

1 **Title**

2 RNAseq analysis of rodent spaceflight experiments is confounded by sample collection
3 techniques

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20 **Summary**

21 To understand the physiological changes that occur in response to spaceflight, mice are
22 transported to the International Space Station (ISS) and housed for variable periods of
23 time before euthanasia on-orbit or return to Earth. Sample collection under such difficult
24 conditions introduces confounding factors that need to be identified and addressed. We
25 found large changes in the transcriptome of mouse tissues dissected and preserved on-
26 orbit compared to tissues from mice euthanized on-orbit, preserved, and dissected after
27 return to Earth. Changes due to preservation method eclipsed those between flight and
28 ground samples making it difficult to identify spaceflight-specific changes. Follow-on
29 experiments to interrogate the roles of euthanasia methods, tissue and carcass
30 preservation protocols, and library preparation methods suggested that differences due
31 to preservation protocols are exacerbated when coupled with polyA selection. This has
32 important implications for the interpretation of existing datasets and the design of future
33 experiments.

34 **Keywords**

35 Tissue preservation, RNAseq, Spaceflight, Mouse, Euthanasia, Liver, Muscle, Rodent
36 Research, ISS, RNAlater, Quadricep, NASA

37 **Introduction**

38 Spaceflight places multiple stresses upon the human body including altered gravity
39 fields and exposure to cosmic radiation, which lead to health risks for spacefaring
40 humans (Institute of Medicine, 2008). Decades of research on astronauts has begun to
41 reveal how humans respond to the spaceflight environment (Garrett-Bakelman et al.,
42 2019) but physiological monitoring of astronauts is still limited. Thus rodent models have
43 been essential for advancing our understanding of how mammals — including humans
44 — respond to spaceflight. This includes the impact of spaceflight on muscle structure
45 (Shen et al., 2017; Spatz et al., 2017; Tascher et al., 2017), liver (Beheshti et al., 2019;
46 Jonscher et al., 2016) and immune functions (Pecaut et al., 2017; Rettig et al., 2017;
47 Ward et al., 2018).

48 Despite success of the rodent model, sample collection under such difficult conditions
49 introduces confounding factors that need to be identified and addressed. These are
50 related to hardware limitations, small sample size, and severe restraints on astronaut
51 crew availability. Successful experiments must work within these constraints to produce
52 meaningful insights. In response, the first Rodent Research (RR) mission established
53 new capabilities for conducting reliable long-duration experiments using rodents with
54 on-orbit sample collection. Animals can either be euthanized onboard the ISS by or
55 returned to Earth alive. Both approaches introduce confounding factors. The former is
56 experimentally challenging but preserves the sample during exposure to microgravity,
57 while the latter exposes the animal to re-entry stresses and sampling occurs only after a

58 variable lag between landing and euthanasia — essentially sampling re-adaptation to
59 Earth conditions in addition to the response to spaceflight. Inconsistent handling of
60 samples necessitates a clear understanding of how dissection and preservation
61 protocols affect downstream data generation.

62 We previously showed, using transcriptomic, proteomic and immunohistochemical data
63 from the Rodent Research-1 (RR-1), Rodent Research-3 (RR-3) and Space
64 Transportation System (STS)-135 missions, that lipotoxic pathways are activated in
65 rodent liver in two different strains of mice that were flown for as long as 42 days in
66 space (Beheshti et al., 2019). Because animals in the RR-1 and RR-3 experiments were
67 euthanized in space, this work suggested that space alone was the most likely cause for
68 similar changes previously observed in liver samples from mice flown during the STS-
69 135 experiments where animals returned live to Earth (Jonscher et al., 2016). The
70 lipotoxic effect is stronger with duration and may have ramifications for astronauts'
71 health during long missions. This analysis did not include two flight and two ground
72 animals from RR-1 as these animals were dissected immediately after euthanasia on-
73 orbit as opposed to the rest which were first returned to Earth as intact frozen carcasses
74 for later dissection.

75 Here we now compare RNA-sequencing (RNAseq) datasets generated from livers
76 preserved using these distinct protocols. We find large changes in the transcriptome of
77 tissues dissected and preserved on-orbit compared to tissues from mice euthanized on-
78 orbit, preserved intact by freezing on-orbit, and dissected after return to Earth. To

79 identify and mediate how the preservation method could have such a large effect on
80 differential gene expression (DGE) results, we performed follow-on experiments to
81 interrogate the role of euthanasia methods, tissue and carcass preservation protocols,
82 and library preparation methods on DGE changes. Our findings have important
83 implications for interpreting existing datasets and the design of future experiments.

84 **Results**

85 ***Preservation method is the primary driver of gene expression variance in RR-1*** 86 ***liver samples***

87 To assess gene expression differences in liver samples from the RR-1 NASA Validation
88 mission (Cadena et al., 2019; Globus and Galazka, 2015; Ronca et al., 2019), RNA was
89 extracted from livers dissected from spaceflight (FLT) and ground control (GC) animals
90 either immediately after euthanasia (immediate preservation, I), or from frozen
91 carcasses after partial thawing (carcass preservation, C), and sequenced following
92 polyA selection. Principal component analysis (PCA) revealed preservation method (C
93 vs. I) as the primary driver of variance among samples rather than spaceflight (FLT vs.
94 GC) (Figure 1A). Furthermore, there was an order of magnitude difference in the
95 number of differentially expressed genes (DEGs) identified in FLT versus GC carcass
96 samples than was observed in FLT versus GC immediate samples, and only 4 DEGs
97 overlapped between the two preservation methods (Figure 1B). Gene set enrichment
98 analysis of FLT versus GC C- (Figure 2A) and I-derived (Figure 2B) samples showed no

99 overlap in enriched gene ontology (GO) terms (Figure 2C), showing that any gene
100 expression changes in the liver as a result of spaceflight exposure were confounded by
101 the sample preservation method used.

102 Since livers from only 2 FLT and 2 GC animals in the RR-1 NASA Validation mission
103 were preserved via the immediate method, RNA from livers prepared via the immediate
104 method from two additional studies, the RR-1 CASIS mission (Cadena et al., 2019;
105 Globus et al., 2015; Ronca et al., 2019) and a ground-based preservation and storage
106 study (Choi et al., 2016; GeneLab, 2016) were also sequenced following polyA
107 selection. Despite multiple different experimental factors in RR-1 NASA, RR-1 CASIS,
108 and the ground-based preservation studies, PCA continued to show preservation
109 method as the primary driver of variance among samples in these datasets (Figure S1).

110 ***Carcass-preserved samples exhibit less uniform transcript coverage than***
111 ***immediate-preserved samples***

112 To further investigate the observed differences in preservation method, RR-1 NASA
113 FLT and GC liver samples derived from the carcass preservation method were grouped
114 together (all-C) and FLT and GC liver samples derived from the immediate preservation
115 method were grouped together (all-I). DGE was evaluated in all-C vs. all-I samples.
116 Many more genes were differentially expressed in all-C vs. all-I samples than in either
117 FLT-C vs. GC-C or in FLT-I vs. GC-I samples, further supporting preservation method
118 as the primary driver of variance in RR-1 NASA liver samples (Figure 3A). Gene set
119 enrichment analysis revealed that several of the gene ontologies enriched in carcass

120 samples (when compared with immediate samples) involved RNA regulation and
121 processing (Figure 3B). Despite similarly high RNA Integrity Number (RIN) values
122 (Figure S2), carcass samples exhibited significantly less 5' gene body coverage than
123 immediate samples (Figure 3C&D).

124 ***Expression of genes involved in 5'-methylguanosine decapping and polyA***
125 ***removal is affected by preservation condition***

126 Given the differences in gene body coverage between carcass and immediate samples,
127 we evaluated the expression of 5'-methylguanosine decapping and polyA removal
128 genes in these groups from the RNAseq data. In mammals, eight genes have
129 decapping activity in vitro and/or in cells: Dcp2 (Nudt20), Nudt3, Nudt16, Nudt2, Nudt12,
130 Nudt15, Nudt17, Nudt19. In addition, Dxo acts on partially capped mRNA's (Grudzien-
131 Nogalska and Kiledjian, 2017). Two of these genes — Dxo and Nudt2 — were
132 significantly more expressed in the carcass samples, while another two — Nudt15 and
133 Dcp2 (Nudt20) — were significantly more expressed in immediate samples (Figure S3).
134 Removal of polyA tails from mRNA is catalyzed by two complexes. The first, CCR4-
135 NOT, consists of CNOT1, CNOT2, CNOT3, CNOT4, CNOT9, CNOT10, CNOT6,
136 CNOT6L, CNOT7, and CNOT8. The second, PAN2-PAN3, consists of PAN2 and PAN3
137 (Siwaszek et al., 2014). In the case of the 10 subunit CCR4-NOT complex, we observed
138 5 genes that were more highly expressed in the immediate group (Cnot1, Cnot2, Cnot9,
139 Cnot6, Cnot6l) and 3 that were more highly expressed in the carcass group (Cnot3,

140 Cnot10, Cnot7) (Figure 3E). In the case of the PAN2-PAN3 complex, both Pan2 and
141 Pan3 were more highly expressed in the carcass group (Figure 3E).

142 ***Samples sequenced following ribodepletion exhibit more uniform transcript***
143 ***coverage than samples prepared with polyA selection***

144 The polyA selection library preparation method, which was initially used to evaluate
145 gene expression differences in RR-1 NASA Validation mission liver samples requires
146 intact RNA to minimize bias ([Kumar et al., 2017](#); [Petrova et al., 2017](#)). Since our data
147 suggest that the carcass samples were more degraded than the immediate samples
148 (Figure 3C&D), the total RNA isolated from the RR-1 carcass liver samples was used to
149 prepare libraries with the ribodepletion method to minimize transcript integrity bias, then
150 re-sequenced. PCA showed a more distinct separation of FLT and GC carcass samples
151 when the samples were prepared via the ribodepletion method (Figure 4A) than by
152 polyA selection (FLT-C and GC-C samples in Figure 1A). DEGs were identified in FLT
153 vs. GC carcass samples prepared with the ribodepletion method and compared with
154 those from poly-A prepared carcass samples. Although hundreds of DEGs in FLT vs.
155 GC carcass samples overlap between ribodepleted and polyA prepared samples, more
156 DEGs were identified in FLT vs. GC samples prepared with the ribodepletion method
157 (Figure 4B), suggesting this method may be more sensitive. There was no overlap of
158 enriched gene ontology terms in FLT vs. GC samples processed by ribodepletion and
159 polyA enrichment (Figure 4C&D).

160 Next, transcript integrity was evaluated in the ribodepletion-prepared FLT and GC
161 carcass samples and compared with polyA selection-prepared carcass samples.
162 Samples prepared with the polyA selection method exhibited less coverage of the 5'
163 portion of transcripts compared to ribodepletion-prepared samples (Figure 4E&F). Thus
164 ribodepletion was used to further investigate the effects of preservation method
165 (Carcass vs. Immediate) on gene-expression in a ground study.

166 ***Total RNA sequencing mitigates the impact of preservation method on gene***
167 ***expression changes in the liver***

168 We designed a ground-based tissue preservation study to determine the best approach
169 to mitigate the impact of preservation method on gene expression, and to identify other
170 confounding variables important for interpreting data from other RR missions. We tested
171 euthanasia and preservation techniques used in different RR missions and compared
172 them to standard laboratory protocols for tissue preservation. In addition to liver
173 samples, we also analyzed quadriceps to determine whether sample preservation
174 methods also confounded DGE analysis in this tissue.

175 Mice of the same age, sex, strain, and source as those used in the RR-1 NASA
176 Validation mission were used in the ground-based tissue preservation study. Mice were
177 evenly divided into one of 6 groups as shown in Figure S4. The mice in groups 1-4 were
178 euthanized with pentobarbital/phenytoin (Euthasol®) as in RR-1 (Choi et al., 2020), then
179 subjected to various preservation protocols to evaluate the phenomena observed in RR-
180 1 NASA carcass and immediate liver samples. Livers and quadriceps were dissected

181 immediately after euthanasia from mice in group 1. These tissues were divided into
182 thirds and preserved in one of three ways: 1) freezing in dry ice to mimic the cold block
183 that was used to freeze the immediate liver samples in the RR-1 NASA mission, 2)
184 submersion in LN2, or 3) with RNA*later*TM. After preservation, all tissues were stored at -
185 80 °C until further processing.

186 Although it is common practice to dissect mice immediately after euthanasia, due to
187 limitations in crew time for spaceflight experiments, immediate dissection is not always
188 possible. Thus, most tissues are preserved *in situ* within the carcass. We therefore
189 sought to determine the most effective way to preserve carcasses that would minimize
190 unintended gene expression changes in tissues preserved *in situ*. Mice in groups 2-4
191 were used to test three different carcass preservation methods: 1) slow freezing in dry
192 ice (DI) to mimic the most common method of carcass preservation used in RR
193 missions to date, 2) snap freezing by submersion in liquid nitrogen (LN2), and 3)
194 segmenting the carcass into thirds and preserving in RNA*later*TM, mimicking the
195 preservation method used in the Rodent Research-7 mission. After preservation, all
196 carcasses were stored at -80 °C until further processing.

197 Carcasses from mice in groups 2-4 were partially thawed, and quadriceps and livers
198 were dissected, then snap frozen, and stored at -80 °C until RNA extraction to mimic the
199 protocol most commonly implemented when carcasses return from spaceflight missions,
200 including RR-1. A summary of all liver and quadriceps tissues evaluated in the present
201 ground-based tissue preservation study are summarized in Tables S1 and S2,

202 respectively. Total RNA was extracted from all liver and quadriceps tissue samples and
203 prepared for sequencing using the ribodepletion method and sequenced.

204 Global gene expression and transcript integrity were evaluated in liver samples from
205 groups 1-4 to identify differences in DGE resulting from the carcass and immediate
206 preservation protocols. PCA showed overlap among immediate samples despite
207 differences in tissue preservation methods (Figure 5A). Similar to RR-1 carcass and
208 immediate samples (Figures 1A & S1), the dry ice-preserved carcass and immediate
209 samples, which mimic the RR-1 preservation conditions, clustered away from each
210 other, albeit to a much lesser degree than that observed with the RR-1 samples (Figure
211 5A). Furthermore, the dry ice-preserved carcass samples exhibited less 5' gene body
212 coverage than the dry ice-preserved immediate samples (Figure 5B). Although this
213 observation is consistent with that observed in RR-1 carcass and immediate samples
214 (prepared using the polyA selection method) (Figure 3C&D), the difference was less
215 dramatic. Therefore, using the ribodepletion method appears to partially alleviate the
216 differences observed in transcript integrity between carcass and immediate samples.

217 ***Carcass preservation by LN2 or RNAlater™ immersion most closely mimic***
218 ***standard tissue preservation protocols***

219 Livers dissected from carcasses preserved in either RNAlater™ or LN2 exhibit more
220 overlap with immediate preserved liver samples than those from carcasses preserved in
221 dry ice (Figure 5A). Unlike livers dissected from slow (dry ice) or snap (LN2) frozen
222 carcasses, livers dissected from carcasses preserved in RNAlater™ showed no

223 difference in 5' to 3' transcript coverage when compared with livers dissected
224 immediately after euthanasia (Figure 5B). These data suggest that carcass
225 segmentation and preservation in RNA $later$ TM may protect the liver from transcript
226 degradation when preserved *in situ*.

227 We next assessed the effects of various carcass freezing methods on gene expression
228 changes in the liver when compared with livers that were dissected immediately after
229 euthanasia then preserved in either RNA $later$ TM or LN2. Only a few genes were
230 differentially expressed between livers dissected immediately and preserved on dry ice,
231 in RNA $later$ TM, or in LN2 (Table S3). Similarly, pairwise gene set enrichment analysis
232 showed no significantly enriched GO terms between these tissue preservation methods
233 (Table S3), suggesting that for immediately dissected livers, the tissue preservation
234 method had minimal impact on gene expression. Livers dissected from slow (dry ice)
235 frozen carcasses, which most closely mimics the carcass preservation method used in
236 RR-1 NASA and several other RR missions (including RR-3 and Rodent Research-6),
237 exhibited the most DEGs when compared with immediately dissected livers preserved
238 in either LN2 or RNA $later$ TM (Tables 1 and S4, respectively). In contrast, livers dissected
239 from carcasses preserved in either RNA $later$ TM or LN2 exhibited hundreds fewer DEGs
240 when compared with immediately dissected livers preserved in either LN2 or
241 RNA $later$ TM (Tables 1 and S4, respectively). These data indicate that carcass
242 segmentation and preservation in RNA $later$ TM or preserving carcasses by submersion in
243 LN2 more closely mimic the common preservation methods used in terrestrial

244 laboratories, than does the slow freeze carcass preservation method used in the RR-1
245 NASA Validation study.

246 ***The impact of preservation method on gene expression is tissue dependent***

247 To determine if the observed differences in gene expression due to carcass
248 preservation method is unique to the liver, gene expression and transcript integrity was
249 also evaluated in quadriceps from mice in groups 1-4 (Figure S4 and Table S2). PCA
250 showed more overlap among carcass and immediate quadriceps samples (Figure 5C)
251 than among carcass and immediate liver samples (Figure 5A), suggesting that gene
252 expression in the quadriceps is less sensitive to preservation methods. Unlike liver
253 samples, almost no significant differences were observed in 5' to 3' gene body coverage
254 in quadriceps samples prepared using different preservation methods (Figure 5D).

255 Fewer DEGs were identified in carcass vs. immediate quadriceps samples than carcass
256 vs. immediate liver samples for almost every preservation method tested (Tables 2, S5,
257 S6), further supporting that gene expression in the quadriceps is less sensitive to
258 different types of preservation methods. Although there are fewer differences over-all,
259 similar to what was observed in liver samples, cutting the carcass into thirds then
260 preserving in RNA^{later}TM resulted in the fewest DEGs when compared with immediate
261 dissection followed by tissue preservation in LN2 or RNA^{later}TM (Tables 2 and S6).

262 ***Gene expression in select tissues was not affected by the method of euthanasia***

263 Since the most common euthanasia method used in RR missions to date is
264 intraperitoneal (IP) injection of ketamine/xylazine and the most common euthanasia
265 method used in standard laboratories is CO₂ inhalation, these methods were used to
266 euthanize mice in groups 5 and 6, respectively, to determine if euthanasia method is
267 another confounding variable that could affect gene expression in select tissues (Figure
268 S4, Tables S1 and S2). Gene expression was evaluated in livers and quadriceps
269 dissected from mice in groups 2, 5, and 6 (Figure S5A-D). PCA showed no distinct
270 differences in global gene expression in liver (Figure S5A) or quadriceps (Figure S5B)
271 samples dissected from mice euthanized with different methods. Pairwise differential
272 gene expression analysis and gene set enrichment analysis also identified few, if any,
273 DEGs and enriched GO terms among liver (Figure S5C) and quadriceps (Figure S5D)
274 samples. These data suggest that the types of euthanasia methods evaluated here do
275 not impact gene expression in select tissues.

276 ***Discussion***

277 Herein, we show that protocols used to preserve mouse carcasses on-orbit have large
278 effects on gene expression patterns as measured by RNAseq. Indeed, changes in gene
279 expression due to preservation condition overwhelmed those due to spaceflight. Gene
280 set enrichment analysis showed that many GO terms enriched due to carcass
281 preservation were involved in RNA processing. This correlated with reduced transcript
282 integrity (relatively poor coverage of the 5' end of transcripts) in samples from carcasses

283 preserved on-orbit when these were sequenced with a polyA enrichment RNAseq
284 protocol.

285 While RNAseq following polyA selection can more efficiently quantify gene expression
286 ([Kumar et al., 2017](#)), ribodepletion methods are more effective on degraded RNA
287 samples ([Li et al., 2014](#); [Schuierer et al., 2017](#)). However, while the RNA used in this
288 study was of good quality (RIN > 7) we observed a severe bias in transcript coverage
289 following polyA selection depending upon the tissue preservation condition utilized.

290 Specifically, samples taken from carcasses that were slow-frozen on-orbit exhibited a
291 lower 5' to 3' coverage ratio as compared to samples taken from immediately dissected
292 tissues. While resequencing of the carcass flight samples with a ribodepletion protocol
293 produced a more even 5' to 3' coverage ratio, our follow-on studies that directly
294 compared slow carcass freezing to immediate dissection revealed a similar (albeit
295 reduced) 5' to 3' coverage bias. Taken together, this suggests that slow carcass
296 freezing causes transcript degradation that in-turn leads to reduced 5' coverage.

297 mRNA degradation starts with the removal of the polyA tail, at which point degradation
298 continues either from the 3' end *via* the exosome complex, or the 5' end following
299 removal of the 5'-methylguanosine cap. Deadenylation of cytoplasmic mRNA is the rate
300 limiting step in mRNA degradation and is catalyzed by one of two complexes. The
301 CCR4-NOT complex, which consists of 10 subunits (CNOT1, CNOT2, CNOT3, CNOT4,
302 CNOT6, CNOT6l, CNOT7, CNOT8, CNOT9, CNOT10), and the PAN2-PAN3
303 deadenylation complex consisting of two subunits (PAN2, PAN3) ([Siwaszek et al.,](#)

304 2014). We observed transcriptional changes to multiple subunits in each of these
305 complexes when comparing carcass and immediate samples. Most striking was the
306 coordinate upregulation of both Pan2 and Pan3 in the carcass samples from RR-1,
307 which suggest an increase in PAN2-PAN3 deadenylation activity, which could result in
308 loss of polyA tails in some transcripts. This could lead to poor mRNA capture by our
309 polyA enrichment protocol and result in some of the differences seen between the polyA-
310 enrichment and ribodepletion protocols.

311 Three proteins - Dcp2 (Nudt20), Nudt3, Nudt16 - have decapping activity both in vitro
312 and in cells, while an additional five – Nudt2, Nudt12, Nudt15, Nudt17, Nudt19 – have
313 decapping activity in vitro. In addition, the Dxo family of proteins acts on partially capped
314 mRNA's (Grudzien-Nogalska and Kiledjian, 2017). While regulation of these proteins is
315 complex and involves subcellular localization and post-translation modification, we
316 observed evidence for altered expression of these decapping enzymes: Dxo and Nudt2
317 were more abundant in carcass samples, while Nudt15 and Dcp2 (Nudt20) were more
318 abundant in immediate samples. While these changes are not coordinated, they do
319 point to altered decapping activity within the carcass samples. As decapping proceeds
320 mRNA degradation via the 5' exonuclease, XRN1, this could alternatively explain the
321 relatively poor 5' transcript coverage seen in both polyA enriched and ribodepleted
322 carcass liver samples. Additional experimentation will be necessary to confirm the
323 changes to decapping and deadenylation enzymes seen here and to understand their
324 role in the 5' to 3' coverage bias observed.

325 We observed a marked difference in the 5' to 3' coverage bias between liver and
326 quadriceps samples. Whereas liver samples were sensitive to carcass preservation *via*
327 slow- or snap-freeze, quadricep samples were not. There are a number of possible
328 explanations for this. First, it could be due to the surface exposure of the quadriceps,
329 which would lead to more rapid quenching of biochemical processes. Second, inherent
330 differences in the transcript pool, mRNA half-lives, and enzymatic complement of liver
331 and quadriceps could offer a biological answer. While we cannot distinguish between
332 these mechanisms, our observations are consistent with previous results showing that
333 post-mortem changes to mRNA is tissue-dependent (Inoue et al., 2002; Lee et al.,
334 2005; Miyatake et al., 2004).

335 The poor transcript integrity in slow frozen carcasses sequenced with a polyA
336 enrichment protocol was not evident in pre-sequencing QC analyses. Indeed, all
337 samples had RIN values > 7 and there was no correlation between the gene expression
338 differences and RIN. This distinguishes our results from previous studies showing a
339 strong correlation between RIN values and loss of 5' coverage (Davila et al., 2016;
340 Sigurgeirsson et al., 2014). Therefore, additional pre-sequencing QC analyses capable
341 of detecting these issues would be useful. Low throughput sequencing is rapid,
342 decreasing in cost, and being adopted as a QC step, but does not provide the coverage
343 necessary to detect the biases seen here.

344 Alternatively, if effective pre-sequencing QC metrics cannot be developed, a number of
345 analytical approaches could be utilized. In one category are methods that calculate

346 additional metrics such as mRIN ([Feng et al., 2015](#)) and TIN ([Wang et al., 2016](#)) to
347 allow assessment transcript integrity and exclusion of problematic samples. In a second
348 category are processes that account for variable transcript integrity by considering only
349 reads that occur near the 3' end of transcripts ([Sigurgeirsson et al., 2014](#)), controlling for
350 the effects of RIN using a linear model framework ([Gallego Romero et al., 2014](#)), or by
351 calculating idealized coverage curves on a gene-by-gene basis and using these for
352 normalization ([Xiong et al., 2019](#)). Additional analyses are necessary to determine if
353 these approaches can mitigate the issue observed here.

354 While we do not have a complete picture of the mechanisms resulting in the apparent
355 gene expression change resulting from slow carcass freezing, we were able to identify
356 effective mitigation strategies. Foremost among these is the utilization of a ribodepletion
357 protocol in place of polyA enrichment. In this study, ribodepletion resulted in more even
358 gene body coverage and was not as sensitive to slow freezing of carcasses. This is in
359 agreement with previous studies which found that ribodepletion is less prone to bias
360 introduced by poor RNA quality ([Li et al., 2014](#)) and less prone to 3' coverage bias
361 ([Schuierer et al., 2017](#)). Beyond this, we found that two carcass preservation methods
362 generated acceptable results, with few DEGs and enriched GO terms when compared
363 to the immediate dissection of tissues and preservation in liquid nitrogen — the *de facto*
364 gold standard. The first is rapid freeze of carcasses in liquid nitrogen and subsequent
365 storage at -80 °C, followed by partial thaw, dissection and tissue preservation in liquid
366 nitrogen. While this led to some loss of 5' transcript coverage, it had the fewest DEGs

367 (515, adj. $p < 0.05$ & $|\text{Log}_2 \text{FC}| > 1.5$) and no enriched GO terms ($\text{FDR} < 0.25$, $\text{NOM} <$
368 0.01) when compared to immediate dissection. Alternatively, segmentation of carcasses
369 and immersion in *RNAlater* and subsequent storage at -80°C , followed by partial thaw,
370 dissection and tissue preservation in liquid nitrogen resulted in better maintenance of 5'
371 transcript coverage but an increased number of DEGs (1952, adj. $p < 0.05$ & $|\text{Log}_2 \text{FC}|$
372 > 1.5) although no GO terms were enriched ($\text{FDR} < 0.25$, $\text{NOM} < 0.01$). As euthanasia
373 protocols can change serum biomarkers ([Pierozan et al., 2017](#)) and mRNA expression
374 levels ([Staib-Lasarzik et al., 2014](#)), we were reassured to find that the euthanasia
375 protocols used here did not affect gene expression in liver or quadriceps.

376 To conclude, our results indicate that care must be taken in choosing sample
377 preservation protocols that preserve transcriptional patterns and other embedded
378 information, but that are also feasible in resource constrained environments such as
379 those found in space.

380 ***STAR Methods***

381 ***Rodent Research-1 (RR-1) Study***

382 **Spaceflight Mission**

383 Rodent Research-1 (RR-1) was the first mission in which animals were maintained on
384 the ISS for a long duration mission in the Rodent Habitat modified from heritage Animal
385 Enclosure Module (AEM) hardware. Complete details were published previously (Choi
386 et al., 2020). In short, RR-1 consisted of two experiments: ISS National Lab study (RR-1

387 CASIS) and NASA Validation study (RR-1 NASA). In the ISS National Lab Study, ten
388 32-week-old female C57BL/6NTac mice (Taconic Biosciences, Rensselaer, NY) were
389 flown to space for 20-21 days then euthanized via IP injection of pentobarbital/phenytoin
390 (Euthasol®) and dissected onboard the ISS. Livers were dissected then inserted into
391 cryovials, which were then frozen in a cold stowage container that was pre-chilled to -
392 130 °C before transferring to the Minus Eighty-Degree Laboratory Freezer (MELFI) at
393 the end of each dissection session (-80 °C). In the NASA Validation study, ten 16-week-
394 old female C57BL/6J mice (Jackson laboratories, Bar harbor, ME) were flown to the ISS
395 for 37 days before euthanasia and subsequent dissection. Due to crew time constraint,
396 only two (out of ten) mice were dissected immediately after euthanasia via IP injection
397 of pentobarbital/phenytoin (Euthasol®) to recover spleen and liver tissues on the ISS.
398 Isolated livers were preserved by using the same method as the ISS National Lab study
399 livers. The remaining eight animals were euthanized, then intact carcasses were
400 wrapped in aluminum foil, put in Ziploc bags, placed in a pre-chilled cold stowage
401 container and stored in the MELFI. For both the ISS National Lab study and the NASA
402 Validation study, there were respective cohorts of age-matched basal animals which
403 were euthanized one day after launch as a baseline control as well as age-matched
404 ground control animals kept in an ISS Environmental Simulator at Kennedy Space
405 Center (KSC) on a 4-day delay to mimic spaceflight conditions. In addition, the NASA
406 Validation study also had a cohort of age-matched vivarium control animals that were
407 housed in the vivarium cages and followed the same experimental timeline and process

408 as the spaceflight animals. A timeline indicating major events in the RR-1 mission is
409 shown in Supplemental Figure 6.

410 **Sample Collection**

411 The frozen intact carcasses from the NASA Validation study were partially thawed then
412 dissected at NASA Ames Research Center upon return to Earth. One lobe of liver from
413 each carcass was removed, immediately homogenized in RLT buffer (Qiagen, Valencia,
414 CA) followed by snap freezing the tissue homogenates in LN₂. Quadriceps were snap
415 frozen upon collection. Tissues were stored at -80 °C until extraction.

416 **RNA Isolation**

417 RNA was isolated from all liver and quadriceps samples using the following methods.
418 For the liver samples, RNA was extracted with the AllPrep DNA/RNA Mini Kit (Qiagen,
419 Valencia, CA) following the manufacturer's protocol. Briefly, homogenization buffer was
420 made by adding 1:100 volume ratio of beta-mercaptoethanol to RLT buffer and kept on
421 ice until use. Approximately 30 mg of tissue was cut using a sterile scalpel and
422 immediately placed in 800 µL of the RLT buffer solution. Each sample was then
423 homogenized for approximately 20 seconds at 21,000 RPM using a Polytron PT1300D
424 handheld homogenizer with a 5 mm standard dispersing aggregate tip (Kinematica,
425 Bohemia, NY). Homogenates were centrifuged for 3 minutes at room temperature at
426 15,000 RPM to remove cell debris. The supernatant from each sample was used to
427 isolate and purify RNA following the manufacturer's protocol including on-column DNase

428 treatment with RNase-free DNase (Qiagen, Valencia, CA). RNA was eluted twice per
429 sample in 30 μ L RNase- and DNase-free H₂O per elution. For quadriceps samples,
430 RNA was extracted using TRIzol reagents (Thermo Fisher Scientific, Waltham, MA)
431 according to the manufacturer's protocol, and the isolated RNA samples were then
432 treated on column with RNase-free DNase (Qiagen, Valencia, CA) and RNeasy Mini kit
433 (Qiagen, Valencia, CA). Concentration and absorbance ratios of all the isolated liver
434 and quadriceps RNA samples were measured using the NanoDrop 2000
435 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA quality was
436 assessed using the Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano Kit or
437 Agilent RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, CA).

438 **Library Preparation and RNA-Sequencing**

439 Samples with RNA Integrity Number (RIN) of 6 or above were sent to the University of
440 California (UC), Davis Genome Center where the libraries were constructed and RNA-
441 sequencing was performed. All the RR-1 RNA-sequencing data analyzed in this
442 manuscript were obtained from the NASA GeneLab Data Repository
443 (<https://genelab.nasa.gov/>), including GLDS-47, GLDS-48, and GLDS-168. The RR-1
444 liver RNA samples were sequenced twice. First, libraries were generated using the
445 Illumina TruSeq Stranded RNA library prep kit (Illumina, San Diego, CA) after polyA
446 selection, and sequencing was done with 50 bp single end reads on the Illumina HiSeq
447 3000 platform (GLDS-47 and GLDS-48). Second, selected RNA samples were spiked
448 in with ERCC ExFold RNA Spike-In Mixes (Thermo Fisher Scientific, Waltham, MA)

449 before shipping to the UC Davis Genome Center. Ribosomal RNA was removed with
450 the Illumina RiboZero Gold ribodepletion kit then RNA sequencing libraries were
451 constructed using the KAPA RNA HyperPrep kit (Roche, Basel, Switzerland) and the
452 sequencing was done with 150 bp paired end reads on the Illumina HiSeq 4000
453 platform (GLDS-168).

454 ***Ground-based Tissue Preservation Study***

455 **Animals**

456 20- to 21-week-old female C57BL/6J mice (Jackson laboratories, Bar harbor, ME) were
457 shipped to the NASA Ames Research Center Animal Care Facility and were randomly
458 housed in the standard vivarium cages with up to five mice per cage. The animals were
459 acclimated for five days before the start of procedures to ensure recovery from the
460 transportation stress. During acclimation, the animals were maintained on a 12h
461 light/dark cycle and were provided with standard chow and water access *ad libitum*.
462 One day before euthanasia, animal body weights were measured and used to distribute
463 the animals into six groups (n=6/group) with similar average body weights. Animal
464 health status, water and food intake were monitored daily. The study followed
465 recommendations in the Guide for the Care and Use of Laboratory Animals (2011) and
466 was approved on February 8, 2018 by the Institutional Animal Care and Use Committee
467 (IACUC) at NASA Ames Research Center (Protocol number NAS-17-006-Y1).

468 **Animal Euthanasia and Dissection**

469 The detailed descriptions and rationale of each group are as follows as well as outlined
470 in Figure S4 and Tables S1 and S2. Group 1 (M1-M6) animals were euthanized by
471 intraperitoneal injection of pentobarbital/phenytoin (Euthasol®) (80 mg in 0.2 ml) (Virbac,
472 West Lake, TX) followed by cervical dislocation. Dissection was performed immediately
473 after euthanasia without freezing the carcasses. Left lobes of livers and quadriceps
474 were subdivided into three sections and each tissue section was preserved either by
475 freezing on dry ice, snap freezing in liquid nitrogen, or preserved in RNA/ater™ solution
476 (Thermo Fisher Scientific, Waltham, WA). For the tissue sections preserved in
477 RNA/ater™, tissue sections were submerged in RNA/ater™ at 4 °C for 3 days then
478 frozen and stored at -80 °C. Note that this is the only group of animals that were
479 dissected upon euthanasia. Carcasses from animals in subsequent groups were
480 preserved intact using various methods then dissected at a later date. Group 1 tissue
481 sections that were preserved by freezing on dry ice most closely mimics the process
482 that was used to generate RR-1 NASA and CASIS immediate samples.

483 Group 2 (M7-M12) animals were euthanized by intraperitoneal injection of
484 pentobarbital/phenytoin (Euthasol®) followed by cervical dislocation. The carcasses
485 were wrapped in foil and preserved by freezing on dry ice, similar to the intact carcass
486 preservation method used for the RR-1 NASA Validation Study. Once frozen, the
487 carcasses were stored at -80 °C. On the day of dissection, mouse carcasses were
488 removed from the -80 °C freezer and thawed at room temperature for 15 to 20 minutes

489 prior to dissection. Left lobes of livers were removed and divided into two: one piece
490 was snap frozen in liquid nitrogen then stored at -80 °C; the other piece was
491 homogenized in RLT buffer (Qiagen, Valencia, CA) and the tissue homogenate was
492 snap frozen in liquid nitrogen then stored at -80 °C for 70 days before RNA extraction to
493 simulate the process used to generate the RR-1 NASA “carcass” liver samples. This
494 extended storage did not result in a substantial number of DEG (Figure S5E&F).
495 Quadriceps were snap frozen in liquid nitrogen after dissection then stored at -80 °C.

496 Group 3 (M13-M18) animals were euthanized by intraperitoneal injection of
497 pentobarbital/phenytoin (Euthasol®) followed by cervical dislocation. The intact
498 carcasses were wrapped in foil and preserved by snap freezing in liquid nitrogen
499 followed by storage at -80 °C. On the day of dissection, mouse carcasses were
500 removed from the -80 °C freezer and thawed at room temperature for 15 to 20 minutes
501 prior to dissection. Left lobes of livers and quadriceps were collected and snap frozen
502 in liquid nitrogen then stored at -80 °C.

503 Group 4 (M19-M24) animals were euthanized by intraperitoneal injection of
504 pentobarbital/phenytoin (Euthasol®) followed by cervical dislocation. The carcasses
505 were sectioned into 3 sections, head, chest, and abdomen with tail removed and
506 discarded and each part was submerged in RNA^{later}™ solution (Thermo Fisher
507 Scientific, Waltham, WA) and placed at 4 °C for 3 days to allow thorough permeation
508 before being stored at -80 °C. On the day of dissection, mouse carcasses were
509 removed from the -80 °C freezer and thawed at room temperature for 15 to 20 minutes

510 prior to dissection. Left lobes of livers and quadriceps were collected and snap frozen
511 in liquid nitrogen then stored at -80 °C. This group was used to simulate the procedure
512 done in the RR-7 mission and to test if gene expression signals could be better
513 preserved using an RNA-specific preservative.

514 Group 5 (M25-30) animals were euthanized by intraperitoneal injection of
515 ketamine/xylazine (10mg/mL / 3mg/mL in 0.3mL PBS) followed by cervical dislocation.
516 The intact carcasses were wrapped in foil and preserved by freezing on dry ice then
517 stored at -80 °C. On the day of dissection, mouse carcasses were removed from the -
518 80 °C freezer and thawed at room temperature for 15 to 20 minutes prior to dissection.
519 Left lobes of livers and quadriceps were collected and snap frozen in liquid nitrogen
520 then stored at -80 °C. The euthanasia and preservation methods used in this group
521 mimics the process used to generate RR-3 carcass liver samples (Smith et al., 2017).
522 Ketamine/xylazine is currently the most common euthanasia method used in RR
523 missions.

524 Group 6 (M31-M36) animals were euthanized by carbon dioxide inhalation followed by
525 cervical dislocation. The carcasses were wrapped in foil and preserved by freezing on
526 dry ice then stored at -80 °C. On the day of dissection, mouse carcasses were removed
527 from the -80 °C freezer and thawed at room temperature for 15 to 20 minutes prior to
528 dissection. Left lobes of livers and quadriceps were collected and snap frozen in liquid
529 nitrogen then stored at -80 °C. This group represents the euthanasia method commonly

530 used in terrestrial laboratories and was used to evaluate any effects on gene expression
531 due to the drug-induced euthanasia methods that have been used in RR missions.

532 **RNA Isolation**

533 RNA was isolated from partial left liver lobe and partial quadriceps muscle tissues using
534 the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA). Briefly, homogenization buffer
535 was made by adding 1:100 volume ratio of beta-mercaptoethanol to RLT buffer and
536 kept on ice until use. On average, 48.85 mg of left liver lobe and 20.08 mg of left or right
537 quadriceps was cut using a sterile scalpel and immediately placed in 600 μ L of the RLT
538 buffer solution. Complete tissue dispersion was achieved using the hand-held Polytron
539 PT1300D homogenizer with 5 mm standard dispersing aggregate by implementing 20
540 second homogenization periods at a speed of 20,000 RPM. Homogenized samples
541 were centrifuged for 3 minutes at room temperature at 15,000 RPM to remove cell
542 debris. The supernatant from each sample was used to isolate and purify RNA following
543 the manufacturer's protocol. RNA was treated with RNase-Free DNase (Qiagen,
544 Valencia, CA) and eluted in 50 μ L of RNase- and DNase-free H₂O molecular grade
545 water. RNA concentration was measured using Qubit 3.0 Fluorimeter (Thermo Fisher
546 Scientific, Waltham, MA). RNA quality was assessed using the Agilent 2100 Bioanalyzer
547 with the Agilent RNA 6000 Nano Kit or Agilent RNA 6000 Pico Kit (Agilent
548 Technologies, Santa Clara, CA).

549 **Library Preparation and Sequencing**

550 Three microliters of Mix 1 or Mix 2 of ERCC ExFold RNA Spike-In (Thermo Fisher
551 Scientific, Waltham, MA) at a dilution of 1:100 was added to 1.5 µg aliquots of RNA
552 immediately after extraction. The two mixes were randomly distributed within the six
553 experimental groups. In addition, Universal Human and Mouse Reference RNA samples
554 (Agilent Technologies, Santa Clara, CA) were included as control samples in the library
555 construction and sequencing.

556 Library construction was performed using 500 ng of ERCC-spiked total RNA with an
557 average RIN of 7.8 for liver samples and 9.8 for quadriceps samples. Total RNA was
558 depleted of the ribosomal fraction and libraries were constructed with TruSeq Stranded
559 Total RNA with Ribo-Zero Gold kit (Illumina, San Diego, CA). Libraries were indexed
560 using 1.5 µM Unique Dual Index adapters with Unique Molecular Identifiers (Integrated
561 DNA Technologies, Coralville, IA) and 15 cycles of amplification were performed to
562 reach desired library concentration. Library size was assessed with 4200 TapeStation
563 (Agilent Technologies, Santa Clara, CA), targeting average size of 300 nt.

564 Libraries were multiplexed then quantified using Universal qPCR Master Mix (Kapa
565 Biosystems, Wilmington, MA). The library pool was sequenced on an iSeq 100
566 (Illumina, San Diego, CA) to assess sample quality and pool balancing before large-
567 scale sequencing. The final library pool (with 1% PhiX spike-in for instrument control)
568 was sequenced on a NovaSeq 6000 using one S4 and one S2 Reagent Kit (Illumina,

569 San Diego, CA), paired-end and 149 bp reads, targeting 60 million clusters for each
570 experimental sample.

571 ***RNA Sequencing Data Analysis***

572 Raw RNA sequence data from the RR-1 NASA Validation flight liver (GLDS-48 and
573 GLDS-168) samples, RR-1 CASIS liver samples (GLDS-47), and the ground-based
574 studies designed to simulate and assess spaceflight euthanasia, carcass and tissue
575 preservation, and/or storage protocols, GLDS-49, GLDS-235, and GLDS-236 were
576 analyzed using the GeneLab standard RNAseq analysis pipeline. First, adapters were
577 removed with Cutadapt (v2.3) (Martin, 2011) using the Trim Galore! (v0.6.2) wrapper.
578 Raw and trimmed read quality were evaluated with FastQC (v0.11.8), and MultiQC
579 (v1.7) was used to generate MultiQC reports. *Mus musculus* STAR and RSEM
580 references were built using STAR (v2.7.1a) and RSEM (v1.3.1) (Li and Dewey, 2011),
581 respectively, with ensembl genome version mm10-GRCm38
582 (*Mus_musculus.GRCm38.dna.toplevel.fa*), and the following gtf annotation file:
583 *Mus_musculus.GRCm38.96.gtf*. Trimmed reads were aligned to the *Mus musculus*
584 STAR reference with STAR (v2.7.1a) (Dobin et al., 2013) and aligned reads were
585 quantified using RSEM (v1.3.1) (Li and Dewey, 2011).

586 The following samples were used for downstream analyses; GLDS-47: FLT and GC;
587 GLDS-48: FLT-C, FLT-I, GC-C, and GC-I; GLDS-49: LN2-3d, LN2-1y, DI-1y; GLDS-
588 168: RR1-FLT-wERCC and RR1-GC-wERCC; for GLDS-235 and GLDS-236, all
589 samples indicated in Figure S4 and Tables S1 and S2 were included. For each GLDS

590 dataset, quantification data from select samples were imported to R (v3.6.0) with
591 tximport (v1.14.0) (Soneson et al., 2016) and normalized with DESeq2 (v1.26.0) (Love
592 et al., 2014). All ERCC genes were removed prior to normalization. Differential
593 expression analysis was performed in R (v3.6.0) using DESeq2 (v1.26.0) (Love et al.,
594 2014); all groups were compared using the Wald test and the likelihood ratio test was
595 used to generate the F statistic p-value. Gene annotations were assigned using the
596 following Bioconductor and annotation packages: STRINGdb (Szklarczyk et al., 2019),
597 PANTHER.db (Muller, 2017), and org.Mm.eg.db (Carlson, 2017).

598 ***Transcript Integrity Analysis***

599 The geneBody_coverage.py function from RSeQC (v3.0.1) (Wang et al., 2012) was
600 used to assess coverage across the median 1000 expressed genes across all datasets.
601 A transcript integrity metric was defined as the ratio between the coverage in a window
602 corresponding to position 10-30% and 80-100% (relative to the entire gene length) and
603 used in boxplots. To determine significance between groups the nonparametric Mann–
604 Whitney U test was used as distributions were not normal.

605 ***Gene Set Enrichment Analysis***

606 Pairwise gene set enrichment analysis (GSEA) was performed on the normalized
607 counts from select samples in GLDS-48, GLDS-168, GLDS-235, and GLDS-236 using
608 the C5: Gene Ontology (GO) gene set (MSigDB v7.1) as described (Subramanian et al.,
609 2005). All comparisons were performed using the phenotype permutation, except those

610 involving GLDS-48 immediate samples, which used the gene set permutation due to low
611 sample size. The ranked lists of genes were defined by the signal-to-noise metric, and
612 the statistical significance were determined by 1000 permutations of the gene set. FDR
613 ≤ 0.25 and FDR ≤ 0.05 were considered significant for comparisons using the
614 phenotype and gene set permutations, respectively, according to the authors'
615 recommendation.

616 ***Data Availability***

617 All sequencing data is available at NASA GeneLab (www.genelab.nasa.gov) (Ray et al.,
618 2019). polyA enrichment-based data from the RR-1 NASA Validation Mission samples
619 are at GLDS-48 (Globus and Galazka, 2015). polyA enrichment-based data from the
620 RR-1 CASIS samples are at GLDS-47 (Globus et al., 2015). Data from ground-based
621 study of tissue storage conditions are at GLDS-49 (GeneLab, 2016). Ribodepletion-
622 based resequencing data from the RR-1 NASA Validation Mission samples are at
623 GLDS-168 (Galazka, 2018). Liver data from ground-based freezing study are at GLDS-
624 235 (Galazka, 2019a). Quadriceps data from ground-based freezing study are at GLDS-
625 236 (Galazka, 2019b).

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632 **Author Contributions**

633 Formal analysis, A.S.M.B, H.F, J.M.G; Investigation, K.C., R.B.C, O.M., S.L.P, V.B.,
634 M.T.D, Y.C., S.R.; Project administration, A.M.SB., S.V.C., J.M.G.; Visualization,
635 A.M.SB, J.M.G; Writing—original draft, A.M.SB, S.V.C, J.M.G, S.L.P; Writing—review
636 and editing, Y.C., A.M.SB, S.S.R., J.M.G., S.L.P; Conceptualization, S.L.P., J.M.G,
637 S.V.C, S.S.R.; Data curation, A.M.SB., S.L.P., Y.C.

638 **Declaration of Interests**

639 The authors declare no competing interests.

640 **References**

641 Beheshti, A., Chakravarty, K., Fogle, H., Fazelinia, H., Silveira, W.A. da, Boyko, V.,
642 Polo, S.-H.L., Saravia-Butler, A.M., Hardiman, G., Taylor, D., et al. (2019). Multi-omics
643 analysis of multiple missions to space reveal a theme of lipid dysregulation in mouse
644 liver. *Sci Rep* 9, 19195.

645 Cadena, S.M., Zhang, Y., Fang, J., Brachat, S., Kuss, P., Giorgetti, E., Stodieck, L.S.,
646 Kneissel, M., and Glass, D.J. (2019). Skeletal muscle in MuRF1 null mice is not spared

647 in low-gravity conditions, indicating atrophy proceeds by unique mechanisms in space.
648 Sci Rep 9, 9397.

649 Carlson, M. (2017). org.Mm.eg.db (Bioconductor).

650 Choi, S., Ray, H.E., Lai, S.-H., Alwood, J.S., and Globus, R.K. (2016). Preservation of
651 Multiple Mammalian Tissues to Maximize Science Return from Ground Based and
652 Spaceflight Experiments. PLoS ONE 11, e0167391.

653 Choi, S.Y., Saravia-Butler, A., Shirazi-Fard, Y., Leveson-Gower, D., Stodieck, L.S.,
654 Cadena, S.M., Beegle, J., Solis, S., Ronca, A., and Globus, R.K. (2020). Validation of a
655 New Rodent Experimental System to Investigate Consequences of Long Duration
656 Space Habitation. Sci Rep 10, 2336.

657 Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P.,
658 Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner.
659 Bioinformatics 29, 15–21.

660 Galazka, J. (2018). RR-1 and RR-3 mouse liver transcriptomics with and without ERCC
661 control RNA spike-ins.

662 Galazka, J. (2019a). Transcriptomic analysis of liver from mice subjected to simulated
663 spaceflight euthanasia, freezing, and tissue preservation protocols.

664 Galazka, J. (2019b). Transcriptomic analysis of quadriceps from mice subjected to
665 simulated spaceflight euthanasia, freezing, and tissue preservation protocols.

666 Garrett-Bakelman, F.E., Darshi, M., Green, S.J., Gur, R.C., Lin, L., Macias, B.R.,
667 McKenna, M.J., Meydan, C., Mishra, T., Nasrini, J., et al. (2019). The NASA Twins
668 Study: A multidimensional analysis of a year-long human spaceflight. *Science* 364.
669 GeneLab, N. (2016). Multi-omic investigations of mouse liver subjected to simulated
670 spaceflight freezing and storage protocols.
671 Globus, R., and Galazka, J.M. (2015). Rodent Research-1 (RR1) NASA Validation
672 Flight: Mouse liver transcriptomic, proteomic, and epigenomic data (NASA GeneLab).
673 Globus, R., Cadena, S., and Galazka, J. (2015). Rodent Research-1 (RR1) National
674 Lab Validation Flight: Mouse liver transcriptomic, proteomic, and epigenomic data.
675 Grudzien-Nogalska, E., and Kiledjian, M. (2017). New insights into decapping enzymes
676 and selective mRNA decay. *Wiley Interdiscip Rev RNA* 8.
677 Guo, Y., Zhao, S., Sheng, Q., Guo, M., Lehmann, B., Pietenpol, J., Samuels, D.C., and
678 Shyr, Y. (2015). RNAseq by Total RNA Library Identifies Additional RNAs Compared to
679 Poly(A) RNA Library. *BioMed Research International* 2015, 1–9.
680 Institute of Medicine (2008). Review of NASA’s Human Research Program Evidence
681 Books: A Letter Report (Washington, D.C.: National Academies Press).
682 Jonscher, K.R., Alfonso-Garcia, A., Suhaimi, J.L., Orlicky, D.J., Potma, E.O., Ferguson,
683 V.L., Bouxsein, M.L., Bateman, T.A., Stodieck, L.S., Levi, M., et al. (2016). Spaceflight
684 Activates Lipotoxic Pathways in Mouse Liver. *PLoS ONE* 11, e0152877.

- 685 Kumar, A., Kankainen, M., Parsons, A., Kallioniemi, O., Mattila, P., and Heckman, C.A.
686 (2017). The impact of RNA sequence library construction protocols on transcriptomic
687 profiling of leukemia. *BMC Genomics* *18*, 629.
- 688 Li, B., and Dewey, C.N. (2011). RSEM: accurate transcript quantification from RNA-Seq
689 data with or without a reference genome. *BMC Bioinformatics* *12*, 323.
- 690 Li, S., Tighe, S.W., Nicolet, C.M., Grove, D., Levy, S., Farmerie, W., Viale, A., Wright,
691 C., Schweitzer, P.A., Gao, Y., et al. (2014). Multi-platform assessment of transcriptome
692 profiling using RNA-seq in the ABRF next-generation sequencing study. *Nat Biotechnol*
693 *32*, 915–925.
- 694 Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and
695 dispersion for RNA-seq data with DESeq2. *Genome Biol* *15*, 550.
- 696 Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput
697 sequencing reads. *EMBnet j.* *17*, 10.
- 698 Muller, J. (2017). PANTHER.db (Bioconductor).
- 699 Pecaut, M.J., Mao, X.W., Bellinger, D.L., Jonscher, K.R., Stodieck, L.S., Ferguson, V.L.,
700 Bateman, T.A., Mohny, R.P., and Gridley, D.S. (2017). Is spaceflight-induced immune
701 dysfunction linked to systemic changes in metabolism? *PLoS ONE* *12*, e0174174.

- 702 Petrova, O.E., Garcia-Alcalde, F., Zampaloni, C., and Sauer, K. (2017). Comparative
703 evaluation of rRNA depletion procedures for the improved analysis of bacterial biofilm
704 and mixed pathogen culture transcriptomes. *Sci Rep* 7, 41114.
- 705 Ray, S., Gebre, S., Fogle, H., Berrios, D.C., Tran, P.B., Galazka, J.M., and Costes, S.V.
706 (2019). GeneLab: Omics database for spaceflight experiments. *Bioinformatics* 35,
707 1753–1759.
- 708 Rettig, T.A., Ward, C., Pecaut, M.J., and Chapes, S.K. (2017). Validation of Methods to
709 Assess the Immunoglobulin Gene Repertoire in Tissues Obtained from Mice on the
710 International Space Station. *Gravit Space Res* 5, 2–23.
- 711 Ronca, A.E., Moyer, E.L., Talyansky, Y., Lowe, M., Padmanabhan, S., Choi, S., Gong,
712 C., Cadena, S.M., Stodieck, L., and Globus, R.K. (2019). Behavior of mice aboard the
713 International Space Station. *Sci Rep* 9, 4717.
- 714 Shen, H., Lim, C., Schwartz, A.G., Andreev-Andrievskiy, A., Deymier, A.C., and
715 Thomopoulos, S. (2017). Effects of spaceflight on the muscles of the murine shoulder.
716 *FASEB J.* 31, 5466–5477.
- 717 Siwaszek, A., Ukleja, M., and Dziembowski, A. (2014). Proteins involved in the
718 degradation of cytoplasmic mRNA in the major eukaryotic model systems. *RNA Biology*
719 11, 1122–1136.

720 Smith, R., Cramer, M., Globus, R., and Galazka, J. (2017). Rodent Research-3-CASIS:
721 Mouse liver transcriptomic, proteomic, and epigenomic data.

722 Sonesson, C., Love, M.I., and Robinson, M.D. (2016). Differential analyses for RNA-seq:
723 transcript-level estimates improve gene-level inferences. *F1000Res* 4, 1521.

724 Spatz, J.M., Ellman, R., Cloutier, A.M., Louis, L., van Vliet, M., Dwyer, D., Stolina, M.,
725 Ke, H.Z., and Bouxsein, M.L. (2017). Sclerostin antibody inhibits skeletal deterioration in
726 mice exposed to partial weight-bearing. *Life Sci Space Res (Amst)* 12, 32–38.

727 Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A.,
728 Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., et al. (2005). Gene set
729 enrichment analysis: A knowledge-based approach for interpreting genome-wide
730 expression profiles. *Proceedings of the National Academy of Sciences* 102, 15545–
731 15550.

732 Sultan, M., Amstislavskiy, V., Risch, T., Schuette, M., Dökel, S., Ralser, M., Balzereit,
733 D., Lehrach, H., and Yaspo, M.-L. (2014). Influence of RNA extraction methods and
734 library selection schemes on RNA-seq data. *BMC Genomics* 15, 675.

735 Szklarczyk, D., Gable, A.L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J.,
736 Simonovic, M., Doncheva, N.T., Morris, J.H., Bork, P., et al. (2019). STRING v11:
737 protein–protein association networks with increased coverage, supporting functional
738 discovery in genome-wide experimental datasets. *Nucleic Acids Research* 47, D607–
739 D613.

- 740 Tascher, G., Brioché, T., Maes, P., Chopard, A., O’Gorman, D., Gauquelin-Koch, G.,
741 Blanc, S., and Bertile, F. (2017). Proteome-wide Adaptations of Mouse Skeletal Muscles
742 during a Full Month in Space. *J. Proteome Res.* *16*, 2623–2638.
- 743 Wang, L., Wang, S., and Li, W. (2012). RSeQC: quality control of RNA-seq
744 experiments. *Bioinformatics* *28*, 2184–2185.
- 745 Ward, C., Rettig, T.A., Hlavacek, S., Bye, B.A., Pecaut, M.J., and Chapes, S.K. (2018).
746 Effects of spaceflight on the immunoglobulin repertoire of unimmunized C57BL/6 mice.
747 *Life Sci Space Res (Amst)* *16*, 63–75.
- 748 Zhao, W., He, X., Hoadley, K.A., Parker, J.S., Hayes, D., and Perou, C.M. (2014).
749 Comparison of RNA-Seq by poly (A) capture, ribosomal RNA depletion, and DNA
750 microarray for expression profiling. *BMC Genomics* *15*, 419.
- 751 (2011). Guide for the care and use of laboratory animals.

752 **Figure Legends**

753 ***Figure 1. Gene expression differences in RR-1 NASA Validation flight liver***
754 ***samples prepared via polyA selection (GLDS-48).*** A) Principal component analysis of
755 global gene expression in RR-1 NASA spaceflight (FLT) and respective ground control
756 (GC) liver samples dissected immediately after euthanasia (I) or from frozen carcasses
757 (C). Percent variance for each principal component (PC) is shown. B) Venn diagram
758 showing the number of similar and unique differentially expressed genes, spaceflight

759 (FLT) vs. ground control (GC), in Carcass (blue) and Immediate (red) samples (adj. p-
760 value < 0.05).

761 **Figure 2. Enriched Gene Ontology (GO) terms between the RR-1 flight and ground**

762 **groups.** A) Enriched GO terms between the flight and ground control immediate

763 samples (FLT-I vs. GC-I) identified by Gene Set Enrichment Analysis (get set

764 permutation). B) Enriched GO terms between the flight and ground control carcass

765 samples (FLT-C vs. GC-C) identified by Gene Set Enrichment Analysis (gene set

766 permutation). In both A and B, positive or negative enrichment scores indicate higher

767 expression in FLT-C or GC-C samples, respectively. Dot size indicates number of

768 genes within GO term. Dot color indicates FDR. GO terms displayed met the thresholds

769 of FDR < 0.05, NOM p < 0.01, gene set size > 40. C) Venn diagram of the number of

770 enriched GO terms identified in Carcass (blue) and Immediate (red) samples when

771 comparing FLT and GC samples. GO terms in Venn diagram met the threshold of FDR

772 < 0.05, NOM p < 0.01.

773 **Figure 3. Gene expression changes and transcript integrity in Carcass vs.**

774 **Immediate RR-1 NASA liver samples.** A) Volcano plot showing 2,934 differentially

775 expressed genes in all carcass (FLT-C and GC-C, all-C) versus all immediate (FLT-I

776 and GC-I, all-I) samples (adj. p-value < 0.05 and $1.5 < \text{Log}_2 \text{ fold change} < -1.5$). B)

777 Common parent terms of enriched GO terms identified by Gene Set Enrichment

778 Analysis of all carcass versus all immediate samples (phenotype normalized, FDR <

779 0.3, NOM p < 0.01). C) Gene body coverage in Carcass (black) and Immediate (red)

780 FLT and GC samples. D) The percent coverage of the 5' and 3' shaded regions in panel
781 C were used to calculate the 5' to 3' transcript integrity ratio for each sample. All
782 Carcass and Immediate samples are grouped together (** = $p < 0.01$, Mann–Whitney U
783 test). E) Average expression of poly(A) removal genes in Carcass (blue) and Immediate
784 (red) groups from RNAseq data. Cnot1, Cnot2, Cnot3, Cnot4, Cnot9, Cnot10, Cnot6,
785 Cnot6l, Cnot7, and Cnot8 are part of the CCR4-NOT complex. Pan2 and Pan3 are part
786 of the PAN2-PAN3 complex. Error bars indicate standard deviation (* = adj. $p < 0.05$, **
787 = adj. $p < 0.01$, *** = adj. $p < 0.001$, ns = not significant, Wald test).

788 **Figure 4. Evaluation of gene expression and transcript integrity in RR-1 NASA**
789 **carcass-dissected FLT and GC samples prepared via polyA selection and**
790 **ribodepletion methods.** A) Principal component analysis of global gene expression in
791 RR-1 NASA spaceflight (FLT) and ground control (GC) liver samples dissected from
792 frozen carcasses and prepared via ribodepletion. Percent variance for each principal
793 component (PC) is shown. B) Venn diagram of differentially expressed (DE) genes
794 between spaceflight (FLT) and ground control (GC) samples prepared with ribodepletion
795 (black) or polyA selection (red) methods (adj. p -value < 0.05). C) Venn diagram of the
796 number of similar and unique enriched GO terms identified in ribodepleted (black) and
797 polyA selected (red) prepared samples (NOM $p < 0.01$, FDR < 0.5 , phenotype
798 permutation). D) Enriched GO terms between the flight and ground control carcass
799 samples prepared with ribodepletion identified by Gene Set Enrichment Analysis
800 (phenotype permutation). Positive or negative enrichment scores indicate higher

801 expression in FLT or GC samples, respectively. Dot size indicates number of genes
802 within GO term. Dot color indicates FDR. GO terms displayed met the thresholds of
803 $FDR < 0.5$, $NOM\ p < 0.01$, $1.6 < NES < -1.6$. E) Gene body coverage of ribodepleted
804 and polyA-selected FLT and GC carcass samples. F) The percent coverage of the 5'
805 and 3' shaded regions were used to calculate the transcript integrity ratio for each
806 sample (***) = $p < 0.001$, Mann–Whitney U test).

807 **Figure 5. Gene expression and transcript integrity analysis of preservation**
808 **methods for liver and quadriceps samples.** Liver and quadriceps samples were
809 dissected from mice immediately after euthanasia with pentobarbital/phenytoin
810 (Euthasol®) then preserved in dry ice (I_DI), liquid nitrogen (I_LN2), or RNA*later*™
811 (I_RL) before RNAseq analysis. Alternatively, liver and quadriceps samples were
812 dissected from partially thawed frozen carcasses of mice that were euthanized with
813 pentobarbital/phenytoin (Euthasol®) then preserved in dry ice (C_DI), liquid nitrogen
814 (C_LN2), or segmented into thirds and preserved in RNA*later*™ (C_RL) before RNAseq
815 analysis. A) Principal component analysis of liver samples. Percent variance for each
816 principal component (PC) is shown. B) Uniformity of gene body coverage in liver
817 samples. C) Principal component analysis of quadriceps samples. Percent variance for
818 each principal component (PC) is shown. D) Uniformity of gene body coverage in
819 quadriceps samples. (***) = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$, ns = no significance,
820 Mann–Whitney U test).

821 **Tables**

Table 1. Comparisons of carcass preservation methods to immediate liquid nitrogen method on gene expression in livers. The number of differentially expressed genes (DEG) and enriched gene ontology (GO) terms identified by Gene Set Enrichment Analysis (phenotype permutation) were evaluated pairwise in liver samples from different carcass preservation methods compared with immediate samples preserved in liquid nitrogen. For GO terms, number on the left corresponds to the group to the left of the ‘vs.’, and number on the right corresponds to the group to the right of the ‘vs.’ in the “Comparison” column. n numbers, p values, log2 fold changes, and FDR values are indicated. Euth=euthanasia by pentobarbital/phenytoin, I=tissue dissected immediately after euthanasia, C=tissue dissected from frozen carcass that has been partially thawed, DI=dry ice, LN2=liquid nitrogen, RL= RNA/ater™. Data are from GLDS-235.

Comparison	# DEG (adj. p < 0.05)	# DEG (adj. p < 0.05 & Log2 FCI > 1.5)	# Enriched GO terms (NOM p < 0.01)	# Enriched GO terms (FDR < 0.5 & NOM p < 0.01)	# Enriched GO terms (FDR < 0.25 & NOM p < 0.01)
Euth_C_DI (n=6) vs. Euth_I_LN2 (n=6)	3798	3143	16, 0	0, 0	0, 0
Euth_C_LN2 (n=5) vs. Euth_I_LN2 (n=6)	784	515	30, 0	0, 0	0, 0
Euth_C_RL (n=5) vs. Euth_I_LN2 (n=6)	2118	1952	22, 0	0, 0	0, 0

Table 2. Comparisons of carcass preservation methods to immediate liquid nitrogen method on gene expression in quadriceps. The number of differentially expressed genes (DEG) and enriched gene ontology (GO) terms identified by Gene Set Enrichment Analysis (phenotype permutation) were evaluated pairwise in quadriceps samples from different carcass preservation methods compared with immediate samples preserved in liquid nitrogen. For GO terms, the first number corresponds to the group to the left of the ‘vs.’, and the second number corresponds to the group to the right of the ‘vs.’ in the “Comparison” column. n numbers, p values, log₂ fold changes, and FDR values are indicated. Euth=euthanasia by pentobarbital/phenytoin, I=tissue dissected immediately after euthanasia, C=tissue dissected from frozen carcass that has been partially thawed, DI=dry ice, LN2=liquid nitrogen, RL=RNA/ater™. Data are from GLDS-236.

Comparison	# DEG (adj. p < 0.05)	# DEG (adj. p < 0.05 & Log ₂ FCI > 1.5)	# Enriched GO terms (NOM p < 0.01)	# Enriched GO terms (FDR < 0.5 & NOM p < 0.01)	# Enriched GO terms (FDR < 0.25 & NOM p < 0.01)
Euth_C_DI (n=6) vs. Euth_I_LN2 (n=6)	2	0	15, 17	0, 0	0, 0
Euth_C_LN2 (n=6) vs. Euth_I_LN2 (n=6)	36	24	24, 24	0, 22	0, 11 (26 with p < 0.05)
Euth_C_RL (n=6) vs. Euth_I_LN2 (n=6)	2	2	30, 9	1, 2	0, 0

823

824 **Supplemental Information**

825 **Supplemental Figure Legends**

826 **Supplemental Figure 1. Carcass liver samples cluster together and away from immediate liver samples across**
827 **datasets.** Principal component analysis containing data from RR-1 NASA spaceflight (FLT) and respective ground
828 control (GC) carcass (FLT_C_48 and GC_C_48) and immediate (FLT_I_48 and GC_I_48) samples (GLDS-48),
829 RR-1 CASIS FLT (FLT_I_47) and GC (GC_I_47) immediate samples (GLDS-47), and samples from a ground-
830 based study in which livers were dissected immediately after euthanasia then frozen on either dry ice (DI) or
831 submerged in liquid nitrogen (LN2) then stored at -80 °C for either 3 days (LN2_I_3d_49) or 1 year (DI_I_1y_49
832 and LN2_I_1y_49) prior to processing. Related to Figure 1.

833 **Supplemental Figure 2. RNA Integrity Number analysis of Carcass and Immediate Liver samples.** RNA
834 Integrity Numbers (RIN) for spaceflight (FLT) and ground control (GC) immediate (I) and carcass (C) samples
835 plotted against principal component 1 (PC1) calculated from gene expression data. Related to Figures 1&3.

836 **Supplemental Figure 3. Expression of decapping enzymes.** A) Average expression of decapping enzymes in
837 Carcass (blue) and Immediate (red) groups. Error bars indicate standard deviation (* = adj. $p < 0.05$, ** = adj. $p <$
838 0.01 , *** = adj. $p < 0.001$, ns = not significant, Wald test). Related to Figure 3.

839 **Supplemental Figure 4. Freezing study workflow.** Diagram of tissue preservation study to evaluate differences in
840 indicated euthanasia, carcass and tissue preservation methods. Mice were euthanized with either
841 pentobarbital/phenytoin (Euthasol[®]) or ketamine/xylazine injection, or CO₂ inhalation. Intact carcasses were
842 preserved by freezing in liquid nitrogen or on dry ice, or by segmentation (head, chest, abdomen) and immersion in
843 an ammonium sulfate solution (RNAlater[™]). Carcasses were then thawed and livers and quadriceps dissected and
844 preserved in liquid nitrogen or guanidinium thiocyanate solution (Qiagen[®] RLT buffer). Alternatively, livers and
845 quadriceps were dissected immediately and preserved by freezing in liquid nitrogen or on dry ice, or by immersion
846 in an ammonium sulfate solution (RNAlater[™]). Related to Figure 5 and Tables 1&2.

847 **Supplemental Figure 5. Comparison of gene expression and gene ontology in liver and quadriceps samples**
848 **derived from mice euthanized with different methods.** Liver and quadriceps samples dissected from partially
849 thawed frozen carcasses of mice that were euthanized with pentobarbital/phenytoin (Euthasol[®]), ketamine/xylazine,
850 or carbon dioxide inhalation were evaluated for global gene expression differences via principal component analysis
851 (A, liver and B, quadriceps), and the number of differentially expressed genes (DEG) and enriched gene ontology
852 (GO) terms identified by Gene Set Enrichment Analysis via pairwise comparisons (phenotype permutation) (C, liver
853 and D, quadriceps). Liver samples dissected from partially thawed frozen carcasses of mice that were euthanized
854 with pentobarbital/phenytoin (Euthasol[®]), then snap frozen in liquid nitrogen and stored at -80 °C or homogenized in
855 RLT buffer then stored for 70 d at -80 °C were evaluated for E) global gene expression differences via principal
856 component analysis, and F) the number of differentially expressed genes (DEG) and enriched gene ontology (GO)
857 terms identified by Gene Set Enrichment Analysis (phenotype permutation) via pairwise comparisons with
858 immediate samples preserved in liquid nitrogen or RNAlater. For GO terms, the number on the left corresponds to
859 the group to the left of the 'vs.', and number on the right corresponds to the group to the right of the 'vs.' in the
860 "Comparison" column. n numbers, p values, log₂ fold changes, and FDR values are indicated. Euth=euthanasia by
861 pentobarbital/phenytoin (Euthasol[®]), Ket-Xyl= euthanasia by ketamine/xylazine, CO₂=euthanasia by carbon dioxide
862 inhalation, C=tissue dissected from frozen carcass that has been partially thawed, I=tissue dissected immediately
863 after euthanasia, DI=dry ice, LN2=liquid nitrogen, RL=RNAlater[™]. Liver and quadriceps data are from GLDS-235
864 and GLDS-236, respectively. Related to Figure 5 and Tables 1&2.

865 **Supplemental Figure 6. RR-1 mission timeline.** Timeline indicating major events in the Rodent Research-1 (RR-
866 1) mission relative to the launch date (L). A minus sign (-) indicates time in days (d) before launch and a plus sign
867 (+) indicates time in days (d) or months (m) after launch. Age-matched ground control animals were processed on
868 similar timeline but on a 4-day delay to mimic spaceflight conditions. KSC = Kennedy Space Center; ISS =
869 International Space Station; CASIS = Center for the Advancement of Science in Space; NASA = National
870 Aeronautics and Space Administration. Related to Figures 1-4.

871 **Supplemental Figure 7. GLDS-49 Experimental Design.** Diagram of the ground-based preservation study
 872 comparing livers collected using standard laboratory protocols with livers collected from simulated spaceflight
 873 dissection flow and storage methods. Liver samples from twelve-week old female C57BL/6J mice were either snap
 874 frozen (n=3), snap frozen after a 25 min delay and stored for 3 days (n=3), or snap frozen after a 25 min delay and
 875 stored for 1 year (n=3). RNA-seq data were then generated using a polyA enrichment protocol. Related to Figure 1.

876 **Supplementary Tables**

Table S1. Liver samples analyzed in the ground-based tissue preservation study. Related to Figure 5A&B and Table 1.

Group	Euthanasia	Carcass preservation	Tissue preservation	Mouse ID
1	Pentobarbital/phenytoin	None	Liquid nitrogen n=6	M1-M6
			RNAlater™ n=6	
			Dry ice n=6	
2	Pentobarbital/phenytoin	Dry ice	Liquid nitrogen n=6	M7-M12
			RLT buffer 70d at -80 °C n=4	
3	Pentobarbital/phenytoin	Liquid nitrogen	Liquid nitrogen n=5	M13-M18
4	Pentobarbital/phenytoin	RNAlater™ ^a	Liquid nitrogen n=5	M19-M24
5	Ketamine/xylazine	Dry ice	Liquid nitrogen n=6	M25-M30
6	Carbon dioxide	Dry ice	Liquid nitrogen n=6	M31-M36

a. Post carcass segmentation

877

Table S2. Quadriceps samples analyzed in the ground-based tissue preservation study. Related to Figure 5C&D and Table 2.

Group	Euthanasia	Carcass preservation	Tissue preservation	Mouse ID
			Liquid nitrogen n=6	

1	Pentobarbital/phenytoin	None	RNAlater™ n=6	M1-M6
			Dry ice n=6	
2	Pentobarbital/phenytoin	Dry ice	Liquid nitrogen n=6	M7-M12
3	Pentobarbital/phenytoin	Liquid nitrogen	Liquid nitrogen n=6	M13-M18
4	Pentobarbital/phenytoin	RNAlater™ ^a	Liquid nitrogen n=6	M19-M24
5	Ketamine/xylazine	Dry ice	Liquid nitrogen n=6	M25-M30
6	Carbon dioxide	Dry ice	Liquid nitrogen n=6	M31-M36

a. Post carcass segmentation

878

Table S3. Comparisons of immediate preservation methods on gene expression in livers. The number of differentially expressed genes (DEG) and enriched gene ontology (GO) terms identified by Gene Set Enrichment Analysis (phenotype permutation) were evaluated pairwise in liver samples from different immediate preservation methods. For GO terms, the first number corresponds to the group to the left of the ‘vs.’, and second number corresponds to the group to the right of the ‘vs.’ in the “Comparison” column. n numbers, p values, log2 fold changes, and FDR values are indicated. Euth=euthanasia by pentobarbital/phenytoin, I=tissue dissected immediately after euthanasia, DI=dry ice, LN2=liquid nitrogen, RL=RNAlater™. Data are from GLDS-235. Related to Figure 5A&B and Table 1.

Comparison	# DEG	# DEG	# Enriched GO terms	# Enriched GO terms	# Enriched GO terms
	(adj. p < 0.05)	(adj. p < 0.05 & Log2 FC > 1.5)	(NOM p < 0.01)	(FDR < 0.5 & NOM p < 0.01)	(FDR < 0.25 & NOM p < 0.01)
Euth_I_DI (n=6) vs. Euth_I_LN2 (n=6)	16	16	31, 6	7, 0	0, 0
Euth_I_DI (n=6) vs. Euth_I_RL (n=6)	0	0	14, 8	0, 0	0, 0
Euth_I_LN2 (n=6) vs. Euth_I_RL (n=6)	14	14	3, 15	0, 0	0, 0

879

Table S4. Comparisons of carcass preservation methods to immediate RNAlater™ method on gene expression in livers. The number of differentially expressed genes (DEG) and enriched gene ontology (GO) terms identified by Gene Set Enrichment Analysis (phenotype permutation) were evaluated pairwise in liver samples from different carcass preservation methods compared with immediate samples preserved in RNAlater™. For GO terms, the first number corresponds to the group to the left of the ‘vs.’, and the second number corresponds to the group to the right of the ‘vs.’ in the “Comparison” column. n numbers, p values, log2 fold changes, and FDR values are indicated. Euth=euthanasia by pentobarbital/phenytoin, I=tissue dissected immediately after euthanasia, C=tissue dissected from frozen carcass that has been partially thawed, DI=dry ice, LN2=liquid nitrogen, RL= RNAlater™. Data are from GLDS-235. Related to Figure 5A&B and Table 1.

Comparison	# DEG (adj. p < 0.05)	# DEG (adj. p < 0.05 & Log2 FC > 1.5)	# Enriched GO terms (NOM p < 0.01)	# Enriched GO terms (FDR < 0.5 & NOM p < 0.01)	# Enriched GO terms (FDR < 0.25 & NOM p < 0.01)
Euth_C_DI (n=6) vs. Euth_I_RL (n=6)	930	718	40, 1	1, 0	1, 0
Euth_C_LN2 (n=5) vs. Euth_I_RL (n=6)	197	118	90, 0	2, 0	0, 0
Euth_C_RL (n=5) vs. Euth_I_RL (n=6)	131	123	65, 0	0, 0	0, 0

880

Table S5. Comparisons of immediate preservation methods on gene expression in quadriceps. The number of differentially expressed genes (DEG) and enriched gene ontology (GO) terms identified by Gene Set Enrichment Analysis (phenotype permutation) were evaluated pairwise in quadriceps samples from different immediate preservation methods. For GO terms, number on the left corresponds to the group to the left of the ‘vs.’, and number on the right corresponds to the group to the right of the ‘vs.’ in the “Comparison” column. n numbers, p values, log2 fold changes, and FDR values are indicated. Euth=euthanasia by pentobarbital/phenytoin, I=tissue dissected immediately after euthanasia, DI=dry ice, LN2=liquid nitrogen, RL= RNAlater™. Data are from GLDS-236. Related to Figure 5C&D and Table 2.

Comparison	# DEG (adj. p < 0.05)	# DEG (adj. p < 0.05 & Log2 FC > 1.5)	# Enriched GO terms (NOM p < 0.01)	# Enriched GO terms (FDR < 0.5 & NOM p < 0.01)	# Enriched GO terms (FDR < 0.25 & NOM p < 0.01)
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Euth_I_DI (n=6) vs. Euth_I_LN2 (n=6)	2	1	2, 51	1, 2	0, 2
Euth_I_DI (n=6) vs. Euth_I_RL (n=6)	14	9	25, 26	6, 0	2, 0
Euth_I_LN2 (n=6) vs. Euth_I_RL (n=6)	0	0	66, 3	51, 0	0, 0

881

Table S6. Comparisons of carcass preservation methods to immediate RNAlater™ method on gene expression in quadriceps. The number of differentially expressed genes (DEG) and enriched gene ontology (GO) terms identified by Gene Set Enrichment Analysis (phenotype permutation) were evaluated pairwise in quadriceps samples from different carcass preservation methods compared with immediate samples preserved in RNAlater™. For GO terms, number on the left corresponds to the group to the left of the ‘vs.’, and number on the right corresponds to the group to the right of the ‘vs.’ in the “Comparison” column. n numbers, p values, log2 fold changes, and FDR values are indicated. Euth=euthanasia by pentobarbital/phenytoin, I=tissue dissected immediately after euthanasia, C=tissue dissected from frozen carcass that has been partially thawed, DI=dry ice, LN2=liquid nitrogen, RL= RNAlater™. Data are from GLDS-236. Related to Figure 5C&D and Table 2.

Comparison	# DEG (adj. p < 0.05)	# DEG (adj. p < 0.05 & Log2 FC > 1.5)	# Enriched GO terms (NOM p < 0.01)	# Enriched GO terms (FDR < 0.5 & NOM p < 0.01)	# Enriched GO terms (FDR < 0.25 & NOM p < 0.01)
Euth_C_DI (n=6) vs. Euth_I_RL (n=6)	50	27	80, 5	67, 1	33 (41 with p < 0.05), 0
Euth_C_LN2 (n=6) vs. Euth_I_RL (n=6)	282	139	54, 8	4, 0	0, 0
Euth_C_RL (n=6) vs. Euth_I_RL (n=6)	59	40	135, 3	135, 1	34 (41 with p < 0.05), 0

882

883 **Supplemental Materials and Methods**

884 **GLDS-49 Ground-based Freezing Study**

885 To compare standard laboratory protocols for tissue freezing and storage with a spaceflight timeline-simulated liver
886 dissection and long-term storage, liver samples from twelve-week old female C57BL/6J mice (Jackson laboratories,
887 Bar harbor, ME) were either immediately snap frozen (in liquid nitrogen), snap frozen after a 25 min delay and
888 stored for 3 days, or snap frozen after a 25 min delay and stored for 1 year (Figure S7).

889 *Sample Collection*

890 The liver tissues of twelve-week-old C57BL/6J mice (Jackson laboratories, Bar harbor, ME) were received from the
891 Rodent Research project collected in a ground-based preservation and storage study (Choi et al., 2016). Three
892 groups of livers were included: 1) Liver tissues dissected and frozen on dry ice 25 min after euthanizing with
893 pentobarbital/phenytoin (Euthasol®) followed by cervical dislocation. At the time of RNA extraction, the liver
894 tissues had been stored at -80 °C for around 1-year (DI_I_1y_49); 2) Liver tissues dissected 3 min after euthanizing
895 with pentobarbital/phenytoin (Euthasol®) followed by cervical dislocation and snap-freezing in liquid nitrogen. At
896 the time of RNA extraction, the liver tissues had been stored at -80 °C for around 1-year (LN_I_1y_49); 3) Liver
897 tissues dissected 3 minutes after euthanizing with pentobarbital/phenytoin (Euthasol®) followed by cervical
898 dislocation and snap-freezing in liquid nitrogen. At the time of RNA extraction, the liver tissues had been stored at -
899 80 °C for only 3 days (LN_I_3d_49). This group served as a positive control for delayed dissection and long-term
900 storage.

901 *RNA Isolation*

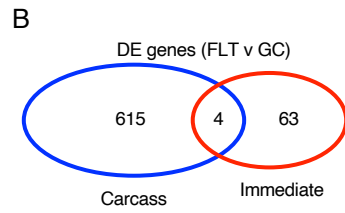
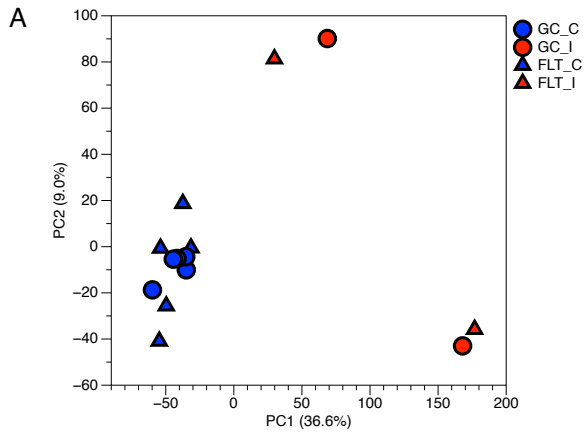
902 RNA was isolated using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's
903 protocol. Briefly, homogenization buffer was made by adding 1:100 volume ratio of beta-mercaptoethanol to RLT
904 buffer and kept on ice until use. Approximately 30 mg of tissue was cut using a sterile scalpel and immediately
905 placed in 800 µL of the RLT buffer solution. Each sample was then homogenized for approximately 20 seconds at
906 21,000 RPM using a Polytron PT1300D handheld homogenizer with a 5 mm standard dispersing aggregate tip
907 (Kinematica, Bohemia, NY). Homogenates were centrifuged for 3 minutes at room temperature at 15,000 RPM to
908 remove cell debris. The supernatant from each sample was used to isolate and purify RNA following the
909 manufacturer's protocol including on-column DNase treatment with RNase-free DNase (Qiagen, Valencia, CA).
910 RNA was eluted twice per sample in 30 µL RNase- and DNase-free H₂O per elution. Concentration and absorbance
911 ratios were measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA
912 quality was assessed using the Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano Kit (Agilent
913 Technologies, Santa Clara, CA).

914 *Library Preparation and RNA-Sequencing*

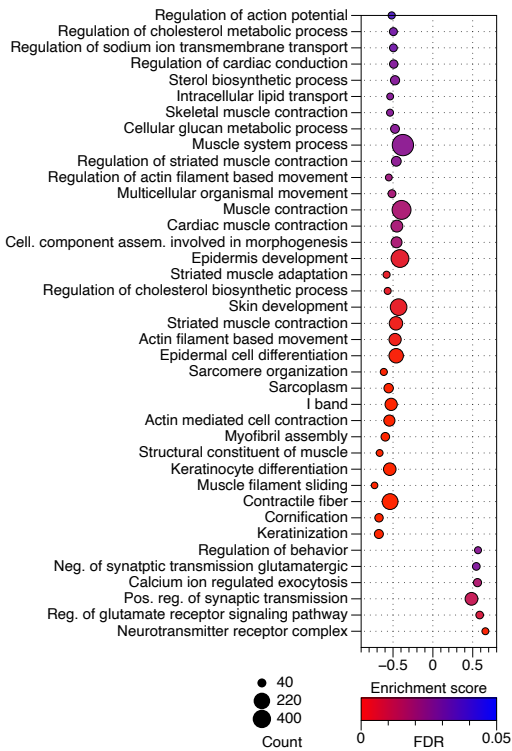
915 Samples with RNA Integrity Number (RIN) of 9 or above were sent to the University of California (UC), Davis
916 Genome Center where the libraries were constructed and RNA-sequencing was performed. Libraries were generated
917 using the Illumina TruSeq Stranded RNA library prep kit (Illumina, San Diego, CA) after polyA selection, and
918 sequencing was done with 50 bp single end reads on the Illumina HiSeq 3000 platform.

919

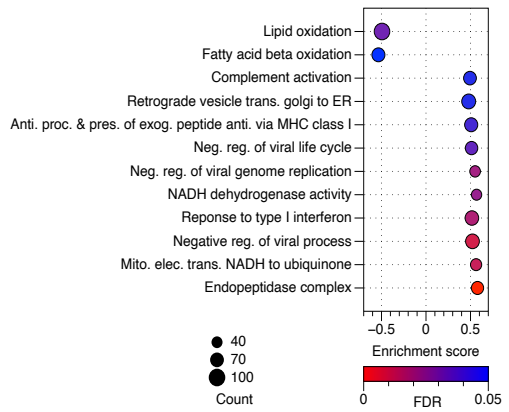
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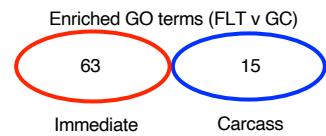
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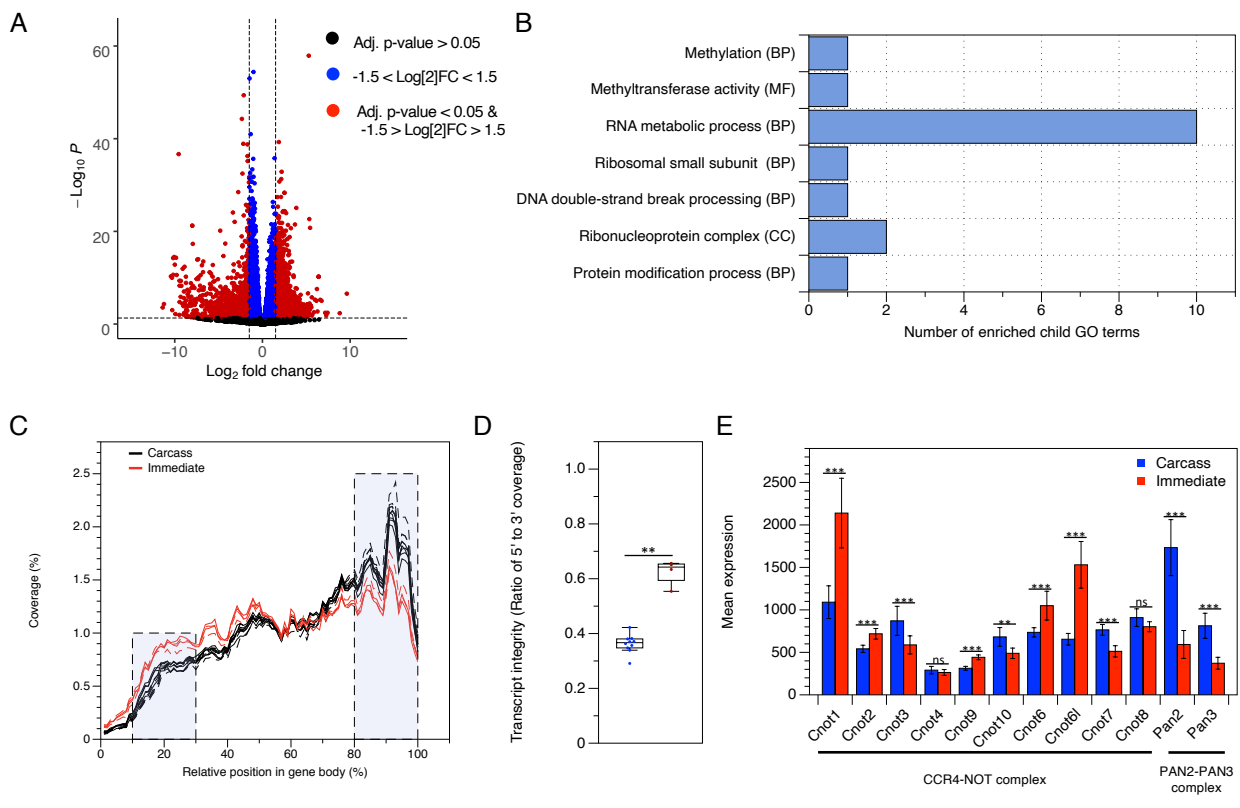


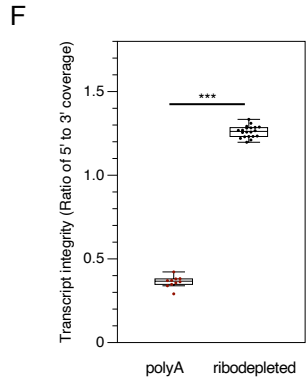
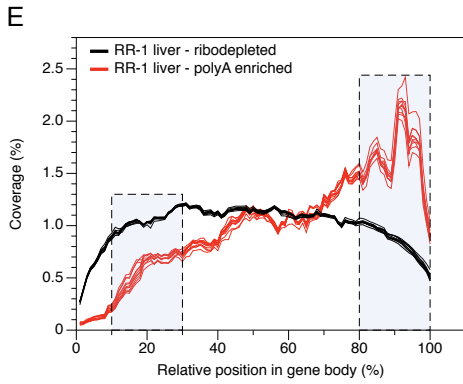
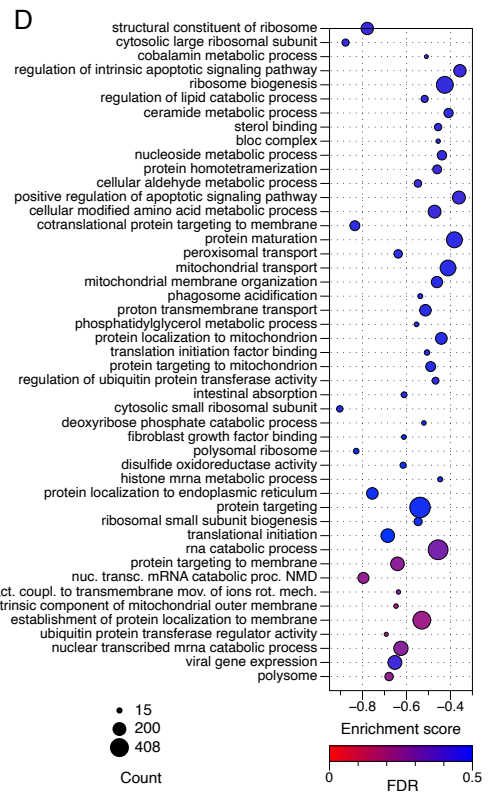
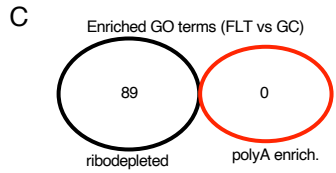
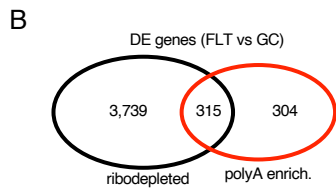
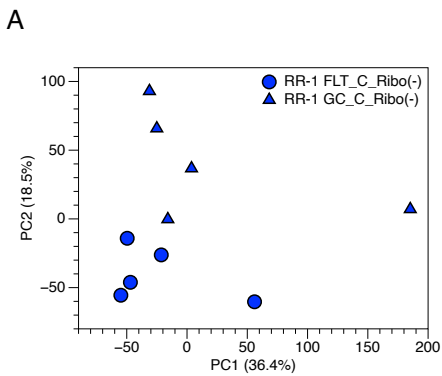
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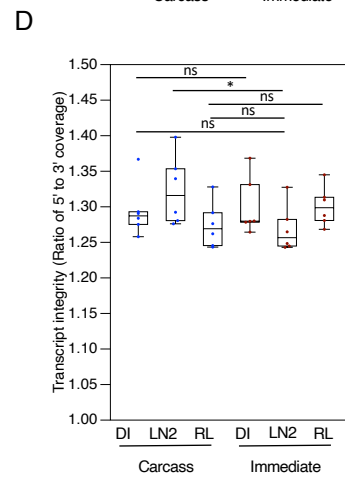
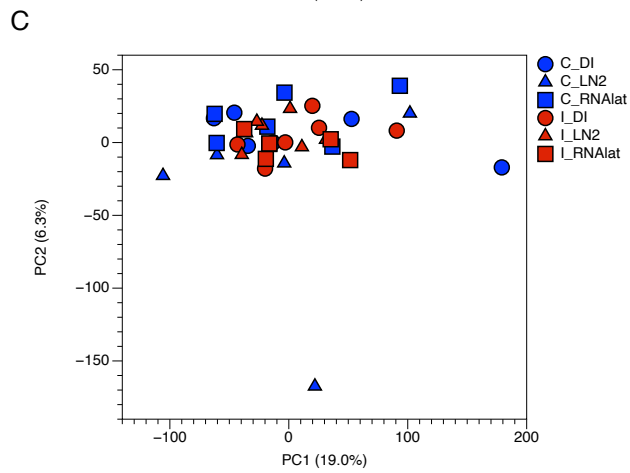
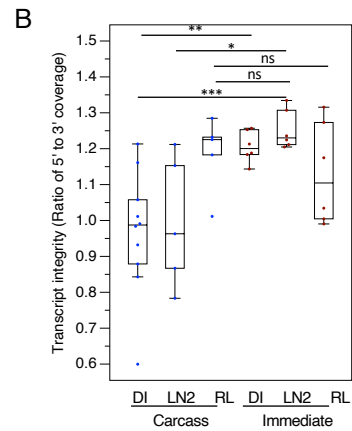
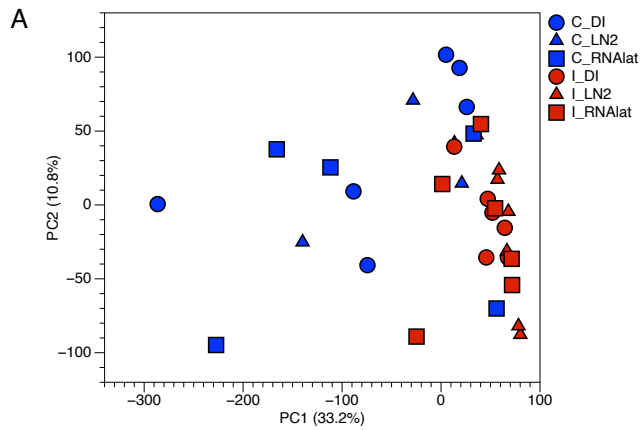


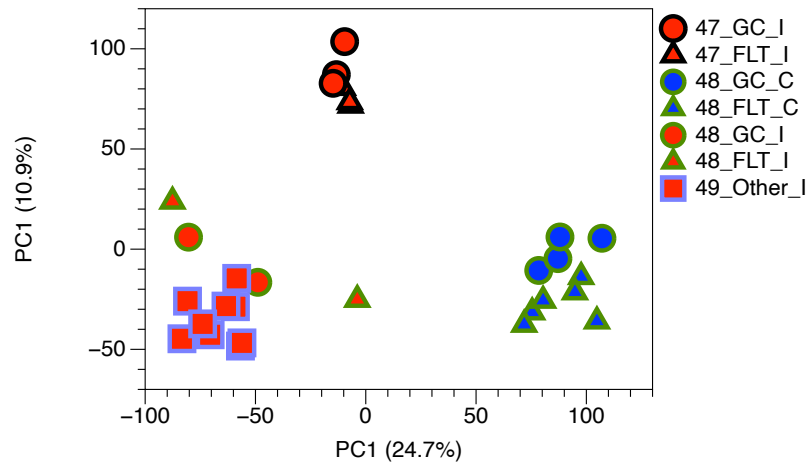
C

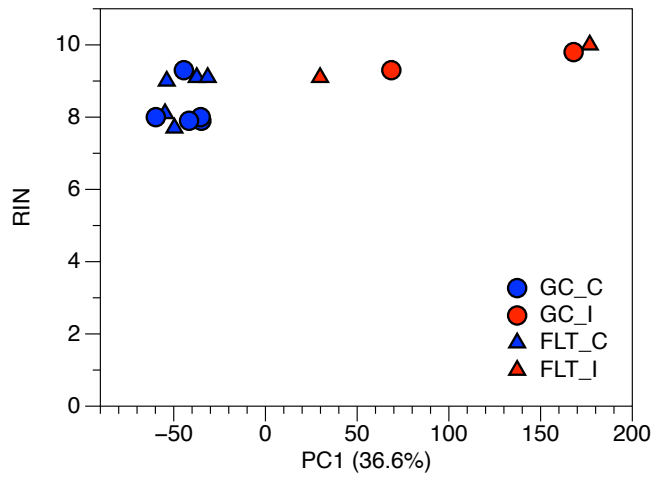


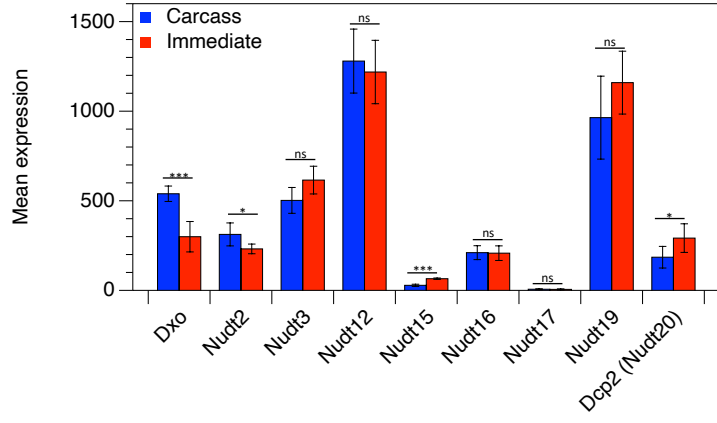


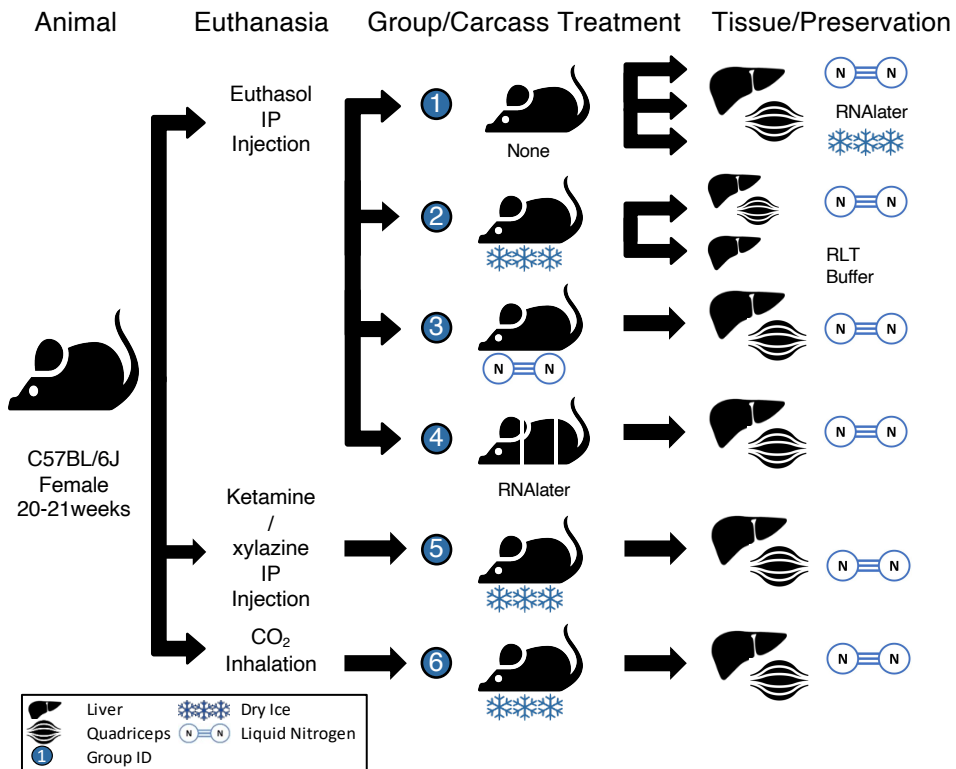




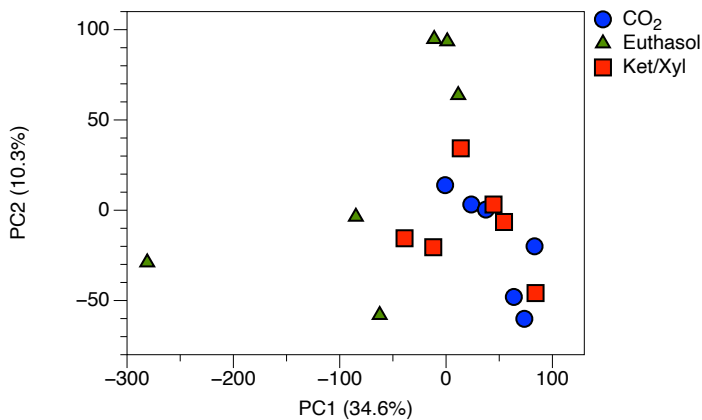




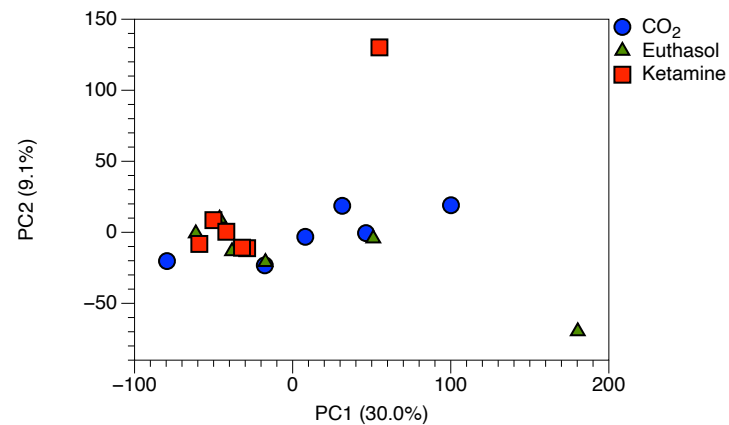




A



B



C

Comparison of euthanasia methods for Carcass liver samples

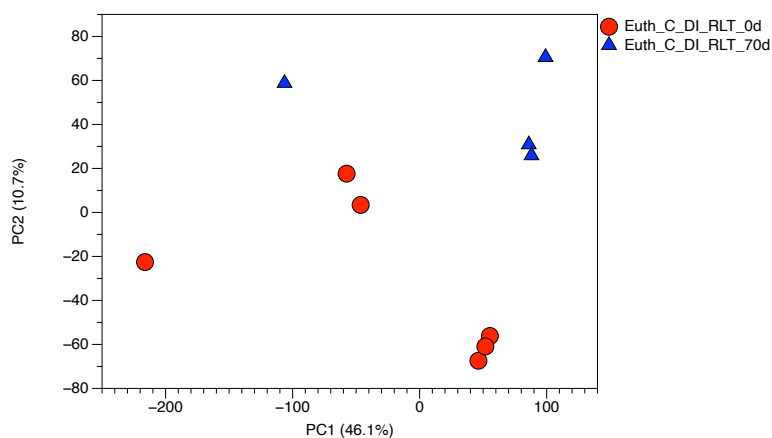
Comparison	# DEG (adj. p < 0.05)	# DEG (adj. p < 0.05 & Log ₂ FCI > 1.5)	# Enriched GO terms (NOM p < 0.01)	# Enriched GO terms (FDR < 0.5 & NOM p < 0.01)	# Enriched GO terms (FDR < 0.25 & NOM p < 0.01)
CO ₂ _C_DI (n=6) v Euth_C_DI (n=6)	192	168	0, 42	0, 0	0, 0
CO ₂ _C_DI (n=6) v Ket-Xyl_C_DI (n=6)	0	0	0, 51	0, 0	0, 0
Euth_C_DI (n=6) v Ket-Xyl_C_DI (n=6)	67	49	37, 0	1, 0	0, 0

D

Comparison of euthanasia methods for Carcass quadriceps samples

Comparison	# DEG (adj. p < 0.05)	# DEG (adj. p < 0.05 & Log ₂ FCI > 1.5)	# Enriched GO terms (NOM p < 0.01)	# Enriched GO terms (FDR < 0.5 & NOM p < 0.01)	# Enriched GO terms (FDR < 0.25 & NOM p < 0.01)
CO ₂ _C_DI (n=6) v Euth_C_DI (n=6)	2	2	8, 23	2, 0	1, 0
CO ₂ _C_DI (n=6) v Ket-Xyl_C_DI (n=6)	0	0	5, 100	2, 99	0, 4
Euth_C_DI (n=6) v Ket-Xyl_C_DI (n=6)	3	3	3, 64	0, 10	0, 0

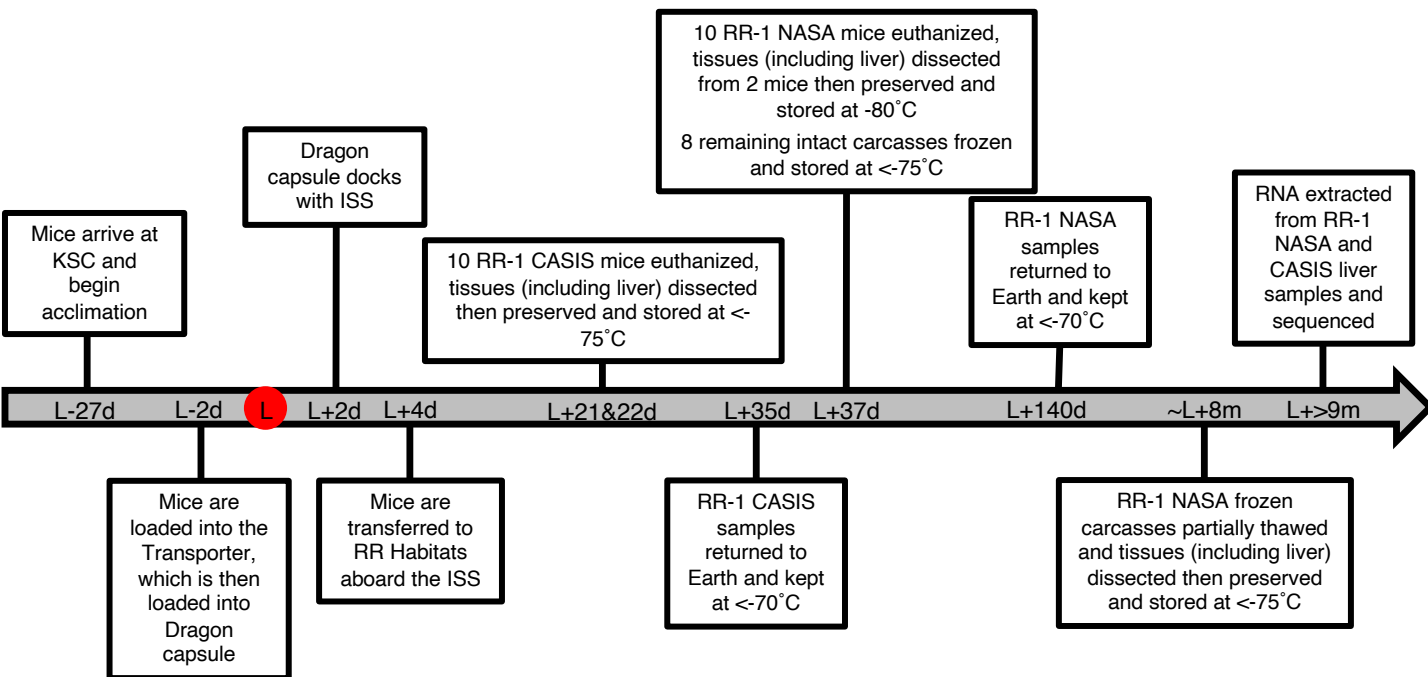
E

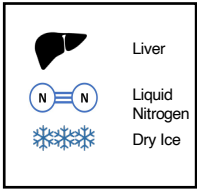


F

Tissue storage methods vs. RNAIat and LN2 preserved Immediate liver samples

Comparison	# DEG (adj. p < 0.05)	# DEG (adj. p < 0.05 & Log ₂ FCI > 1.5)	# Enriched GO terms (NOM p < 0.01)	# Enriched GO terms (FDR < 0.5 & NOM p < 0.01)	# Enriched GO terms (FDR < 0.25 & NOM p < 0.01)
Euth_C_DI_RLT-70d (n=4) v Euth_C_DI_RLT-0d (n=6)	284	174	3, 110	0, 31	0, 2
Euth_C_DI_RLT-70d (n=4) v Euth_I_LN2 (n=6)	1523	1250	67, 14	0, 0	0, 0
Euth_C_DI_RLT-70d (n=4) v Euth_I_RL (n=6)	1003	752	62, 18	3, 0	0, 0
Euth_C_DI_RLT-0d (n=6) v Euth_I_LN2 (n=6)	3798	3143	16, 0	0, 0	0, 0
Euth_C_DI_RLT-0d (n=6) v Euth_I_RL (n=6)	930	718	40, 1	1, 0	1, 0

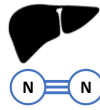




C57BL/6J
Female
12 weeks

Euthasol
IP Injection

ASAP
(~2
minutes)



Store at -80°C for 3
days

Store at -80°C for 1 year

25
minutes



Store at -80°C for 1 year