Longitudinal metabolomic profiles reveal sex-specific adjustments to long-duration spaceflight and return to Earth

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Abstract
Spaceflight entails a variety of environmental and psychological stressors that may have long-term physiological and genomic consequences. Metabolomics, an approach that investigates the terminal metabolic outputs of complex physiological alterations, considers the dynamic state of the human body and allows the identification and quantification of downstream metabolites linked to upstream physiological and genomic regulation by stress. Employing a metabolomics-based approach, this study investigated longitudinal metabolic perturbations of male (n = 40) and female (n = 11) astronauts on 4–6-month missions to the International Space Station (ISS). Proton nuclear magnetic resonance (1H-NMR) spectroscopy followed by univariate, multivariate and machine learning analyses were used on blood serum to examine sex-specific metabolic changes at various time points throughout the astronauts’ missions, and the metabolic effects of long-duration space travel. Space travel resulted in sex-specific changes in energy metabolism, bone mineral and muscle regulation, immunity, as well as macromolecule maintenance and synthesis. Additionally, metabolic signatures suggest differential metabolic responses—especially during the recovery period—with females requiring more time to adjust to return to Earth. These findings provide insight into the perturbations in glucose and amino acid metabolism and macromolecule biosynthesis that result from the stressors of long-duration spaceflight. Metabolomic biomarkers may provide a viable approach to predicting and diagnosing health risks associated with prolonged space travel and other physiological challenges on Earth.

Keywords Microgravity · Human space travel · Space exploration · Sex differences · Metabolic biomarkers

Introduction
Space travel affects a host of physiological and psychological functions of the human body. Adverse health outcomes linked to human space exploration have been mainly attributed to factors such as microgravity, galactic cosmic radiation, and isolation or confinement in a spacecraft [1, 2]. Astronauts on missions to the International Space Station (ISS) experience environmental stressors such as altered light/dark cycles affecting sleep, rapid accelerations, vibrations, noise, elevated ambient carbon dioxide exposure [1, 3] in addition to motion sickness and vestibular imbalances.
Moreover, astronauts face significant psychological stresses such as anxiety, high workload, limited communication with family and friends on Earth, and the knowledge of the scope of isolation in which they are subjected [4]. Long-term spaceflight may lead to vision impairment [5], bone resorption and demineralization [6], skeletal muscle atrophy [7], cardiac muscle dysfunction and atrophy [8, 9], delayed wound healing, immune system dysfunction and cancer susceptibility [10], metabolic alterations, as well as oxidative stress which may damage DNA structure and, consequently, affect protein synthesis [1, 11, 12]. Spaceflight-induced impacts also include cytoskeletal alterations which may impair vesicle and organelle trafficking having implications for signal transduction, hormone regulation, and cellular metabolism [13]—along with associated perturbations in gene expression [7, 14]. As recent space exploration efforts increasingly focus on exploration-class missions, such as to Mars, it is critical to understand temporal profiles of space travel-induced metabolic and physiological changes to identify means of monitoring and protecting crew health.

Long-duration space flights may gradually facilitate physiological deconditioning [15] amongst other physiological disturbances [16, 17] resulting in metabolic changes that may be identifiable through longitudinal metabolomic studies. Metabolomics provides a unique, quantitative approach to investigating metabolic end-products of dynamic systems indicative of phenotypic responses to adverse experiences [18, 19]. Despite the potential for prediction and prevention of adverse health outcomes linked to prolonged spaceflight, few metabolomic studies in space travelers have been undertaken to date. Multiscale omics data from the NASA Twins Study showed cell type- and context-specific responses in fatty acid metabolism in a 50-year-old, male astronaut who spent nearly a year in space [20, 21]. Their findings showed a mid-flight spike in gene expression followed by a return to pre-flight levels in the astronaut which suggests up-stream epigenetic regulation [20]; however, this may also be attributed to changes in diet or exercise patterns as the astronaut altered his regular exercise regimen mid-flight. In ground-based spaceflight simulation studies, proton nuclear magnetic resonance (1H-NMR) metabolomics revealed progressive reduction in metabolic diversity and an increase in the number of affected metabolic pathways in response to just three weeks of bed rest and/or hypoxia [22]. These changes, in addition to depression symptoms, insulin resistance and other adverse health outcomes observed, appeared to be mitigated by physical exercise [22].

The global, untargeted, and non-destructive nature of 1H-NMR spectroscopy is ideal for the quantitative study of complex metabolome alterations linked to physical and psychological stress in astronauts during long-duration spaceflight. The present study is the first to use a 1H-NMR spectroscopy approach to identify down-stream metabolic fingerprints associated with space travel in blood serum samples from male and female astronauts. Using a longitudinal time-series analysis based on univariate, multivariate, and machine learning analysis, the present study was able to identify metabolic perturbations both common and sex-specific to male and female astronauts while they were visiting the ISS as well as after their return to Earth. The analyses provide potential biomarkers that may be suitable for prediction and prevention of adverse health outcomes during prolonged spaceflight.

Materials and methods

The data reported herein represent findings from 3 NASA Human Research Program supported projects: Biochemical Profile, Nutritional Status Assessment (Nutrition SMO), and Dietary Intake Can Predict and Protect Against Changes in Bone Metabolism During Spaceflight and Recovery (ProK). While many papers have been published from the nominal findings from these studies, the work described here represents extended analyses from the samples collected from those projects, and none of the data reported herein have been previously published. The additional work was reviewed and approved by the NASA Johnson Space Center IRB, as well as by the biomedical ethics boards from International Partner Space Agencies. Participating astronauts provided written informed consent for the additional analyses of their samples before testing began, in addition to their original consent to participate in the research.

The data reported here are from a subset of the three studies’ astronaut complement, the inclusion criterion being that the participants must have had enough sample (serum) remaining for NMR analysis to be conducted properly.

Subjects

Blood samples (n = 335) were collected from male (n = 40) and female (n = 11) astronauts who participated in 4–6-month-long missions to the ISS between 2006 and 2018. The average age at launch was 49 years (range 39–60 years) in males and 45 years (range 38–57 years) in females (see Table 1). The average flight duration for males and females was 161 (58–215) and 164 (91–290) days, respectively. Previous cumulative number of days in space (including short and long-duration missions) for any astronaut who had visited space before the mission at which samples were collected were on average 96.8 days (13–362 days) in 12/40 males, and 117 days (11–277 days) in 6/11 females. Astronauts were provided individual exercise programs, including both aerobic and resistance exercise before, during, and after flight [23, 24]. Astronauts’ diets were generally not controlled or monitored before flight. During flight, astronauts...
Table 1 Cohort information for both male and female NASA astronauts included in this study showing the average age at launch, average duration of the flight, and average previous number of days in space prior to the mission at which samples were taken for this study (± standard deviation), as well as the ranges detailing minima and maxima for each descriptor.

|                | Males (n = 40) | Females (n = 11) |
|----------------|----------------|------------------|
| Age at launch  | 49 ± 5.0       | 45 ± 5.4         |
| Flight duration (days) | 161 ± 31.1 | 164.2 ± 52.72   |
| Previous number of days in space (n = 12) | 96.8 ± 105 | 171 ± 106      |

Sample collection, handling, and storage

Sample collection, handling, and storage adhered to previously published protocols [26]. Blood samples were collected using standard phlebotomy techniques into evacuated serum gel separator tubes, centrifuged at 3800 rpm for 30 min and then frozen at −96 °C on the ISS. All samples were collected after at least 8 h of fasting and not exercising, typically between 0700 and 0900 h Greenwich Mean Time (GMT) while on ISS, and local time when in Houston before and after flight, except for the R + 0 blood collection time point [26]. The inflight samples were typically returned to Earth from the ISS within 6–12 months after collection and were kept frozen until arrival at the Nutritional Biochemistry Lab at the Johnson Space Center. While these samples were processed and analyzed for the primary study objectives, care was taken to minimize the number of freeze–thaw cycles with, at most, two cycles occurring before transfer to the University of Lethbridge for metabolomic analysis.

Sample processing differences occurred between the L-10 and FD samples and the L-45 to R + 0/30 samples. Postflight (R + 0/30) samples, were collected, allowed to clot, and centrifuged to obtain serum which was subsequently pipetted into separate cryotubes for storage at −80 °C. In contrast, inflight (FD) samples were collected, allowed to clot, and centrifuged, but the collection tubes were directly frozen at −96 °C (inflight) after centrifugation as the serum could not be pipetted into a separate cryotube. While the L-10 samples are designated as such, in many cases they were collected at the same time as the L-45 samples; thus, L-10 samples were collected and processed similarly to inflight (FD) samples (frozen at −80 °C on Earth after centrifugation with no transfer of the serum to a separate tube) whereas L-45 samples were collected and processed similarly to postflight (R + 0/30) samples. Differences between the L-45 and L-10 collections were confirmed by additional preliminary statistical tests (data not shown), and to avoid the consideration of confounding effects due to sample processing/storage instead of the variable of interest—space flight—the L-45 samples were compared to the R + 0/30 samples and the L-10 samples were compared to the FD samples.

NMR sample preparation

After arrival at the University of Lethbridge, all effort was made to continue to minimize freeze–thaw cycles of the serum samples. Once thawed, 200 µL of each serum sample was pipetted into microcentrifuge tubes, added to 360 µL saline buffer, and pipetted into Amicon Ultra 3 kDa filters pre-rinsed with d2H2O ten times to remove residual glycerol. The buffer was prepared by combining dH2O (Milli-Q water), 0.9% w/v sodium chloride.
Fig. 1 Multivariate Bayesian time-series analyses for the top four most significant metabolites in the comparison pre-launch to return to Earth to recovery (L-45 to R + 0 to R + 30) for males (left) as ranked by Hotelling’s $T^2$ values of 11.20, 11.12, 7.66, 6.19 for A, C, E, G, respectively, and the corresponding bins in females (right). The Hotelling’s $T^2$ values corresponding to the only comparative female bin that was significant is 4.39 for D. The y-axis corresponds to the labelled time points. The y-axis corresponds to metabolite abundance relative to all subjects across all time points. Note that 3-hydroxyisooavanic acid, ethanol and propylene glycol in males showed a reduction in relative abundance from pre-flight to return to Earth, and then an increase from return to Earth to recovery 30 days later—whereas lactic acid revealed the opposite relationship. In females, lactic acid remained relatively constant from pre-flight to return to Earth to recovery.

and 0.05% w/v potassium formate and calibrated to a pH 7.4. Following this, 40 µL of D$_2$O with 0.02709% w/v 3-trimethylsilyltetredeuteriopropionate (TSP) was added. Filters and samples were then centrifuged at 12,000 rpm for 5 min at 4 °C. Following centrifugation, 550 µL of the supernatants were pipetted into 5 mm glass NMR tubes and scanned by NMR immediately. After data acquisition, each sample was pipetted back into appropriately labelled centrifuge tubes and stored at −80 °C [27].

### NMR data acquisition and processing

$^1$H-NMR spectra were obtained using a 700 MHz Bruker Avance III HD NMR Spectrometer equipped with a 5 mm triple resonance TBO_Z probe. The one-dimensional spectra were acquired at 22 °C using the NOESY gradient pulse-water pre-saturation pulse sequence (noesygppr1d) with the following parameters: mixing time of 10 ms 128k data points (TD); sweet width (SW) of 20.52 ppm, acquisition time (AQ) of 4.56 s, transmitter offset (o1p) of 4.6 ppm; recycle delay (D1) of 1 s: 128 scans (NS). Spectra were then processed using zero-filling to 256 k points and line broadening with a 0.3 Hz exponential multiplication. The water pre-saturation offset (o1p) and the 90-degree pulse were determined prior to data acquisition on each sample. Once the spectra had been collected, TopSpin was used for manual phase shifting, baseline correction, and chemical shift was referenced relative to TSP as an internal standard (0.00 ppm). Data were then imported into MATLAB (MathWorks) in an ascii format where groups of comparisons were created. The data were normalized to the total area of all bins, log-transformed and Pareto-scaled [28]. MATLAB was used for alignment of the spectra and Dynamic Adaptive Binning [29] followed by manual adjustment to correct for errors. These data processing steps yielded a total of 173 and 140 bins for the male and female samples, respectively.

### Statistical analysis

All data bins were subjected to both supervised and unsupervised multivariate tests after ensuring normality. For the unsupervised models, Principal Component Analysis (PCA) was used to identify class differences (not shown) [30]. For the supervised multivariate models, Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) and Partial Least Squares Discriminant Analysis (PLS-DA) (not shown), which both rely on class membership to visualize separation [30], as well as Variable Importance Analysis based on random Variable Combinations (VIAVC) [31] were undertaken. VIAVC is an algorithm which performs cross-validations to select the most significant bins to be used for comparisons between discrete groups. Data were visualized using variables determined to be significant by either the paired $t$-test ($p < 0.05$) [32] or VIAVC Best Subset testing. All univariate tests carried out were Bonferroni–Holm corrected for multiple comparisons using the total number of variables being analyzed. The data were subjected to multivariate empirical Bayesian time-series analysis (MEBA) [33, 34] to visualize metabolite perturbations over time using MetaboAnalyst 4.0 [35]. These were conducted using three groupings of the data sets, including the L-10 to all FDs, all the FDs to each other (not shown), and L-45 to R + 0 to R + 30 because of the sample processing differences.

### Metabolite identification and pathway topology

Chenomx NMR Suite 8.5 [36] and the Human Metabolome Database (HMDB) [27, 37–40] were used for metabolite identification of the bins determined to be significant by both paired $t$-test and VIAVC best subset testing ($p < 0.05$). The complete list of metabolites identified as significantly altered in any of the time-point comparisons was used for pathway topology analysis. This was accomplished using Metabo-Analyt 4.0 [41, 42] by selecting the hypergeometric test as the enrichment method and for over-representation analysis, the relative-betweenness centrality for the topology analysis, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database for *Homo sapiens* as the pathway library [37, 43, 44].

### Results

#### Multivariate Bayesian time-series analysis (MEBA) revealed characteristic sex-specific profiles

Of the 173 bins created for males, and the 140 bins created for females, Fig. 1 shows the metabolites corresponding to the top four most-significant bins by MEBA analysis, ranked by Hotelling’s $T^2$ values for males (left) and
the corresponding bins (metabolite) in females (right) in the comparison of pre-flight to return to Earth to recovery (L-45 to R + 0 to R + 30). The Hotelling’s $T^2$ values associated with these figures are quantitative measures of the correlation of one subject’s metabolite (in the bin) relative to the other subjects’ metabolite fluctuations over time. Thus, a low Hotelling’s $T^2$ value indicates low correlation and vice versa [33]. 3-hydroxyisovaric acid (Fig. 1A), ethanol (Fig. 1E), and propylene glycol (Fig. 1G) in males showed a reduction in relative abundance from L-45 to R + 0, and then an increase from R + 0 to R + 30. Lactic acid (Fig. 1C) in males revealed the opposite relationship, increasing from L-45 to R + 0 and decreasing from R + 0 to R + 30. Of the top four metabolites for males, only lactic acid was significant in females and appears to remain relatively constant in abundance across the time-series depicted. The trend for lactic acid in females is visualized better in Fig. 2C due to the higher regulation of the bin.

In females the metabolites corresponding to the top four bins determined to be significant by MEBA analysis for L-45 to R + 0 to R + 30 are shown in Fig. 2 (left) along with the corresponding metabolites (bins) in males (right). Pyruvate appears to decrease in relative abundance from L-45 to R + 0, and then increase from R + 0 to R + 30 in females (Fig. 2A), while in males (Fig. 2B) the trends are not discernable due to the rather low correlation between subjects. Lactic acid and glucose appear to have the opposite trend to pyruvate in females (Fig. 2C, E)—increasing in relative abundance from L-45 to R + 0 and then decreasing again from R + 0 to R + 30. In males, lactic acid appears to have the opposite trend to females (Fig. 2D) and, again, the trend in glucose (Fig. 2F) is not explicitly discernable due to the low Hotelling’s $T^2$ value. Valine in females appears to remain constant across the depicted time-series (Fig. 2G) and was not significantly altered in males (Fig. 2H).

The metabolites corresponding to the top four bins, as determined by MEBA, for the comparison pre-flight to all inflight days (L-10 to FD15 to FD30 etc.) in both males and females are included in Supplemental Figure 1 and show no distinct trends over the time series.

Orthogonal partial least squares-discriminant analysis (OPLS-DA) showed a characteristic time course of metabolic profiles during spaceflight

Given that the most dramatic metabolite abundance trends were observed in entry into and exit out of flight, univariate (paired $t$-test) and multivariate (VIAVC) analysis were used to compare the associated time points. Significant bins ($p < 0.05$) were combined and visualized using OPLS-DA scores plots where each plotted point represents a subject’s serum sample collected at the indicated time points. For the entry into space time points (L-10 vs. FD15), OPLS-DA scores plots show the within- and between-group separation for males (Fig. 3A) and females (Fig. 3B). The males (40/173 bins, $p < 0.005$) showed little separation between the serum metabolomes between these time points in addition to some misclassification error. By contrast, the females (7/140 bins, $p < 0.050$) for these time-points showed more group separation and little misclassification (Fig. 2B). Quantitative measures for the variance described by the model and the fit were reflected by $R^2_Y = 0.311$ and $Q^2 = 0.15$ in males, and $R^2_Y = 0.738$ and $Q^2 = 0.567$ in females, both respectively. Note that all discrete comparisons were carried out (L-10 to FD15; L-10 to FD30; L-10 to FD60; L-10 to FD120; L-10 to FD180); however the L-10 to FD30; L-10 to FD60; L-10 to FD120; and L-10 to FD180 for both sexes all had $Q^2$ values less than 0.5—indicating poor quality of the supervised multivariate model—and were excluded for this reason. The male L-10 to FD15 comparison also had a $Q^2$ less than 0.5 but is shown for sex comparison purposes.

The OPLS-DA scores plot for L-45 versus R + 0 time-point samples of males (27/173 bins, $p < 0.05$; Fig. 4A) indicated that between-group separation was poor with several misclassifications but depict tight within-group clustering. Model fit and variance in males were $Q^2 = 0.115$ and $R^2_Y = 0.409$, respectively. Comparisons for L-45 versus R + 30 and R + 0 versus R + 30 in males are not shown as they failed permutation testing. OPLS-DA models for the female time course are also shown in Fig. 4, with L-45 versus R + 0 (20/140 bins, $p = 0.074$; Fig. 4B), L-45 versus R + 30 (14/140 bins, $p < 0.05$; Fig. 4C), and R + 0 versus R + 30 (44/140 bins, $p < 0.05$; Fig. 4D). Figure 4 illustrates relatively clear separation between females for L-45 versus R + 0 and for R + 0 versus R + 30, but less group separation for L-45 versus R + 30. The measures for variance and model fit, respectively, were $R^2_Y = 0.458$ and $Q^2 = 0.12$ (Fig. 4B), $R^2_Y = 0.482$ and $Q^2 = 0.302$ (Fig. 4C), and $R^2_Y = 0.630$ and $Q^2 = 0.207$ (Fig. 4D).

Significantly altered metabolites indicate discrete metabolic changes over time

Significantly altered metabolites were identified across all time-points for both males (Supplemental Table 1) and females (Supplemental Table 2) by both univariate paired $t$-test and multivariate VIAVC Best Subset tests ($p < 0.05$). The time-point comparisons, respective to the sample processing differences, revealed significant changes for 10 days pre-launch to inflight days 15, 30, 60, 120, and 180 (L-10 to FD15, 30, 60, 120, 180); 45 days pre-launch to both return to Earth and recovery (L-45 to both R + 0 and R + 30) as well as return to Earth to recovery (R + 0 to R + 30). Each comparison also indicates up- or down-regulation between the two time-points where negative
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Fig. 2 Multivariate Bayesian time-series analyses for the top four most significant metabolites in the comparison pre-launch to return to Earth to recovery (L-45 to R + 0 to R + 30) for females (left) as ranked by Hotelling’s $T^2$ values of 11.30, 9.86, 9.14, and 8.33 for A, C, E, G, respectively and the corresponding bins in males (right). The Hotelling’s $T^2$ values corresponding to the comparative male bins that were significant are 0.2003, 10.34, and 2.310 for B, D, F, respectively. The $x$-axis corresponds to the labelled time points. The $y$-axis corresponds to metabolite abundance relative to all subjects across all time points. Note that pyruvate appears to decrease in relative abundance from pre-flight to return to Earth, and then an increase from return to Earth to recovery 30 days later in females, while lactic acid and glucose appear to have the opposite trend. In males, lactic acid appears to have the opposite trend to females. Valine in females appears to remain constant across this time-series.
regulations correspond to down-regulated metabolites and positive regulations correspond to up-regulated metabolites.

Based on these discrete metabolic changes seen for both males and females in response to space travel, bar graphs were generated to visualize the differences between sexes. Figure 5 depicts the metabolites significantly altered \( (p < 0.05) \) in the time-point comparisons L-10 to FD15 (A), L-45 to R + 0 (B), L-45 to R + 30 (C), and R + 0 to R + 30 (D) between males and females—corresponding to the most descriptive comparisons by our analysis (Refer to the Multivariate Bayesian Time-Series Analysis (MEBA)... and Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA)... sections). The other discrete comparisons assessed (L-10 to FD30, L-10 to FD60, L-10 to FD120, and L-10 to FD180) are shown in Supplemental Figures 2A-2D.

Comparisons between the metabolic effects seen in males and females revealed that while there are more metabolites significantly altered in males for the time-point comparisons in Fig. 5, the females had more drastic changes in the metabolites identified to be affected as evidenced by the larger (in magnitude) regulations seen in females. As well, metabolites affected in females were mostly down-regulated between the time-points assessed in comparison to males—which generally were mostly upregulated. Moreover, many of the metabolites were similar across all time point comparisons evidenced by repeats of metabolites for each figure—similarly seen in Supplemental Figure 2. Thus, metabolites affected were relatively consistent throughout the time-point comparisons while their regulations are what differed.

Lastly, many metabolites were uniquely affected in both males and females. In females these were 7-methyladenine (L-45 to R + 0), aspartylphenylalanine (R + 0 to R + 30), threonine (L-45 to R + 30, and R + 0 to R + 30), and valine (L-10 to FD15); in males these were paraxanthine (L-10 to all FDs), 2-isopropylmalic acid (L-10 to FD120 and L-45 to R + 30), 3-hydroxyisovaleric acid (R + 0 to R + 30), 3-hydroxyacetoneglutaric acid (L-10 to FD30 and R + 0 to R + 30), 3-methyl-2-oxovaleric acid (L-45 to R + 0), acetylphosphate (L-45 to R + 30 and R + 0 to R + 30), alanine (L-45 to R + 0, L-10 to FD30 and R + 0 to R + 30), creatine (L-10 to FD60, L-45 to R + 0, and R + 0 to R + 30), ethanol (L-45 to R + 0, L-45 to R + 30, R + 0 to R + 30, L-10 to FD15, 30 and 180), formic acid (L-10 to FD60 and 120), isoleucine (L-10 to FD30, 60, and 120), and propylene glycol (L-10 to FD15, 120, and 180, and R + 0 to R + 30).

**Pathway analysis displayed temporal patterns of metabolic functions**

Based on the significantly altered metabolites in both sexes across the time-points, pathway topology analysis was performed to identify metabolic pathways that were perturbed by spaceflight. Significantly perturbed pathways up to \( p < 0.05 \) across all comparisons were identified for males (Supplemental Table 3) and females (Supplemental Table 4).

Pathways that were commonly perturbed in both males and females included glycolysis and gluconeogenesis; caffeine metabolism; glyoxylate and dicarboxylate metabolism; pyruvate metabolism; alanine, aspartate, and glutamate
metabolism; and the branched chain amino acids (BCAA) valine, leucine, and isoleucine biosynthesis. Pathways that were uniquely affected in males included the branched chain amino acid degradation and aminoacyl-tRNA biosynthesis seen both in L-10 to FD30 and FD60. Pathways uniquely affected in females included the pantothenate and CoA biosynthesis seen in L-10 to FD15; the tricarboxylate cycle (TCA) in L-10 to FD60; glycerolipid metabolism in L-10 to FD120 and FD180, as well as R + 0 to R + 30; glycine, serine, and threonine biosynthesis in L-10 to FD60 and L-45 to R + 30; cysteine and methionine metabolism in L-10 to FD60; as well as arginine and proline metabolism in L-10 to FD60.

With respect to the pathways that were commonly affected across the time-points in males (Supplemental Table 3), glycolysis and gluconeogenesis were the top one or two most significantly altered pathways common to every time-point comparison. Moreover, BCAA biosynthesis alteration was common to L-10 to all FDs through to FD120, as well as L-45 to R + 30; pyruvate metabolism was commonly perturbed in the L-10 to FD60, L-10 to FD120, and R + 0 to R + 30 comparisons; aminoacyl t-RNA for both L-10 to FD30 and FD60; alanine, aspartate, and glutamate metabolism in both L-10 to FD30 and R + 0 to R + 30; glyoxylate and dicarboxylate metabolism in both L-10 to FD60 and FD120; caffeine metabolism was uniquely altered between...
Discussion

Spaceflight involves major physical, physiological, and psychological stressors due to entry and exit from space, the in-flight environment, and the recovery period after return to Earth [21, 45]. Previous studies have shown that sex alone influences the baseline metabolome of individuals including when in healthy status without any other external influence [46]. Using univariate, multivariate and machine learning analyses in a ¹H NMR metabolomics-based approach, the present study investigates the notion that sex influences the metabolome of individuals in response to external factors (such as a spaceflight) as well as the general effects of spaceflight on humans through the use of blood from NASA astronauts.

Metabolic changes in serum linked to entry into space

Some of the most affected pathways in both males and females pointed to changes in glycolysis and gluconeogenesis for the discrete comparison of L-10 to FD15, mainly by downregulations of glucose, pyruvate, and ethanol in males (Supplemental Table 1) and glucose, lactate, and pyruvate in females (Supplemental Table 2). Glycolysis functions to catalyze glucose by converting it into lactate, pyruvate, and energy in the form of ATP [45, 47, 48]. Conversely, gluconeogenesis is an anabolic pathway generating glucose whilst also disposing of lactate produced by tissues undergoing glycolysis—particularly in erythrocytes and muscle tissue during exertion [43, 44, 46–52]. As a result, the downregulation of glucose and, consequently, lactate, ethanol, and pyruvate seen in both males and females, may reflect altered energy metabolism [43, 44, 49]. Perturbation in energy production is known to occur for space travellers due to mitochondrial stress and can affect cognitive performance [12, 48, 53]. Our study also suggests that additional energy metabolism pathways are affected in females by entry into space including pyruvate metabolism as well as pantothenate and coenzyme A biosynthesis—indicating that this change may represent a sex-specific indicator for longitudinal assessment.

With respect to pyruvate metabolism, pyruvate is ultimately metabolized in the cytoplasm by tricarboxylic acid (TCA) cycle processes that transport metabolites into mitochondria for ATP production [54]. Thus, disruptions in pyruvate metabolism due to the downregulation of pyruvate and lactate affect tissues with high demand for carbohydrate-metabolism based ATP generation, such as the nervous system and muscle tissue [54]. Perturbations in this pathway may lead to higher mitochondrial reactive oxygen species (ROS) susceptibility and elevated risk of cancers, heart failure, and muscle atrophy [54]. Moreover, pantothenate is the primary pre-cursor for the biosynthesis of CoA which is involved in the TCA cycle amongst other metabolic processes, hence also contributing to ATP production [55–58]. Perturbations in the pantothenate and CoA biosynthesis pathway, due to the upregulation of pantothenate’s precursor valine, may result in ATP production dysfunction [43, 44, 49, 55–59].
Williams et al. [48] have discussed progressive or merely persistent bone demineralization as another major immediate effect of space travel [48, 60]. Various pathways contribute to bone mineralization maintenance, including glycolysis/gluconeogenesis and fatty acid metabolism [61]. Fatty acid metabolism, which is interrelated with pyruvate metabolism, glycolysis, and pantotenate and CoA metabolism, is involved in bone cell differentiation [51, 61]; thus, because of their roles in energy metabolism, bone demineralization, and muscle function, the suggested perturbations in gluconeogenesis, pyruvate metabolism, and pantotenate and CoA biosynthesis, are arguably interrelated both biochemically and functionally [43, 44, 49].

The presented results reflect the expected significant physiological consequences of entry-into spaceflight especially considering its impact on the homeostasis of biofluids (such as blood) which are re-distributed in the body following entry into space [1, 48, 62–64]. Despite this, the MEBA plots show little discrepancy between pre-launch to entry-into space for males and females; however, this model is not necessarily designed to assess discrete comparisons. Hence, OPLS-DA models can be assessed which show better between-group separation for the female metabolomes relative to males—suggesting more drastic changes in the female astronauts’ metabolomes upon entry into space. This suggested effect was quantitatively corroborated by the generally larger regulations (in magnitude) of significantly altered metabolites in females for this time-point comparison.

### Metabolic changes in serum caused by prolonged presence in space

Longitudinal metabolite perturbations from L-10 to FD30 through to FD180 did not reveal clear trends, suggesting that serum metabolomes fluctuated irregularly but not in specific patterns. To validate this observation, a new comparison was run consisting of FD30 versus FD180 samples in an attempt to see non-ambiguous differences between the in-flight (FD) time points. This comparison, however, failed permutation testing in both males and females, hence, validating the observation that the blood metabolome does not provide substantial insight into metabolic changes occurring throughout flight relative to pre-flight. It is possible that the previously described difference in processing of samples may have obscured the ability to observe in-flight metabolic changes more explicitly and is a limitation of the present study. Despite this lack of observable longitudinal patterns, the data clearly suggest glycolysis/gluconeogenesis is the main pathway affected by spaceflight in both males and females. In line with the literature [65–67] energy metabolism is suggested to be continuously affected throughout space travel due to resultant increases in core body temperature (CBT)
or immune responses, for example. Other pathways in males and females propose a role for alanine, aspartate, and glutamate metabolism and glyoxylate and dicarboxylate metabolism in the body’s response to space travel [43, 49, 50, 65, 68], which are also involved in protein synthesis, pathogenesis, and cancer mitigation [68–71]. As well, the appearance of caffeine metabolism, which is interrelated with glyoxylate and dicarboxylate metabolism, is likely due to diet [70–72], especially given the food system used during flight is different than pre- and postflight.

Both BCAA degradation and aminoacyl tRNA biosynthesis were perturbed only in males in the L-10 to FD30 and 60 comparisons. Aminoacyl tRNA biosynthesis involves the biosynthesis of the substrates for translation and, therefore, plays a critical role in protein synthesis [73]. Leucine, alanine, and, to a lesser extent, the other BCAAs, are known to stimulate both hepatic and muscle-tissue protein synthesis [74, 75]. Thus, the perturbations of leucine, isoleucine, and alanine in these pathways may contribute to the altered prevalence of these amino acids in polypeptide primary structure and affect both protein synthesis and muscle function.

A prevalent effect of space flight on humans is skeletal muscle atrophy/fatigue due to loss of mass and utility, which involves BCAA functions [48]. As mammals are unable to synthesize the BCAAs valine, leucine, and isoleucine, they must be supplied exogenously [70, 76, 77]. BCAA degradation, however, which was altered in male astronauts, occurs in skeletal muscle unlike most amino acid metabolic mechanisms—which involve hepatic catabolism [78]. BCAAs serve as substrates for muscle protein synthesis, are involved in energy production, and modulate glucose metabolism [51, 79–82]. Thus, dysregulation in this catabolic pathway can lead to skeletal muscle protein synthesis perturbations, energy production dysregulation, and problems with maintenance of skeletal muscle due to energy requirements for muscle exertion—especially during space travel [79, 80, 83, 84].

With respect to female-specific changes, the tricarboxylic acid (TCA) cycle; glycerolipid metabolism; glycine, serine, and threonine metabolism; cysteine and methionine metabolism; and arginine and proline metabolism were suggested to be potentially affected throughout spaceflight. All these pathways’ functions are interrelated by various physiological requirements [43, 44, 49]. The interrelated pathways can be grouped into triplets unified by a physiological responsibility. First, the TCA cycle, cysteine, and methionine metabolism, and glycerolipid metabolism are involved in energy metabolism/regulation [43, 44, 49, 85–89]. Second, the TCA cycle, glycine, serine, and threonine metabolism, arginine, and proline metabolism, and glycerolipid metabolism are involved in immunity [75, 85, 86, 90–92]. Third, the TCA cycle, glycine, serine, and threonine metabolism, arginine and proline metabolism, and cysteine and methionine metabolism are involved in protein and other macromolecule synthesis and maintenance [43, 44, 49, 61, 75, 87–91, 93–98]. Lastly, the TCA cycle, arginine and proline metabolism, and cysteine and methionine metabolism are involved in epigenetic modification of DNA resulting in the epigenetic modification of DNA regulation [85, 92, 99].

The interrelated pathways can also be grouped into pairs unified by physiological responsibility as the TCA cycle, through ROS production regulation, as well as arginine and proline metabolism are involved in physiological stress resilience [75, 98, 100, 101]. Our analysis also reveals unique effects of pathways suggested to be perturbed in a few cases which are supported effects of space travel. As such, unique to glycerolipid metabolism is its role in body temperature regulation by regulating lipolysis [89, 102]. Additionally, unique to the TCA cycle in the present work is its role in bone mineral maintenance [85, 86]. Thus, the alterations in valine, glucose, lactate, pyruvate, glycerol, and leucine in females throughout flight can lead to physiological effects that are well supported by the literature and the observed effects in relation to space travel, as well as with respect to the sex differences suggested are supported [100, 103]. The vast interrelatedness of the pathways and metabolites suggested to be perturbed through spaceflight by the present study may simplify countermeasures undertaken to mitigate adverse effects on astronauts.

Sex-specific metabolic adaptations to prolonged life in space and re-entry to Earth

It has been shown that the physical and psychological changes acquired through long-duration space travel persist well into an astronaut’s recovery period after returning to Earth. Several studies [104–106] have documented that the physiological recovery process following return to Earth the physiological recovery process is often longer than the time spent in space. This particularly applies to re-building muscle tissue, bone remineralization, and immune regulation amongst other physiological processes as observed in both human and animal cohorts following space flight [61, 62, 104, 105, 107–110].

The OPLS-DA models in females show more separation between L-45 and R + 0—suggesting that the females’ metabolomes are more different between these time-points than males. This observation is corroborated by the metabolic processes significantly perturbed in all three of the comparisons. The well-documented relationship between stress and physical health may explain in part the more drastic metabolic responses in females relative to their male counterparts in these comparisons [100, 103, 111, 112]. Moreover, the effects of space travel persist following a 30-day recovery period on Earth as indicated by persistent changes in many of the metabolites and pathways for both
sexes. For example, glycolysis and gluconeogenesis and other pathways involved in bone mineral homeostasis are affected in the L-45 to R + 30 comparison, suggesting that bone demineralization may still occur a month after returning to Earth, as has been observed [113]. This observation highlights the importance of countermeasures to offset the prolonged effects of long-duration space flight, which can result in life-long adverse health outcomes [61, 62, 106, 108, 109].

The OPLS-DA models suggest that this general persistence in the alterations caused by long-duration space travel beyond return to earth is more prevalent for females. It has been suggested that the extreme physiological and psychological stress of re-entry to the Earth’s environment may dysregulate immune functions [114]. The downregulation of threonine, unique to the comparisons pre-launch/recovery and return to Earth/recovery time-points in females, suggests reduced maintenance of intestinal mucosal integrity which may weaken immune function [115]. Alternatively, down-regulation of threonine may also occur as a consequence of an increased utilization of dietary threonine for intestinal mucosal protein synthesis [115]. Immune dysregulation following return to Earth was also observed in previous cosmonaut missions [114–118] and suggested to be facilitated by altered pathogenicity of bacteria and biofluid redistribution [48].

Limitations

The present data suggest that blood serum in both males and females, specifically with respect to NMR-identifiable metabolites, may be better suited for providing information about metabolite changes for entry-in and exit-out of space rather than throughout flight. Limitations of the present study include the limited cohort size, particularly for females and shortcomings with regards to the approach. Also, landing day (R + 0) samples were not fasted, which could have affected the postflight analyses. Given these were secondary analyses of existing samples, follow-on studies with pristine sample collections for metabolomic analyses are warranted. Psychogios et al. [27] reported that a combination of gas chromatography-mass spectrometry (GC–MS) and liquid chromatography mass spectrometry (LC–MS) may provide the most complete blood serum analysis [119]. Thus, the volume of metabolomics data acquired through this study could be, in part, attributed to the scope of 1H-NMR identifiable metabolites. However, this study ultimately showed that NMR-based metabolomics is appropriate for distinguishing the effects of long-duration space travel on the serum metabolome.

It is possible that the observed metabolic changes reflect up-stream changes in gene expression, epigenetic regulation, and secondary metabolic processes linked to physiological and psychological stress [12, 120, 121]. Thus, future studies may consider analysis of other biofluids and use of other ‘omics’ approaches and technologies to yield more information about the effects of long–duration space travel. Future studies should also aim to assess the longitudinal profile of space travel on astronauts by avoiding the sample processing differences reported herein. Further analyses of these data are warranted, including examination of effects as related to body mass and composition, diet, exercise, menstrual cycle, and other factors.

Conclusions

Nutrition and metabolism are key elements in human health; stress and environmental factors can impinge upon these, increasing health risks. A comprehensive understanding of the metabolic and metabolomic response to space flight will be required to enable the continued exploration beyond our planet. The present findings identify key metabolic pathways affected by spaceflight that may reflect the metabolic response to prolonged stress and/or adaptation to stress. The metabolites and potential pathways perturbed by long-duration space travel overlapped across time points and sexes, and most of the affected metabolites and pathways have roles in energy metabolism, bone-mineral and muscle regulation, immunity, and macromolecule synthesis and maintenance. Additionally, sex differences in the astronauts’ metabolomes suggest differential metabolic responses—especially during the postflight recovery period with females potentially requiring longer readjustment phases than males. As such, the present data may inform the development of countermeasures to mitigate the adverse health consequences of prolonged spaceflight. Such efforts may be aided by the overlap in effects seen.

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Author contributions SZ, SS, MH, TM, and GM designed the study. SZ and SS assisted with sample collection and coordinated data analysis. JS, MG, and TM prepared the samples for 1H NMR and analysed the data. JS, MG, SS, TM, and GM contributed to writing the
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**Availability of data and material** Data are available upon request from the corresponding authors.

**Declarations**

**Conflict of interest** None of the authors declare any competing interests.

**Ethics approval and consent to participate** Participants provided written informed consent prior to sample collection, as well as for the extended analyses reported herein. Approval for this study was granted by Human Participant Research Committee at the University of Lethbridge (# 2021-107), the National Aeronautics and Space Administration (NASA) Johnson Space Center Institutional Review Board (# 0225, 0326, 0797), the Japanese Aerospace Exploration Agency, and the European Space Agency Medical Boards.

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