RESEARCH ARTICLE

Impact of Anesthesia and Euthanasia on Metabolomics of Mammalian Tissues: Studies in a C57BL/6J Mouse Model

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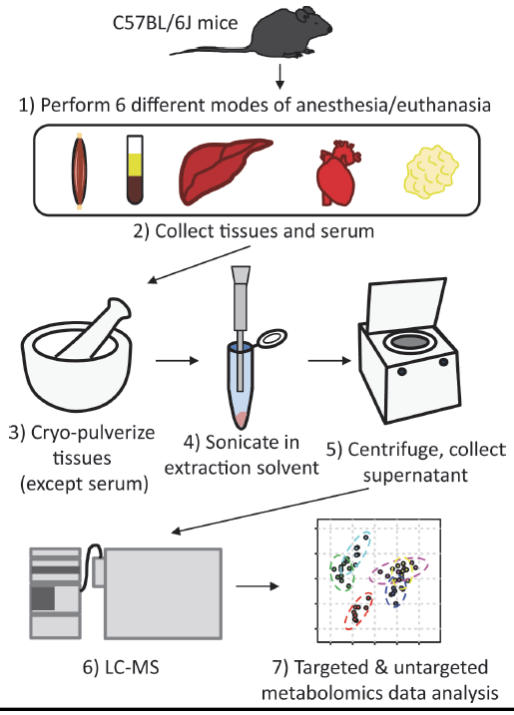
Presented by Katie Gibbs

Sample Preparation is Key

- Sample extraction and instrumental analysis methods are well documented in metabolomics.
- Understanding the changes in metabolome in response to method of sample collection is limited.
- How might mode of anesthesia or euthanasia affect metabolite profiles of collected tissues?

Objective of study

- Systematically examine the effect of commonly used methods of anesthesia and euthanasia on metabolome of tissues in male C57BL/6J mice
- Untargeted and targeted profiling of polar metabolites using HILIC-ESI-MS



Animal Model

C57BL/6J mice

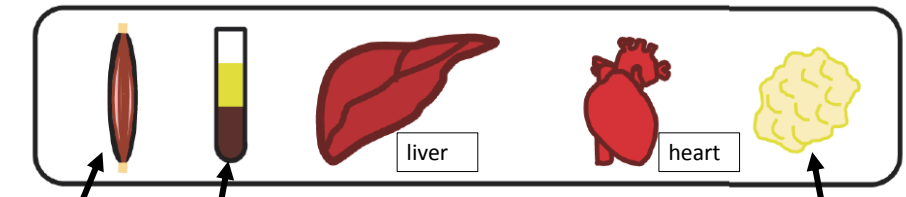
- Male C57BL/6J mice from Jackson Labs
  - 20 weeks of age
  - ~27 g body weight
  - 12:12 light:dark
  - Standard chow and water *ad libitum*
  - Fasted 5 hrs before tissue collection

C57BL/6J mice

n = 8 mice per mode

1) Perform 6 different modes of anesthesia/euthanasia

Mode	Method	Time to collect
Euthanasia	Cervical Dislocation	10 s to death
Euthanasia	100% Carbon dioxide	2.5 min to death
Euthanasia	Isoflurane overdose	2 min to death
Anesthesia	Continuous isoflurane 4% to 2%	1.5 min
Anesthesia	Ketamine (100mg/mL) IP 120 mg/kg dose	20 min
Anesthesia	Pentobarbital (50mg/mL) IP 60mg/kg dose	15 min



2) Collect tissues and serum

Gastrocnemius muscle

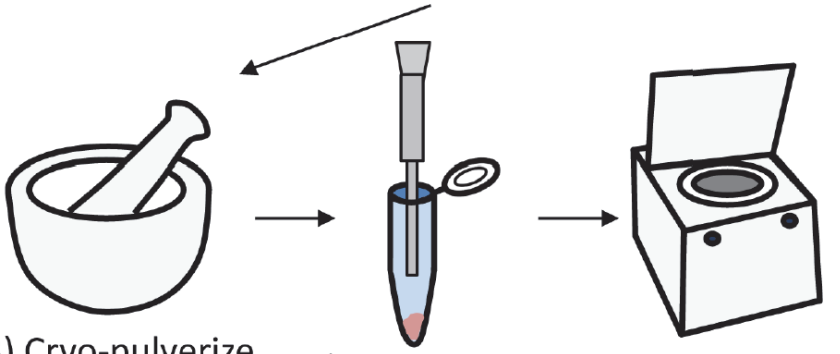
Arterial blood from descending aorta (600 uL)

Epididymal white adipose tissue

Questions:
What type of container?
How long stored at -80C?

- Tissues rinsed rapidly in DI water, blotted, frozen by immersion in liquid nitrogen (10 s)
- Blood allowed to clot on ice for 15 min, centrifuge 15 min @ 3000xg, supernatant frozen
- Collection time of 3 minutes for all tissues and serum; stored at -80C

2) Collect tissues and serum

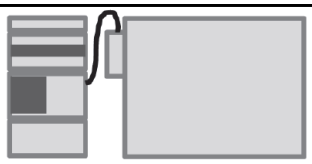


3) Cryo-pulverize tissues (except serum)

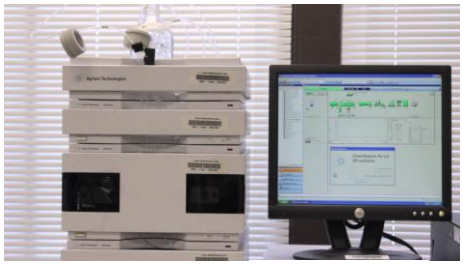
4) Sonicate in extraction solvent

5) Centrifuge, collect supernatant

Tissue Extraction solvent: single-phase mix
7 parts methanol: 2 parts water: 1 part chloroform
Blood serum extraction solvent:
1:1:1 methanol: acetonitrile: acetone
Both solvents contained 13C-labeled internal standards



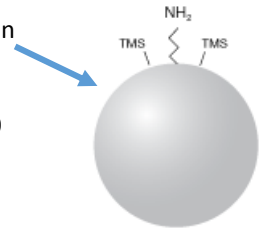
Agilent 1200 LC System

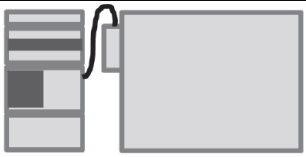


6) LC-MS

Chromatographic method:
HILIC-anion exchange separation
(polar compounds)

Column:	Phenomenex Luna 3µ NH_2 column 2.1 x 150 mm
Mobile Phase A:	acetonitrile
Mobile Phase B:	5 mM ammonium acetate pH 9.9
Gradient:	0 min = linear from 20 to 80% B 15 min = 100% B hold 3 min 18.1 min = return to 20% B 30 min = stop
Injection volume:	25 µL
Flow rate:	0.25 mL/min; column at 25°C, auto sampler 4°C





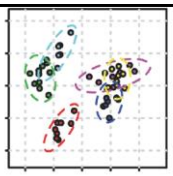
Agilent 6220 TOF MS

6) LC-MS

Time of Flight (TOF):
High resolution mass spec

MS:	Electrospray ionization in negative ion mode
Full scan:	m/z range 50 – 1200 Da
Data acquisition rate:	1 scan/sec
Source parameters:	drying gas temp 350°C, flow rate 10L/min nebulizer pressure 30 psig capillary voltage 3500 V

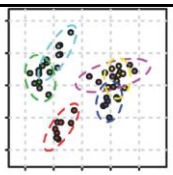
Untargeted metabolite screening: data pre-processing



7) Targeted & untargeted metabolomics data analysis

1. Raw LC-MS data converted from Agilent.d format to mzXML format and imported to MZmine 2.10
2. Mass detection: centroid mass detector noise level at 1.0E3
3. Chromatogram builder to generate peaks
   - min time span 0.2 min, height 1.0E3, m/z tolerance 0.002 m/z or 20 ppm
4. Chromatograms smoothed
   - filter width 5

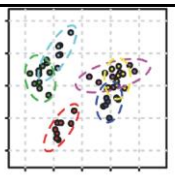
Untargeted metabolite screening: data pre-processing



7) Targeted & untargeted metabolomics data analysis

5. Chromatogram deconvolution performed using noise amplitude algorithm
   - min peak height 5.0E3, peak duration 0 – 25 min, noise amplitude 2.0E3
6. Isotopic peaks grouped
   - m/z tolerance of 20 ppm
   - Retention time tolerance (RTT) 0.1 min
   - max charge of 2
   - representative isotope set as most intense
7. Retention time normalization
   - m/z tolerance of 20 ppm
   - RTT 1.0 min
   - min standard intensity 1.0E4

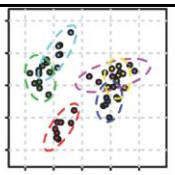
Untargeted metabolite screening: data pre-processing



7) Targeted & untargeted metabolomics data analysis

8. Chromatograms aligned into peak list
   - join aligner
   - m/z tolerance of 0.005 m/z or 50 ppm
   - weight for m/z 50
   - RTT 1.5 min with weight of 50
9. Gap filling with peak finder algorithm
   - intensity tolerance 25%
   - m/z tolerance 20 ppm
   - RTT 1.0 min and RT correction enabled
10. Duplicate peak filter applied
    - remove peaks w/in m/z tolerance of 0.01 m/z or 50ppm
    - RTT 0.5 min

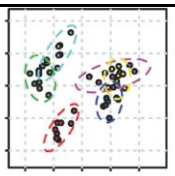
Untargeted metabolite screening: data pre-processing



7) Targeted & untargeted metabolomics data analysis

11. Peak list rows filter
    - only peaks in 75% of all samples
    - 1 peak min per isotope pattern
    - m/z range set automatically
    - RT range 1.0 – 25.0 min
    - peak duration 0.1 – 2.0 min
12. Visual inspection of peak shapes
    - artifacts discarded

Untargeted metabolite screening: statistical analysis

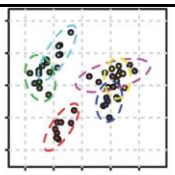


7) Targeted & untargeted metabolomics data analysis

Metabolanalyst

- Upload peak intensity table
- Filtered by interquartile range
- Normalized by median intensity
- Log transformed
- Principal component analysis (PCA)
- Partial least squares discriminant analysis (PLS-DA)

Targeted metabolite analysis



7) Targeted & untargeted metabolomics data analysis

Agilent MassHunter Quantitative Analysis software

- Compared accurate mass and retention time with that of authentic standards analyzed using same method
- Relative quantitation: peak area
- Absolute quantitation: selected metabolites; peak areas measured relative to the peak areas of 13C-labeled internal standards
    - six-point calibration curves for standards

Untargeted Metabolomics

- PCA
- PLS-DA

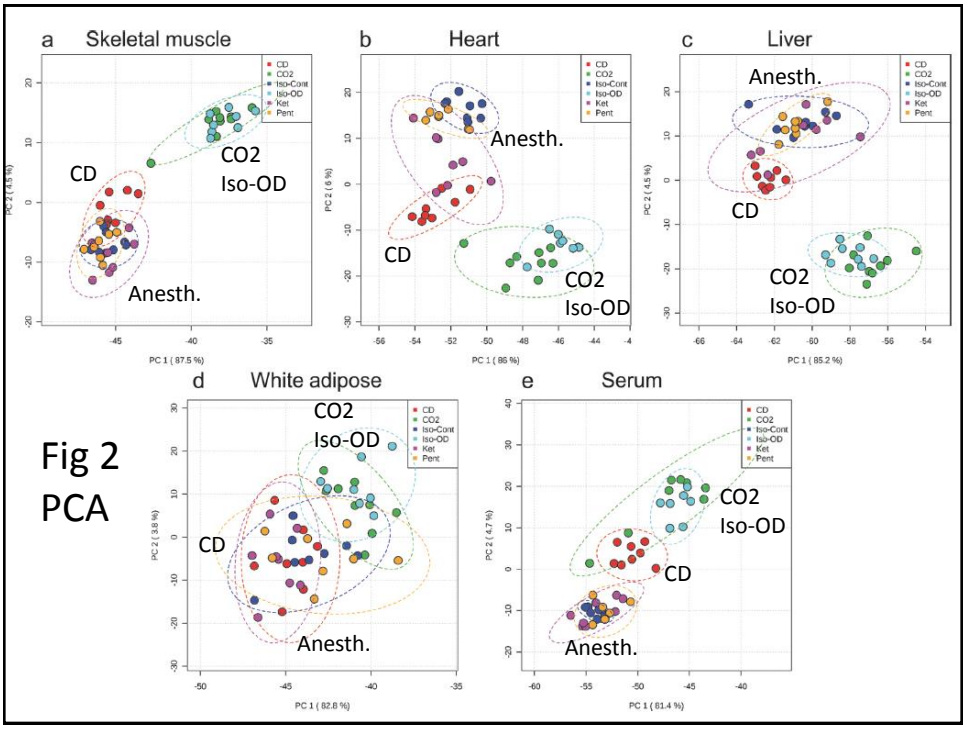


Fig 2
PCA

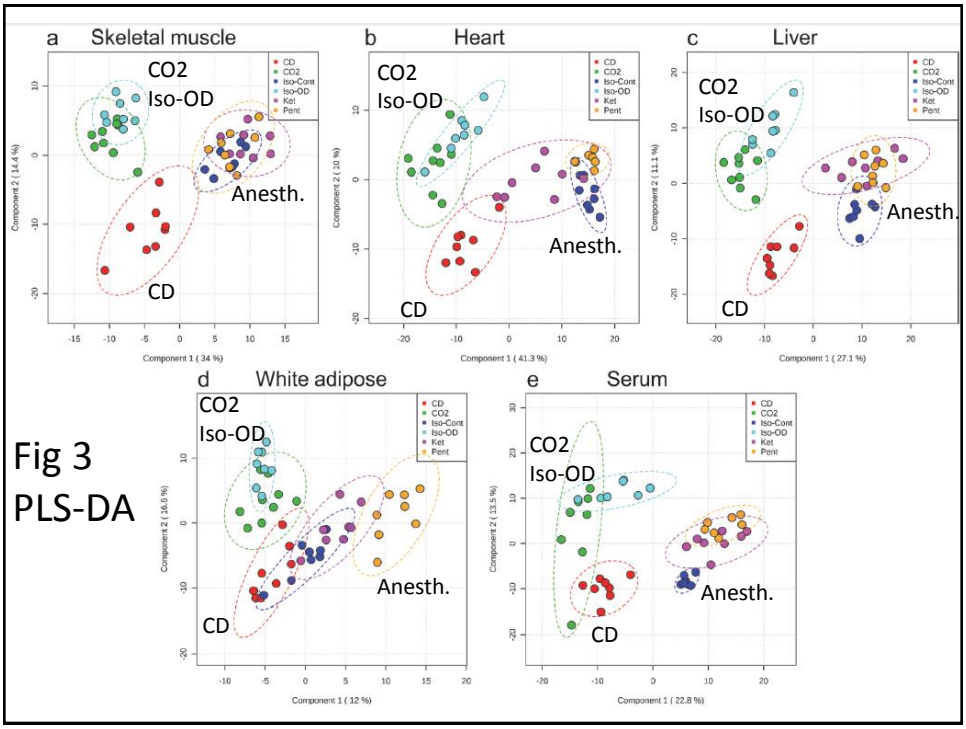


Fig 3
PLS-DA

Untargeted Metabolomics

- Variable importance in projection (VIP) scores
  - Based on PLS-DA classification
  - Higher scores contribute to greater class separation
- Searched m/z values of top features against Human Metabolome Database (HMDB)
  - Mass accuracy of 20 ppm with top 10 listed Table 1

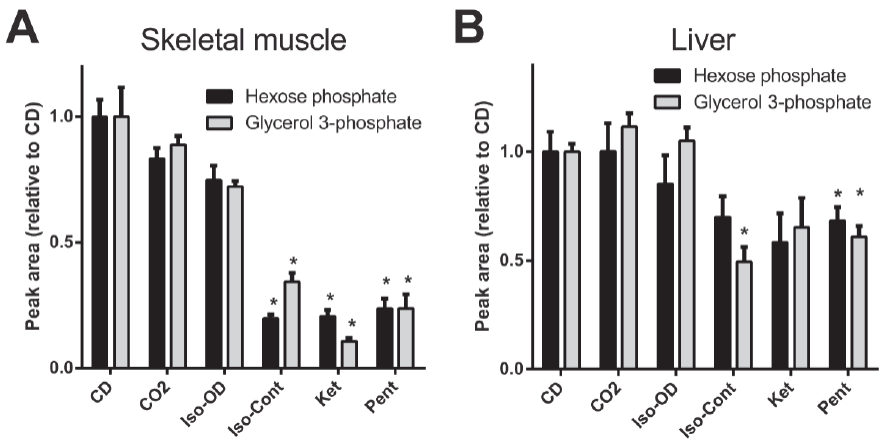
Putative matches

- Skeletal muscle
  - Glycolytic metabolites, phosphocreatinine, phosphocreatine
- Liver and adipose tissue
  - Lipid species
- Common across multiple tissues:
  - Succinic acid, glycerol-3-phosphate, inosine monophosphate, ceramide phosphates
- No validation of untargeted approach

Targeted approach

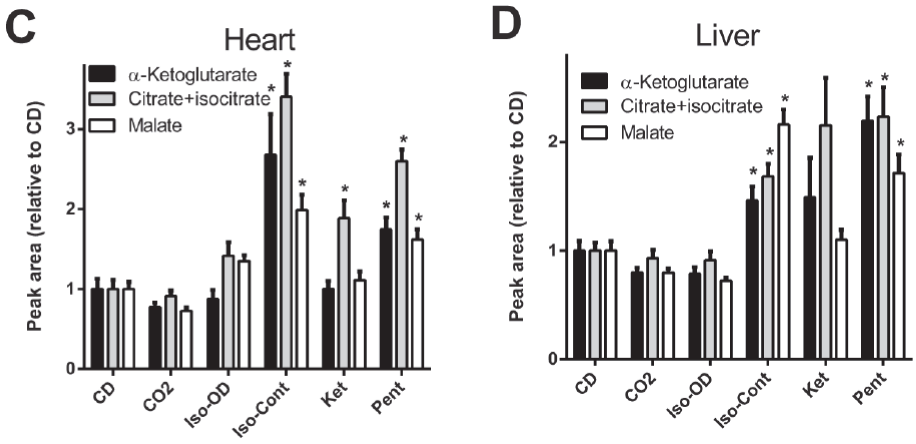
- 112 known polar metabolites
  - Quantitated by peak area
  - Accurate mass and retention time compared to authentic standards previously run
- Absolute concentrations of 21 metabolites that matched 13C-labeled internal standards
- "…data consistent with the effects of hypoxia brought about by the absence of respiration and blood circulation in euthanized animals."

Glycolysis and gluconeogenesis
- higher lactate levels for euthanized mice
- hexose phosphate and glycerol 3-phosphate



*** Figure 5 Metabolites identified by accurate mass and retention time compared to known standards previously run on the HILIC-LC-MS platform

TCA cycle



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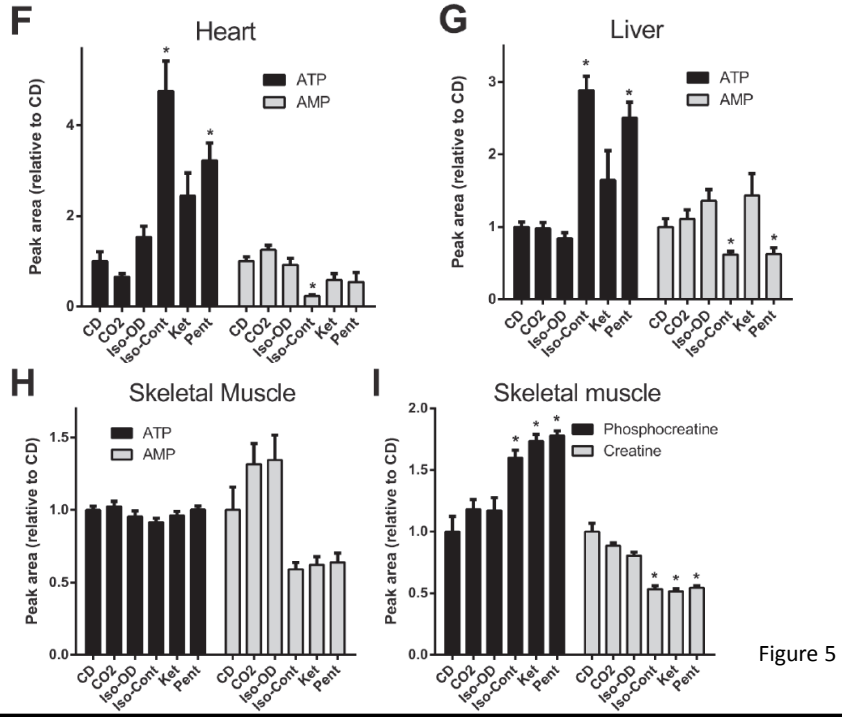

Figure 5

Conclusions

- Results consistent with literature on the effects of anesthesia and/or euthanasia on rodent tissue metabolism.
- Can we believe the data?
  - Putative metabolites from untargeted approach
  - No validation of putative metabolites
  - Targeted approach used in lieu of validation?
  - Many metabolites identified based on previously run standards.