0.12	0.25	0.12	0.002			
-	+		2.40	1.03	2.33	0.866	2.33	0.79	0.866	0.03	0.02	0.08	0.15	0.08	0.15	0.003			
+	-		13730.54	1181	13931.29	0.866	13931.29	1668	0.866	22567.65	8646	18377.72	7304	18377.72	7304	0.005			
-	+		389.44	275.92	329.74	0.345	329.74	295.59	0.345	83.70	103.80	154.03	204.63	154.03	204.63	0.006			
-	-		155.92	60.97	128.70	0.128	128.70	45.60	0.128	87.87	128.86	34.73	16.56	34.73	16.56	0.008			
+	+		118.02	31.02	155.12	0.063	155.12	54.17	0.063	71.58	53.28	93.93	65.82	93.93	65.82	0.029			
-	+		3.04	3.86	2.13	0.345	2.13	2.49	0.345	3.49	2.10	6.16	7.24	6.16	7.24	0.030			
-	-		935.59	92.01	913.05	0.499	913.05	129.43	0.499	1153.77	1130	772.60	696.77	772.60	696.77	0.039			
+	+		18.79	2.77	21.87	0.091	21.87	4.64	0.091	3.79	3.90	5.14	3.48	5.14	3.48	0.046			
+	-		2.85	1.64	3.69	0.176	3.69	1.37	0.176	6.81	20.11	0.33	0.27	0.33	0.27	0.048			

a Change in bears was not significant.

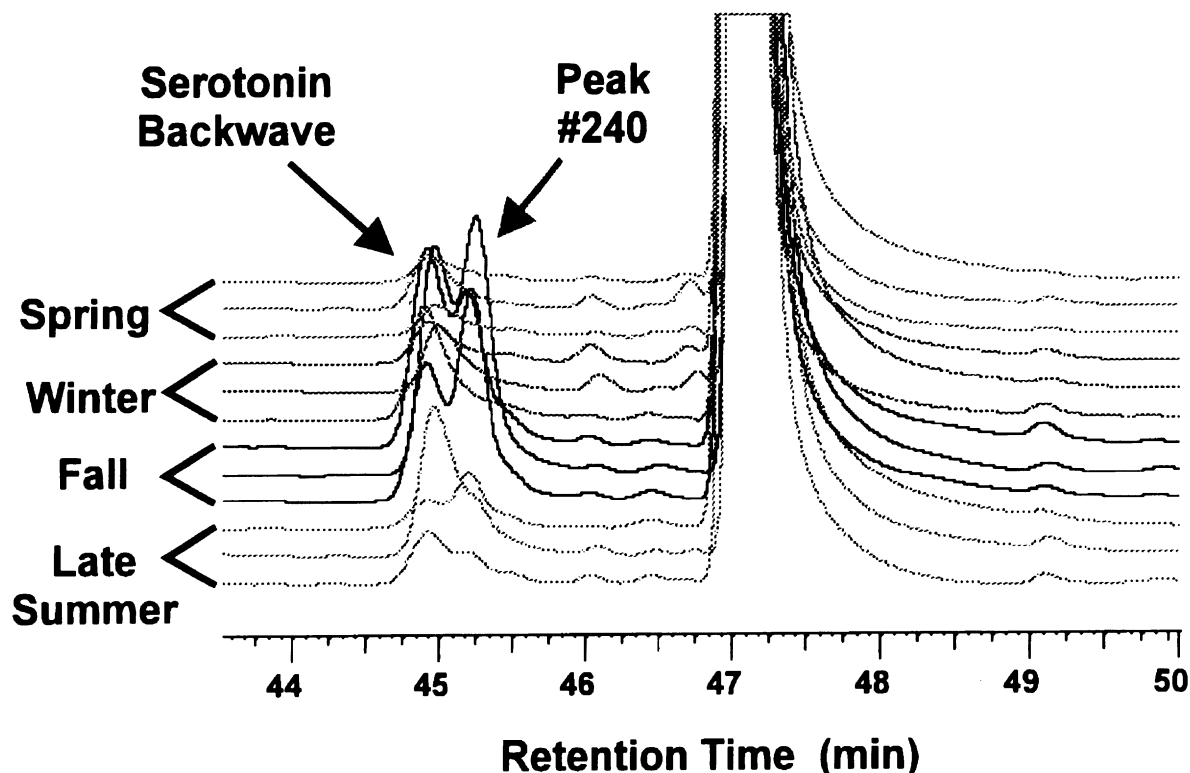
b Change in humans was not significant.

Table 3. Continued.

Unidentified compounds changing in denning bears only <sup>b</sup>														
Retention time (min)	Dominant detector channel	Oxidation potential (milli-V)	Direction of change		Human data						Bear data			
			Human	Bear	Ambulatory (n = 7)		Bedrest (n = 7)		Summer (n = 31)		Winter (n = 63)		Mann-Whitney U-test P	
					Avg	SD	Avg	SD	Avg	SD	Avg	SD		
58	27.70	15	880	-	-	96.37	19.94	83.63	24.20	4287.92	491.98	961.45	1179	6.57 x 10 <sup>-25</sup>
062	9.63	11	600	-	+	146.88	28.76	107.72	35.04	202.22	75.88	293.99	105	1.88 x 10 <sup>-08</sup>
207	36.39	12	670	-	+	150.32	54.35	102.59	19.79	171.96	75.27	4218.76	6956	2.72 x 10 <sup>-06</sup>
233	43.47	15	880	-	+	140.65	46.98	93.80	25.68	141.09	67.48	253.41	142	2.81 x 10 <sup>-06</sup>
176	30.81	12	670	-	+	118.44	41.76	101.83	63.86	62.51	42.83	21.64	19	8.01 x 10 <sup>-06</sup>
311	62.02	16	950	+	+	101.38	37.35	151.24	66.75	107.63	101.32	1819.13	4453	8.93 x 10 <sup>-05</sup>
255	49.49	16	950	+	-	128.93	33.26	176.78	40.02	4380.91	4189	1295.20	2344	2.79 x 10 <sup>-04</sup>
179	31.53	12	670	-	-	142.13	72.36	110.91	94.07	625.21	828.56	144.47	173	3.49 x 10 <sup>-04</sup>
226	42.10	12	670	-	-	261.51	113.31	165.18	51.58	2044.39	2497	630.42	1284	0.001
086	12.89	9	460	-	+	125.65	21.69	96.80	26.93	92.35	33.44	139.72	65.52	0.001
430	85.80	6	250	+	-	67.35	25.29	101.28	55.68	879.07	841.86	241.04	212.22	0.001
059	9.42	7	320	+	+	115.89	34.92	186.05	76.31	381.48	242.60	97.42	44.51	0.001
154	26.58	1	-100	+	-	111.50	93.81	133.12	51.61	237.16	144.39	157.93	96.50	0.001
289	57.63	12	670	+	+	97.50	31.51	281.46	504.48	36.23	9.71	52.87	29.05	0.001
514	100.61	10	530	-	+	131.41	56.69	115.82	41.45	348.42	289.38	543.39	451.64	0.002
185	32.34	1	-100	+	+	107.90	5.73	116.31	9.53	123.23	22.39	143.00	38.04	0.002
307	61.14	15	880	-	-	100.21	92.72	31.90	5.07	19.34	11.26	13.13	8.74	0.002
054	8.35	16	950	-	+	180.98	59.89	123.18	33.30	32.09	32.28	56.80	59.27	0.002
107	19.28	12	670	-	-	111.78	61.45	60.44	20.41	233.25	270.63	86.15	111.07	0.003
272	52.88	16	950	+	+	139.41	86.27	176.81	56.12	178.07	98.93	301.69	423.55	0.012
188	33.08	12	670	-	-	117.19	13.72	102.83	9.17	516.20	946.36	147.94	113.59	0.016
012	4.31	3	40	-	-	511.41	474.99	144.49	62.15	115.69	138.39	60.66	83.81	0.020
222	41.23	12	670	-	-	129.51	43.06	69.06	41.84	39.17	25.83	25.26	26.62	0.031
234	43.76	9	460	-	+	168.09	59.82	88.92	40.14	317.97	495.53	522.89	585.51	0.034
409	81.85	13	740	+	+	140.65	46.98	92.82	25.33	130.88	53.53	194.20	106.46	0.038
378	75.11	12	670	-	-	179.34	85.87	109.69	32.56	2636.38	5036	232.71	175.03	0.045

<sup>a</sup> Change in bears was not significant.

<sup>b</sup> Change in humans was not significant.



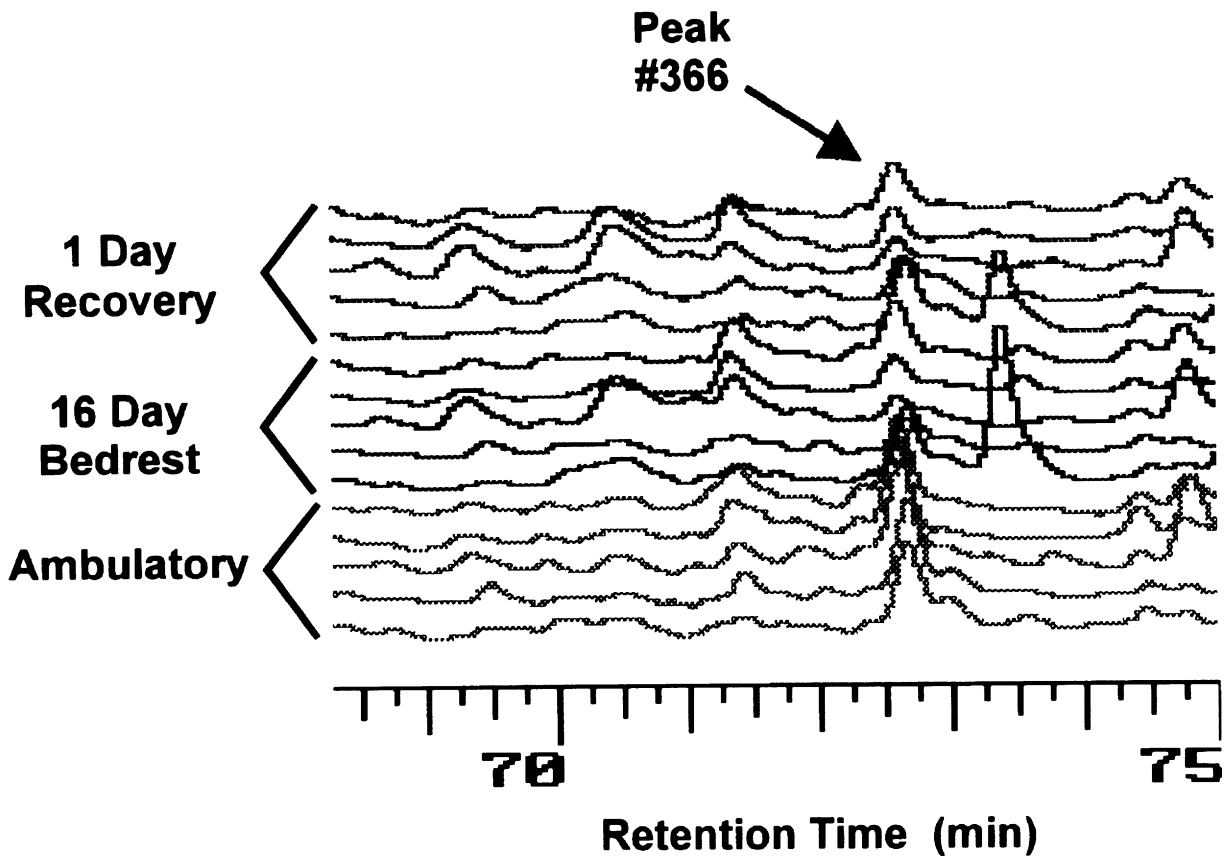
**Fig. 2. Chromatographic trajectory for compound 240 during 4 seasons for 3 individual black bears. Plasma concentration of the substance is higher in the fall relative to other seasons and may be involved in metabolic changes associated with the change from hyperphagia to hypophagia in the bear.**

true (type 2 error). This quandary is philosophically unsatisfying and has long been a point of contention among statisticians. The design of our study diminished the probability of finding immobility-involved compounds by chance alone by highlighting compounds that have a high probability of involvement in immobility-induced osteopenia, as evidenced by changing differentially in 2 species that differ in susceptibility to immobility-induced osteopenia. The probability of error should be quite low for finding compounds by chance alone that change both significantly and differentially between bedrest humans and hibernating bears. For all other compounds, we recognize the risk of type 1 errors. The only solution to this problem lies in verifying these results by repeating the analysis with a new set of samples. That project is underway currently. Nevertheless, in addition to reporting significance levels from Wilcoxon Signed Rank (human) and the Mann-Whitney *U*-tests (bear), we used alternate statistical approaches to confirm suspected compound involvement.

While the approach to statistical analysis of the database presented here is designed to highlight compounds with the highest probability of involvement in immobil-

ity-induced changes, caution must prevail in interpreting results. These data cannot be considered without awareness of current theories of dormancy, starvation, and reproductive biology in the bear. For example, in this study serotonin decreased significantly in humans only. Norepinephrine decreased in both species during immobility, but not significantly. These monoamines have been demonstrated to be the chief central neurotransmitters mediating thermoregulatory responses in deep hibernators (Feldberg and Myers 1964, Glass and Wang 1979, Lin and Pivorun 1990). The bear is not considered a deep hibernator, with body temperature only decreasing to 31.2 C during its somnolent state (Hock 1960). Our data suggest these minor temperature changes in the bear are not accompanied by dramatic peripheral changes in serotonin or norepinephrine. However, these bear samples showed a high degree of variability, and levels of serotonin were higher than expected, indicating possible problems with hemolysis in some samples.

The decrease in peripheral levels of serotonin and norepinephrine in bedrest humans is unexpected, and, at the moment, unexplained. It has been suggested that head tilt-down bedrest inhibits the renin-angiotensin-aldoster-



**Fig. 3. Chromatographic trajectory for compound 366 in NASA HR113 human bedrest experiment. Peak 366 is present in control pre-bedrest samples, significantly diminished by 16 days of bedrest, and begins to recover after 1 day of ambulatory conditions. This compound is a candidate for involvement in immobility-induced metabolic changes in humans.**

one system, which may explain observed decreases in norepinephrine (Gharib et al. 1988) but this theory remains controversial. Inspection of both databases left the impression that changes in humans involved serotonergic pathways more than the dopaminergic pathways, but the opposite was true in wintering bears. Significant differences were seen in 3-O-methyldopa values between summer and winter samples, and time course data (Fig. 4) clearly showed a diminishing concentration of this analyte in the winter months and a recovery in early spring.

It has been proposed that dopamine, norepinephrine, and serotonin are involved in control of the appetite-satiation centers of the brain, the maintenance of a normal eating response, and ultimately in the regulation of obesity and anorexia (Booth 1981, Rolls 1981, Morley and Levine 1983, Smith et al. 1987). Metabolite changes observed here support this hypothesis, as bears exhibit anorexia during hibernation. Decreased serotonin levels and increased dopamine levels in rats result in hy-

perphagia and obesity (Breisch et al. 1976, Wurtman 1988, Abadie et al. 1993, Noach 1994).

Changes observed in tyrosine, vanillic acid, and 4-hydroxyphenylacetic acid metabolism suggest differential control of the phenylalanine and tyrosine pathways. Again, these changes cannot be considered without noting that the bear undergoes drastic metabolic changes that also involve lipid and energy metabolism; phenylalanine and phenylacetate are central to those processes. The bear demonstrates intrinsically that fat metabolism and management is important in successful fasting. Since earlier studies demonstrated increased body protein oxidation and decreased metabolic rates in response to fasting (Addis et al. 1936), numerous studies have shown that obese animals lose less protein and experience a slower decline in metabolic rate during fasting than lean animals (Goodman et al. 1980, Dunn et al. 1982). During fasting, obese Zucker rats experience protein sparing and lose lipid at a higher rate than lean rats. Some of this increased rate of lipid loss is thought to be due to the larger size of obese

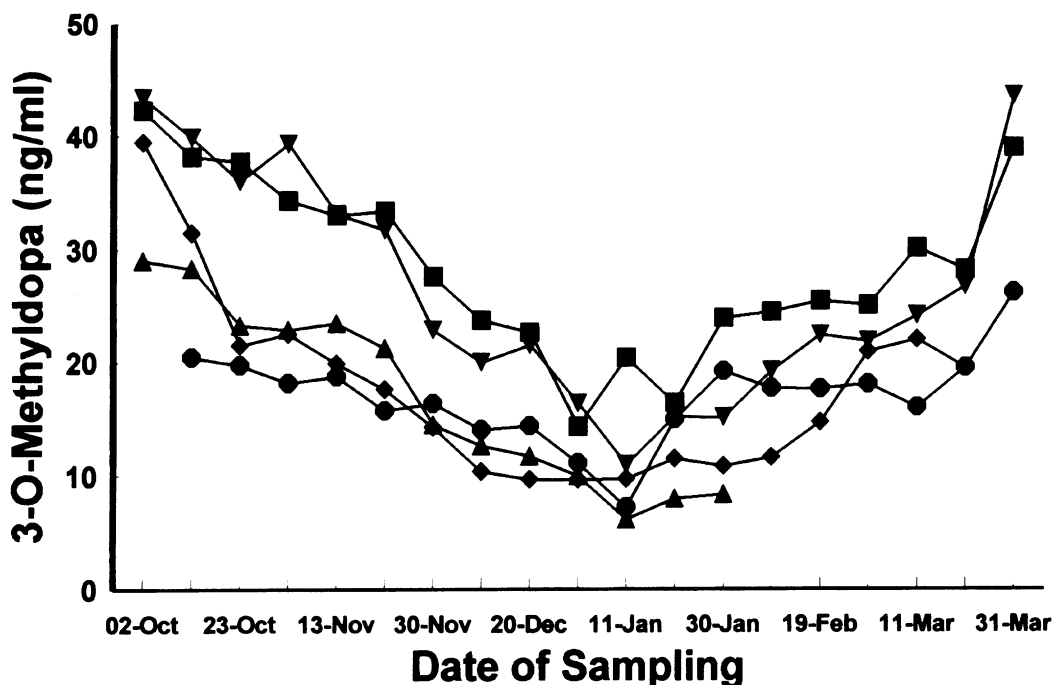


Fig. 4. Serial changes in plasma concentrations of 3-O-methylidopa for 5 captive female black bears maintained in Blacksburg, Virginia.

rats, which require greater daily energy expenditures. Fasting bears gain some advantage by relative immobility during dormancy, which minimizes daily energy expenditure and in turn, perhaps, decreases the rate of lipid metabolism required to prevent potential decline in the rate of protein synthesis.

In most mammals, extended periods of reduced dietary protein result in decreased protein breakdown in muscle and liver. Although the regulatory mechanisms for control of protein breakdown appear to be diffuse, involving ubiquitination and oxidation as initiation steps in proteolysis, there is substantial evidence that the available amino acid mix can significantly influence protein degradation, with alanine playing a coregulatory role influencing the inhibitory efficacy of leucine, tyrosine, glutamine, proline, histidine, tryptophan, and methionine (Mortimore 1987, Mortimore et al. 1988). Perhaps balanced protein synthesis and degradation in wintering bears is more dependent on lipid metabolism and gluconogenesis than is generally thought. Control of amino acid availability may be a key to maintaining protein synthesis in the absence of dietary protein intake, and the term "essential amino acids" takes on new meaning if nitrogen is not lost from the system via the urea cycle. In bedridden humans, with no dietary stress, intervention to avoid loss of lean body mass may be aided by appropriate dietary considerations, adjustment of oxidative states (Harper and Benjamin 1984,

Harper 1986), and adjusting body amino acid constituents to favor protein synthesis and reduce protein degradation. We report here that, of the oxidizable amino acids, tyrosine pathway metabolites change notably during winter dormancy in bears. Until more studies are completed, it must be assumed that there are measurable differences in body amino acid concentrations and that these differences may have profound effects on hormonal balances, energy use, protein metabolism, and bone metabolism.

Significant differences were seen, as expected, in compounds involved in energy metabolism, which may indicate basic shifts in the bear from nicotinamide adenine dinucleotide (NAD) to adenosine triphosphate (ATP) based systems as a result of shifts in lipid biochemistry from storage to utilization strategies. Increased serum concentrations of pyridoxal were observed in the wintering bear. Pyridoxal kinase combines ATP with pyridoxal to form pyridoxal-5-phosphate, which is a required coenzyme for at least 60 enzymes involved in every aspect of amino acid metabolism, including transamination. In humans, pyridoxine (Vitamin B<sub>6</sub>) is an essential nutrient whose deficit leads to derangement of tryptophan metabolism and many ill-defined symptoms.

Changes were observed in tyrosine metabolism between summer and winter bears. Reported changes in metabolites of tyrosine and tryptophan pathways suggest fundamental differences in the dormancy of the bear as

compared to other deep hibernators (Kilduff et al. 1987, Salzman et al. 1985, Lin and Pivorun 1990). The literature suggests that increases in activity of the hypothalamic serotonergic and dopaminergic systems may play a significant role in the deep sleep associated with true hibernators. Increases in homovanillic acid, decreases in 3-O-methyldopa, and the lack of a change in 5-hydroxyindoleacetic acid in the bear during winter are inconsistent with changes seen in true hibernators and may contribute to ease of arousal from winter dormancy.

## SUMMARY

The bear presents a unique opportunity to study starvation mechanisms in which lean body mass is not depleted. This analytical effort and database investigation has highlighted 6 compounds for identification that have a high probability of involvement in immobility-induced changes in human and bear metabolism. These 6 compounds showed differential control between humans and bears with immobility. These results are being confirmed in an ongoing and larger study. Compound 240 is intriguing as it increases dramatically in black bears in autumn. We speculate that it is involved in metabolic changes that occur as the bear becomes anorexic and changes from lipid storage to lipid utilization. Until completion of identification protocols, theories regarding compound 240 remain speculative. If it is a fasting-related metabolite, the decrease in humans during bedrest is unexplained, as no change in appetite or food consumption was evident during NASA's bedrest studies. Our findings demonstrate the effectiveness of metabolic pattern analysis at highlighting compounds not yet identified but involved in the process for further study.

Metabolite patterns can be maintained under field conditions with routine blood collection practices and sampling techniques that avoid hemolysis. Under some field conditions, temperature control may be a critical factor in preventing pattern degradation and diminishing variability in final databases. A small thermos with ice may be sufficient to control the temperature of collected samples in both cold and warm conditions. Extended frozen storage diminishes the usefulness of both human and bear samples for metabolic profiling of oxidizable compounds. Storage at -80 C under nitrogen, while more costly, may diminish the effects of oxidation but does not eliminate sample degradation. Samples collected for these studies need to be analyzed in a timely manner, preferably within 6 months.

The bear is a particularly intriguing subject for biomedical research, with potential application in the study

of obesity, anorexia, osteopenia, atherosclerosis, reproductive biology, and liver and kidney disorders. Modern human society tends to preserve only what has perceived value. The value of biodiversity is sometimes a remote consideration to the public at large until a direct linkage and tantalizing promises of solutions for human problems arise. A cost-effective means to impart value to wild bear populations is to routinely include biomedical research when designing field protocols that require handling bears. Once governments and the public understand the value of both captive and wild bear populations to human biomedical research, perhaps additional efforts to preserve bears and their habitats will be forthcoming.

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