Supplemental Material

Dietary Macronutrients Modulate the Fatty Acyl Composition of Rat Liver Mitochondrial Cardiolipins

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Supplemental inventory

Supplemental Figures

Five supplemental figures, each matches cognate figure in main text, presents data by glycemic index instead of by primary fat. Will be helpful by individuals who want to visually test certain implications of our work

Supplemental Text and Statistical tables

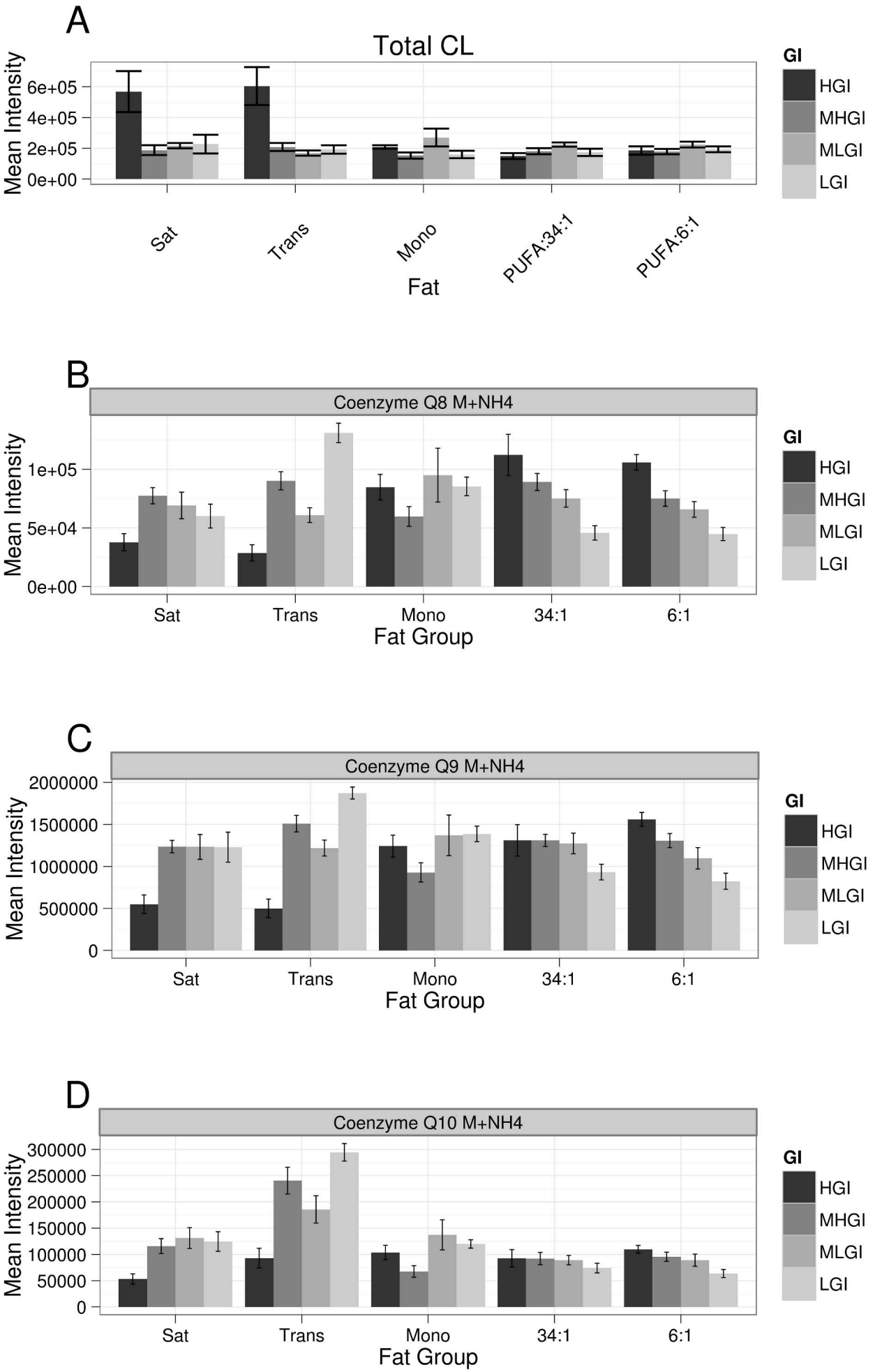
Supplemental Figure Legends

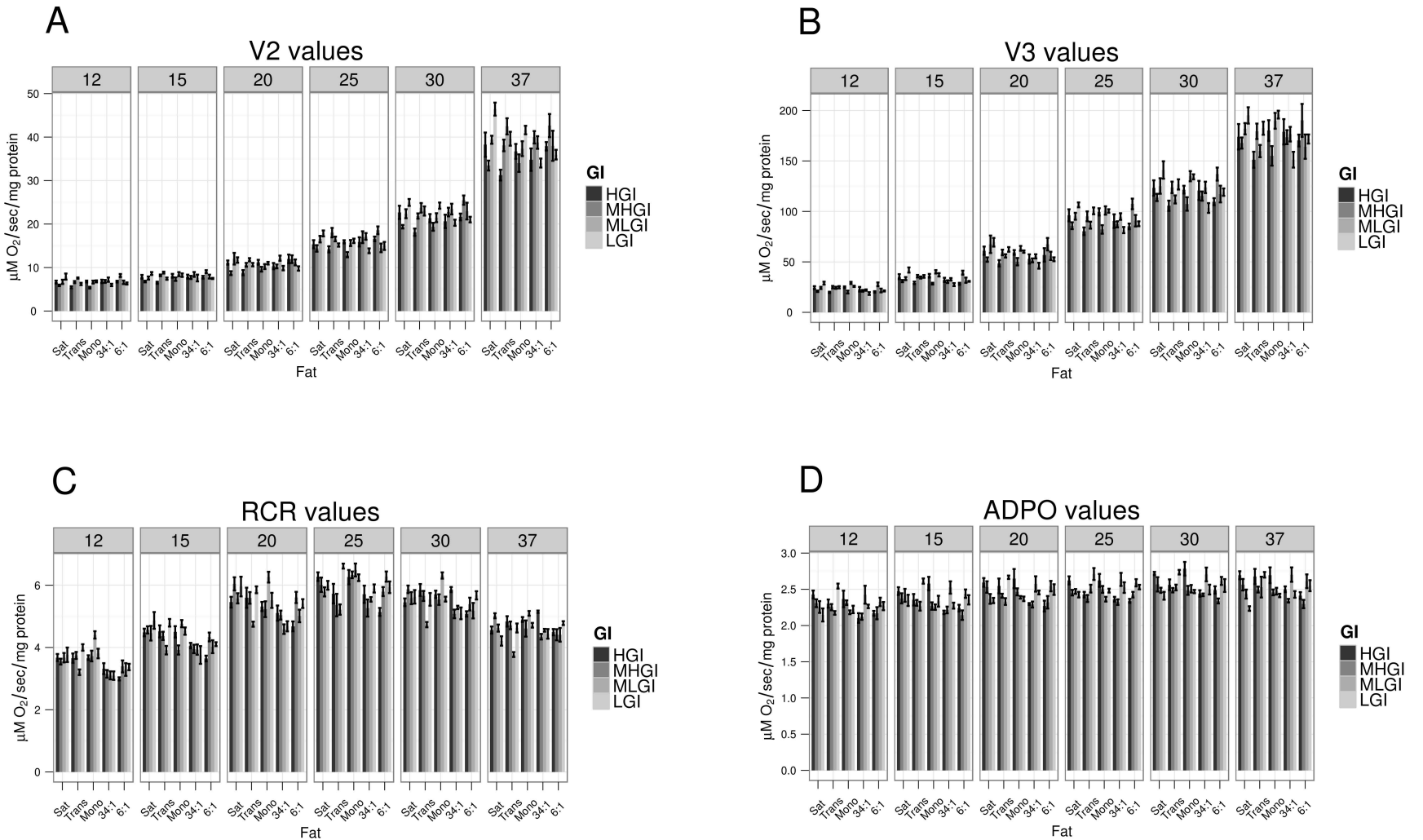
Post-Hoc statistical tables

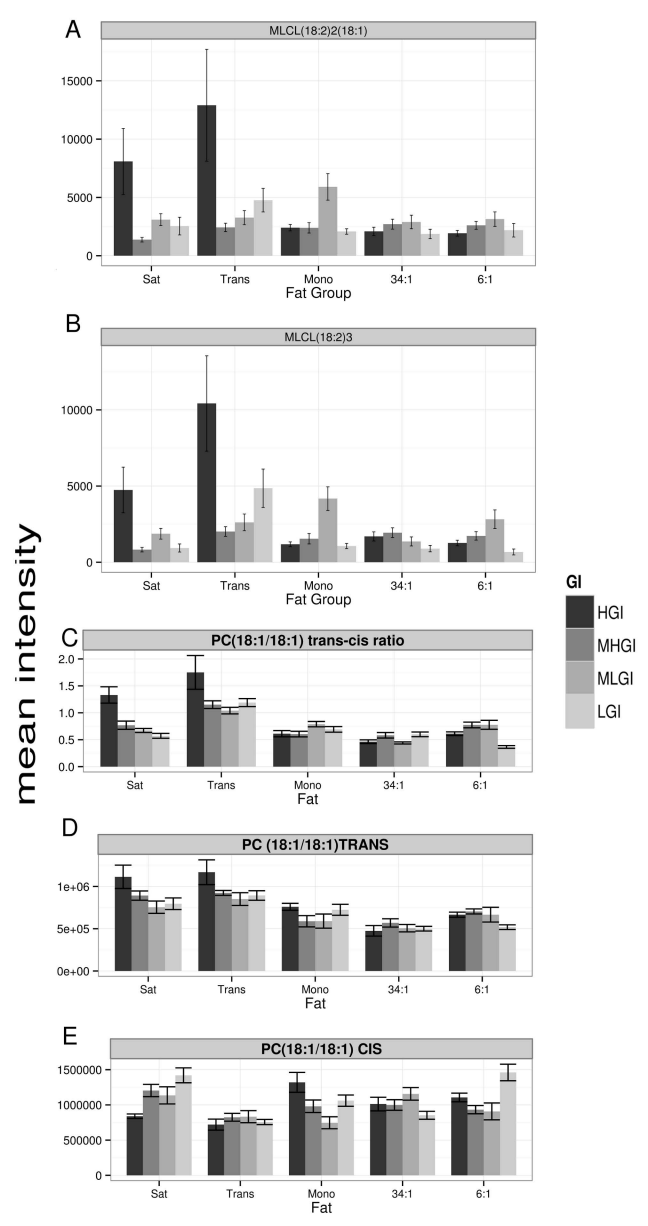
Provided for individuals who wish to consider specific subset analyses

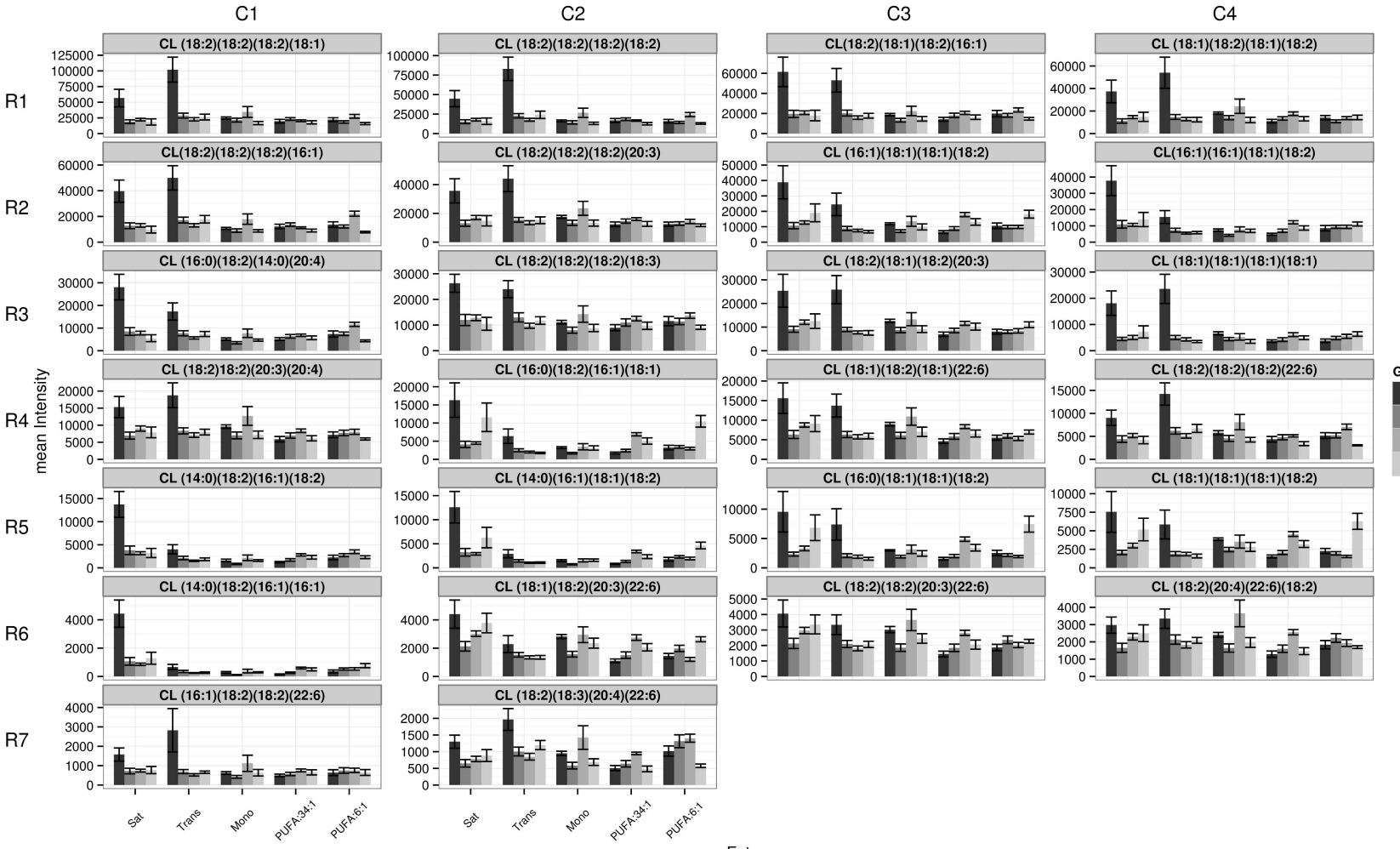
Supplemental Animal protocol

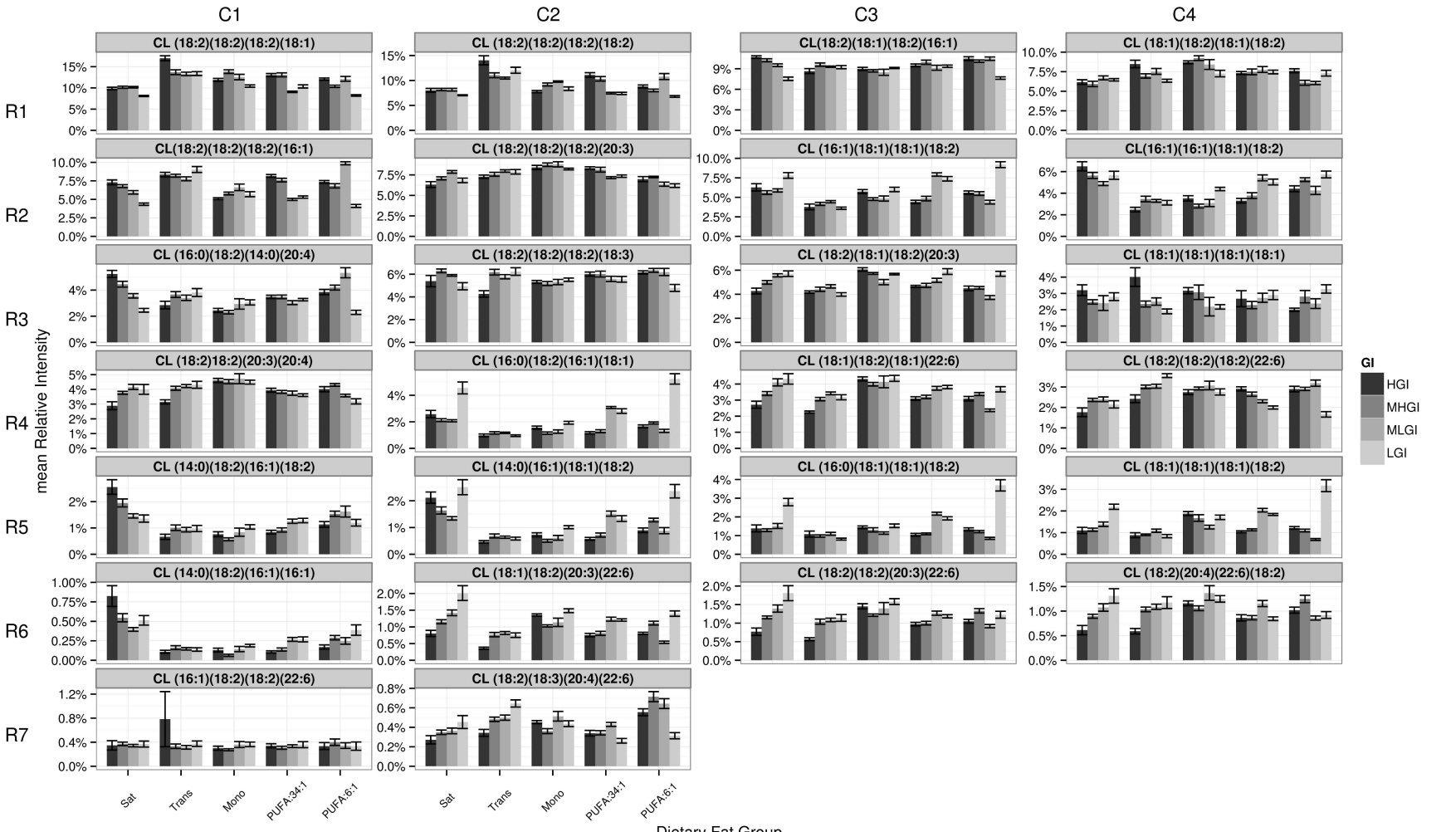
Details of animal work, using ARRIVE criteria


A
Total CL
Mean Intensity
6e+05
4e+05
2e+05
0e+00
Sat
Trans
Mono
PUFA:34:1
PUFA:6:1
Fat
GI
HGI
MHGI
MLGI
LGI
B
Coenzyme Q8 M+NH4
Mean Intensity
1e+05
5e+04
0e+00
Sat
Trans
Mono
34:1
6:1
Fat Group
GI
HGI
MHGI
MLGI
LGI
C
Coenzyme Q9 M+NH4
Mean Intensity
2000000
1500000
1000000
500000
0
Sat
Trans
Mono
34:1
6:1
Fat Group
GI
HGI
MHGI
MLGI
LGI
D
Coenzyme Q10 M+NH4
Mean Intensity
300000
250000
200000
150000
100000
50000
0
Sat
Trans
Mono
34:1
6:1
Fat Group
GI
HGI
MHGI
MLGI
LGI


A
V2 values
12
15
20
25
30
37
µM O2/sec/mg protein
Sat
Trans
Mono
34:1
6:1
Fat
GI
HGI
MHGI
MLGI
LGI
B
V3 values
C
RCR values
D
ADPO values


A
MLCL(18:2)2(18:1)
15000
10000
5000
0
Sat
Trans
Mono
34:1
6:1
Fat Group
B
MLCL(18:2)3
10000
5000
0
Sat
Trans
Mono
34:1
6:1
Fat Group
mean intensity
GI
HGI
MHGI
MLGI
LGI
C
PC(18:1/18:1) trans-cis ratio
2.0
1.5
1.0
0.5
0.0
Sat
Trans
Mono
34:1
6:1
Fat
D
PC (18:1/18:1)TRANS
1e+06
5e+05
0e+00
Sat
Trans
Mono
34:1
6:1
Fat
E
PC(18:1/18:1) CIS
1500000
1000000
500000
0
Sat
Trans
Mono
34:1
6:1
Fat


C1
C2
C3
C4
R1
R2
R3
R4
R5
R6
R7
mean Intensity
CL (18:2)(18:2)(18:2)(18:1)
CL (18:2)(18:2)(18:2)(18:2)
CL(18:2)(18:1)(18:2)(16:1)
CL (18:1)(18:2)(18:1)(18:2)
CL(18:2)(18:2)(18:2)(16:1)
CL (18:2)(18:2)(18:2)(20:3)
CL (16:1)(18:1)(18:1)(18:2)
CL(16:1)(16:1)(18:1)(18:2)
CL (16:0)(18:2)(14:0)(20:4)
CL (18:2)(18:2)(18:2)(18:3)
CL (18:2)(18:1)(18:2)(20:3)
CL (18:1)(18:1)(18:1)(18:1)
CL (18:2)18:2)(20:3)(20:4)
CL (16:0)(18:2)(16:1)(18:1)
CL (18:1)(18:2)(18:1)(22:6)
CL (18:2)(18:2)(18:2)(22:6)
CL (14:0)(18:2)(16:1)(18:2)
CL (14:0)(16:1)(18:1)(18:2)
CL (16:0)(18:1)(18:1)(18:2)
CL (18:1)(18:1)(18:1)(18:2)
CL (14:0)(18:2)(16:1)(16:1)
CL (18:1)(18:2)(20:3)(22:6)
CL (18:2)(18:2)(20:3)(22:6)
CL (18:2)(20:4)(22:6)(18:2)
CL (16:1)(18:2)(18:2)(22:6)
CL (18:2)(18:3)(20:4)(22:6)
Sat
Trans
Mono
PUFA:34:1
PUFA:6:1
Fat
GI
HGI
MHGI
MLGI
LGI


C1
C2
C3
C4
R1
R2
R3
R4
R5
R6
R7
CL (18:2)(18:2)(18:2)(18:1)
CL (18:2)(18:2)(18:2)(18:2)
CL(18:2)(18:1)(18:2)(16:1)
CL (18:1)(18:2)(18:1)(18:2)
CL(18:2)(18:2)(18:2)(16:1)
CL (18:2)(18:2)(18:2)(20:3)
CL (16:1)(18:1)(18:1)(18:2)
CL(16:1)(16:1)(18:1)(18:2)
CL (16:0)(18:2)(14:0)(20:4)
CL (18:2)(18:2)(18:2)(18:3)
CL (18:2)(18:1)(18:2)(20:3)
CL (18:1)(18:1)(18:1)(18:1)
CL (18:2)18:2)(20:3)(20:4)
CL (16:0)(18:2)(16:1)(18:1)
CL (18:1)(18:2)(18:1)(22:6)
CL (18:2)(18:2)(18:2)(22:6)
CL (14:0)(18:2)(16:1)(18:2)
CL (14:0)(16:1)(18:1)(18:2)
CL (16:0)(18:1)(18:1)(18:2)
CL (18:1)(18:1)(18:1)(18:2)
CL (14:0)(18:2)(16:1)(16:1)
CL (18:1)(18:2)(20:3)(22:6)
CL (18:2)(18:2)(20:3)(22:6)
CL (18:2)(20:4)(22:6)(18:2)
CL (16:1)(18:2)(18:2)(22:6)
CL (18:2)(18:3)(20:4)(22:6)
mean Relative Intensity
Sat
Trans
Mono
PUFA:34:1
PUFA:6:1
Dietary Fat Group
GI
HGI
MHGI
MLGI
LGI

Supplemental legends

Figure 1 Supplemental: Diets coupling high glycemic index with saturated or trans fatty acids are associated with elevations in mitochondrial cardiolipins and decreased mitochondrial prenols

Panel A: Total cardiolipins by diet (mean +/- sem, relative intensity in arbitrary units); Panels B,C, and D: Mitochondrial levels of ubiquinone$_8$, ubiquinone$_9$, and ubiquinone$_{10}$. Within a panel, diets are broken down by dietary fat and then, within each dietary fat group, by GI. See text for ANOVA p values and supplemental for post-hoc tests.

Figure 2 Supplemental: Respiration parameters are essentially unchanged across the diets

Panel A: Oxygen consumption in V_2 (with substrate, no ADP); Panel B: Oxygen consumption in V_3 (with substrate and ADP); Panel C: respiratory control ratio defined as V_3/V_2. (RCR as V3/V2); Panel D: ADP/O ratio. Within a panel, diets are broken down by dietary fat and then, within each dietary fat group, by GI. (N~8 for all studies, mean±sem). See text for statistical discussion.

Figure 3 Supplemental: Despite respiratory stability, diets coupling high glycemic index with saturated or trans fatty acids are associated with elevated mitochondrial oxidative stress

Panels A and B: Levels of MLCL $(18:2)_2(18:1)$ and MLCL $(18:2)_3$, respectively; Panel C-E: *trans/cis* ratio (Panel C) of mitochondrial PC (18:1/18:1) *trans* (Panel D) relative to PC (18:1/18:1)*cis* (panel E). Within a panel, diets are broken down by dietary fat and then, within each dietary fat group, by GI. (N=8 for all bars [N=160 total], mean±sem). See text for ANOVA p values and supplemental for post-hoc tests.

Figure 4 Supplemental: The Effects of Macronutrient shifts on CL levels are broad but not uniform

Absolute mass spectrometry signal of each CL is presented in each of 26 panels. The Y-axis scale is varied so as to highlight the inter-CL species changes across the diets, thus each panel has a different Y axis scaling. Abundance is highest in the upper left corner and decreases moving right and then down. Each panel's title includes the CL fatty acid makeup, with the first two and last two located on the same phosphatidylglycerol moiety. Within a panel, diets are broken down by dietary fat and then, within each dietary fat group, by GI. Mean +/- sem, N=8/diet, 160 total.

Figure 5 Supplemental: Changes in the relative abundance of the CL pool

Abundance of each CL as a percentage of the total CLs (normalized at the level of the individual animals) is presented in each of 26 panels (mean +/- sem, N=8/diet, 160 total). Specifics as in Figure 4

Post-Hoc Statistics.

Post-hoc analysis of the specific key ANOVA comparisons of coenzymes Q_8, Q_9, and Q_{10}, the cis and trans 18:1 species and their ratio, and the MLCLs is provided below. Note that, because N=8 for any comparison, power is low, and thus some false negatives (Type II statistical errors) are expected. Specifically, the differences in means would have to be ~ 1.5 SD for typical statistical parameters, α=0.05, β=0.8; for 1 1 StDev difference at α=0.05, the β=0.48, meaning only ~50% of truly different groups would be recognized (determined using PASS 6.0). All values shown are corrected for multiple comparisons by FDR.

Q10 - pvalues with FDR correction

Sat-HGI	vs	Trans-HGI	vs
1.98E-007	Trans-MLGI	2.92E-004	Trans-MLGI
1.17E-003	Mono-MLGI	1.22E-001	Mono-MLGI
2.92E-003	Sat-MLGI	1.71E-001	Sat-HGI
7.19E-003	Sat-LGI	1.89E-001	Sat-MLGI
1.28E-002	Mono-LGI	2.94E-001	Sat-LGI
2.03E-002	Sat-MHGI	3.36E-001	6:1-LGI
4.02E-002	6:1-HGI	3.74E-001	Mono-LGI
7.05E-002	Mono-HGI	4.02E-001	Mono-MHGI
1.44E-001	6:1-MHGI	4.59E-001	Sat-MHGI
1.71E-001	Trans-HGI	5.40E-001	34:1-LGI
1.78E-001	34:1-HGI	5.96E-001	6:1-HGI
1.81E-001	34:1-MHGI	7.44E-001	Mono-HGI
2.22E-001	34:1-MLGI	9.15E-001	34:1-MLGI
2.22E-001	6:1-MLGI	9.15E-001	6:1-MLGI
4.96E-001	34:1-LGI	9.32E-001	6:1-MHGI
6.46E-001	Mono-MHGI	9.81E-001	34:1-MHGI
7.51E-001	6:1-LGI	9.89E-001	34:1-HGI
1.28E-012	Trans-MHGI	4.62E-014	Trans-LGI
1.35E-017	Trans-LGI	8.06E-009	Trans-MHGI

Trans-CIS - pvalues with FDR correction

Sat-HGI	vs	Trans-HGI	vs
8.94E-009	34:1-HGI	1.05E-015	34:1-HGI
1.52E-004	Sat-MHGI	1.53E-004	Trans-LGI
1.69E-004	6:1-MHGI	5.08E-003	Sat-HGI
1.80E-004	6:1-MLGI	1.21E-013	34:1-MHGI
2.45E-004	Mono-MLGI	1.41E-010	Sat-MHGI
5.08E-003	Trans-HGI	1.59E-010	6:1-MHGI
6.14E-002	Trans-MLGI	1.66E-010	6:1-MLGI
2.90E-001	Trans-MHGI	1.86E-006	Trans-MLGI
3.90E-001	Trans-LGI	2.16E-013	34:1-LGI
1.11E-006	34:1-LGI	2.25E-013	Mono-MHGI
1.20E-006	Mono-MHGI	2.40E-013	6:1-HGI
1.44E-006	6:1-HGI	2.40E-013	Mono-HGI
1.442406936E	Mono-HGI	2.42E-010	Mono-MLGI
1.81E-005	Mono-LGI	2.85E-012	Sat-MLGI
2.18E-010	6:1-LGI	4.00E-017	6:1-LGI
3.80E-009	34:1-MLGI	5.55E-016	34:1-MLGI
4.70E-007	Sat-LGI	5.89E-005	Trans-MHGI
6.18E-007	34:1-MHGI	6.08E-012	Mono-LGI
9.61E-006	Sat-MLGI	9.72E-014	Sat-LGI

CoEnzymes

Q8 - pvalues with FDR correction

Sat-HGI	vs	Trans-HGI	vs
9.94E-006	34:1-HGI	6.37E-007	34:1-HGI
6.19E-004	Mono-MLGI	2.29E-004	Trans-MHGI
1.69E-003	Trans-MHGI	2.77E-004	34:1-MHGI
2.01E-003	34:1-MHGI	6.97E-004	Mono-LGI
4.59E-003	Mono-LGI	7.71E-004	Mono-HGI
5.23E-003	Mono-HGI	3.59E-003	Sat-MHGI
1.72E-002	Sat-MHGI	5.48E-003	34:1-MLGI
2.65E-002	34:1-MLGI	5.48E-003	6:1-MHGI
2.66E-002	6:1-MHGI	1.57E-002	Sat-MLGI
6.73E-002	Sat-MLGI	2.67E-002	6:1-MLGI
9.91E-002	6:1-MLGI	6.03E-002	Trans-MLGI
1.79E-001	Trans-MLGI	6.73E-002	Sat-LGI
1.99E-001	Sat-LGI	7.03E-002	Mono-MHGI
2.06E-001	Mono-MHGI	3.42E-001	34:1-LGI
6.07E-001	Trans-HGI	3.67E-001	6:1-LGI
6.53E-001	34:1-LGI	6.07E-001	Sat-HGI
6.98E-001	6:1-LGI	3.14E-009	Trans-LGI
4.83E-005	6:1-HGI	4.71E-006	6:1-HGI
4.89E-008	Trans-LGI	6.60E-005	Mono-MLGI

Cis Trans PC(18:1)(18:1)

CIS - pvalues with FDR correction

Sat-HGI	vs	Trans-HGI	vs
1.01E-004	Sat-LGI	1.24E-003	Sat-MHGI
1.32E-003	Mono-HGI	3.56E-003	34:1-MLGI
1.63E-002	Sat-MHGI	6.01E-003	Sat-MLGI
3.91E-002	34:1-MLGI	1.05E-002	6:1-HGI
5.28E-002	Sat-MLGI	2.56E-002	Mono-LGI
8.51E-002	6:1-HGI	5.57E-002	34:1-HGI
1.58E-001	Mono-LGI	6.93E-002	34:1-MHGI
2.85E-001	34:1-HGI	9.10E-002	Mono-MHGI
3.30E-001	34:1-MHGI	1.75E-001	6:1-MHGI
3.85E-001	Mono-MHGI	2.43E-001	6:1-MLGI
4.72E-001	Trans-HGI	4.20E-001	34:1-LGI
5.77E-001	6:1-MHGI	4.72E-001	Sat-HGI
5.77E-001	Mono-MLGI	5.04E-001	Trans-MLGI
6.21E-001	Trans-LGI	5.38E-001	Trans-MHGI
6.75E-001	6:1-MLGI	8.29E-001	Trans-LGI
9.26E-001	Trans-MHGI	8.77E-001	Mono-MLGI
9.33E-001	34:1-LGI	4.09E-006	6:1-LGI
9.56E-001	Trans-MLGI	4.84E-006	Sat-LGI
3.93E-005	6:1-LGI	6.62E-005	Mono-HGI

Q9 - pvalues with FDR correction

Sat-HGI	vs	Trans-HGI	vs
1.01E-004	Mono-MLGI	1.09E-004	34:1-HGI
2.71E-004	34:1-HGI	1.09E-004	34:1-MHGI
2.71E-004	34:1-MHGI	1.09E-004	6:1-MHGI
2.71E-004	6:1-MHGI	2.16E-004	34:1-MLGI
4.52E-004	34:1-MLGI	3.64E-004	Mono-HGI
8.41E-004	Mono-HGI	3.80E-004	Sat-MHGI
8.88E-004	Sat-MHGI	3.95E-004	Sat-MLGI
9.30E-004	Sat-MLGI	4.08E-004	Sat-LGI
9.65E-004	Sat-LGI	1.82E-003	Trans-LGI
1.16E-003	Trans-MLGI	3.85E-003	6:1-MLGI
8.37E-003	6:1-MLGI	4.48E-002	34:1-LGI
8.19E-002	34:1-LGI	4.66E-002	Mono-MHGI
8.19E-002	Mono-MHGI	1.50E-001	6:1-LGI
2.18E-001	6:1-LGI	2.14E-001	Trans-MHGI
8.74E-001	Trans-HGI	8.74E-001	Sat-HGI
1.65E-006	6:1-HGI	9.94E-001	Trans-MLGI
4.89E-010	Trans-LGI	2.46E-005	Mono-LGI
5.53E-006	Trans-MHGI	3.44E-005	Mono-MLGI
7.23E-005	Mono-LGI	7.77E-007	6:1-HGI

TRANS - pvalues with FDR correction

Sat-HGI	vs	Trans-HGI	vs
8.26E-008	34:1-HGI	2.51E-008	34:1-HGI
1.42E-004	6:1-HGI	1.58E-004	Mono-LGI
1.42E-004	6:1-MLGI	4.37E-004	Sat-MLGI
4.82E-004	6:1-MHGI	4.82E-004	Mono-HGI
8.61E-004	Mono-LGI	1.38E-003	Sat-LGI
2.16E-003	Sat-MLGI	6.76E-003	Trans-MLGI
2.40E-003	Mono-HGI	1.96E-002	Sat-MHGI
6.68E-003	Sat-LGI	2.01E-002	Trans-LGI
2.75E-002	Trans-MLGI	3.99E-002	Trans-MHGI
6.22E-002	Sat-MHGI	6.78E-001	Sat-HGI
6.38E-002	Trans-LGI	1.77E-005	6:1-HGI
1.14E-001	Trans-MHGI	1.77E-005	6:1-MLGI
6.78E-001	Trans-HGI	3.75E-007	34:1-MHGI
2.47E-007	34:1-LGI	4.37E-008	34:1-LGI
2.88E-007	34:1-MLGI	4.37E-008	34:1-MLGI
3.31E-006	34:1-MHGI	6.11E-008	6:1-LGI
4.06E-007	6:1-LGI	7.74E-007	Mono-MHGI
7.56E-006	Mono-MHGI	7.74E-007	Mono-MLGI
7.62E-006	Mono-MLGI	8.76E-005	6:1-MHGI

MonoLysoCL PC(18:2)2(18:1) - pvalues with FDR correction

Sat-HGI	vs	Trans-HGI	vs
6.66E-007	1:1-HGI	1.58E-009	1:1-HGI
1.40E-006	Sat-MHGI	2.36E-009	Sat-MHGI
2.19E-006	1:1-LGI	2.85E-009	1:1-LGI
2.19E-006	1:1-MLGI	2.85E-009	1:1-MLGI
4.88E-006	34:1-LGI	5.08E-009	34:1-LGI
5.35E-006	6:1-HGI	5.26E-009	6:1-HGI
7.87E-006	34:1-HGI	6.73E-009	34:1-HGI
7.87E-006	Mono-LGI	6.73E-009	Mono-LGI
1.06E-005	6:1-LGI	8.34E-009	6:1-LGI
1.21E-005	1:1-MHGI	9.06E-009	1:1-MHGI
1.99E-005	Mono-HGI	1.29E-008	Mono-HGI
1.99E-005	Mono-MHGI	1.29E-008	Mono-MHGI
1.99E-005	Trans-MHGI	1.29E-008	Trans-MHGI
2.94E-005	Sat-LGI	1.75E-008	Sat-LGI
3.49E-005	6:1-MHGI	2.00E-008	6:1-MHGI
4.97E-005	34:1-MHGI	2.64E-008	34:1-MHGI
9.68E-005	34:1-MLGI	4.57E-008	34:1-MLGI
0.00019379	Sat-MLGI	8.15E-008	Sat-MLGI
0.00021761	6:1-MLGI	8.93E-008	6:1-MLGI
0.00031367	Trans-MLGI	1.27E-007	Trans-MLGI
0.02258717	Trans-LGI	1.11E-005	Trans-LGI
0.23146439	Mono-MLGI	0.00023597	Mono-MLGI

MonoLyso CardioLipins

Note: Sat-HGI Diets not compared with Trans-HGI Diets

MonoLysoCL PC(18:2)3 - pvalues with FDR correction			
Sat.HGI	**vs**	**Trans.HGI**	**vs**
9.34E-006	1:1-LGI	1.84E-013	1:1-HGI
9.34E-006	1:1-MLGI	1.84E-013	1:1-LGI
9.34E-006	6:1-LGI	1.84E-013	1:1-MLGI
9.68E-006	Sat-MHGI	1.84E-013	6:1-LGI
1.06E-005	1:1-HGI	1.84E-013	Sat-MHGI
1.24E-005	34:1-LGI	1.90E-013	34:1-LGI
1.47E-005	Sat-LGI	2.02E-013	Sat-LGI
2.70E-005	Mono-LGI	3.57E-013	Mono-LGI
4.75E-005	Mono-HGI	5.93E-013	Mono-HGI
6.65E-005	6:1-HGI	8.33E-013	6:1-HGI
0.00010511	34:1-MLGI	1.31E-012	34:1-MLGI
0.00022188	Mono-MHGI	3.11E-012	Mono-MHGI
0.00032351	1:1-MHGI	4.66E-012	1:1-MHGI
0.00043001	34:1-HGI	5.89E-012	34:1-HGI
0.0005034	6:1-MHGI	6.61E-012	6:1-MHGI
0.00092738	Sat-MLGI	1.31E-011	Sat-MLGI
0.00121532	34:1-MHGI	1.75E-011	34:1-MHGI
0.00173662	Trans-MHGI	2.47E-011	Trans-MHGI
0.02088736	Trans-MLGI	5.02E-010	Trans-MLGI
0.04067298	6:1-MLGI	1.32E-009	6:1-MLGI
0.68042905	Mono-MLGI	7.28E-007	Mono-MLGI
0.9547594	Trans-LGI	1.29E-005	Trans-LGI

ARRIVE Criteria

TITLE

1 Provide as accurate and concise a description of the content of the article as possible.

Dietary Macronutrients Modulate the Fatty Acyl Composition of Rat Liver Mitochondrial Cardiolipins

ABSTRACT

2 Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.

The interaction of dietary fats and carbohydrates on liver mitochondria were examined in male FBNF1 rats fed 20 different low-fat, isocaloric diets. Animal growth rates and mitochondrial respiratory parameters were essentially unaffected, but mass spectrometry-based, mitochondrial lipidomics profiling revealed increased levels of cardiolipins (CLs), a family of phospholipids essential for mitochondrial structure and function, in rats fed saturated or trans fat-based diets with a high glycemic index. These mitochondria showed elevated monolysocardiolipins (consistent with oxidative damage to CL), elevated ratio of trans PC (18:1/18:1) to cis PC (18:1/18:1) (a marker of thiyl radical stress), and decreased ubiquinone Q9, implying a low-grade mitochondrial redox abnormality. Extended analysis demonstrated: (i) dietary fats and, to a lesser extent, carbohydrates induce changes in the relative abundance of specific CL species; (ii) FA incorporation into mature CLs undergoes both positive (~400-fold) and negative (≥2.4-fold) regulation, and; (iii) dietary lipid abundance and incorporation of FAs into both the CL pool and specific mature tetra-acyl CLs are inversely related, suggesting previously unobserved compensatory regulation. This study reveals previously unobserved complexity/regulation of the central lipid in mitochondrial metabolism.

INTRODUCTION: Background

3 a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale.

Cardiolipins (CLs) are a subclass of phospholipids unique to mitochondria. Each CL is a dimeric phospholipid consisting of two phosphatidyl head groups, connected on a glycerol backbone, and four fatty acyl (FA) chains [1-3]. Unlike most membrane phospholipids, CLs are predominantly found in the mitochondrial inner membrane and at contact sites between the inner and outer mitochondrial membrane [3]. CLs comprise ~ 25% of total phospholipids in the mitochondrial inner membrane [1-3]. Mammalian CL has been reported to contain primarily (~85%) 18:2 (linoleic) acyl side chains [2,4-5] that mediate the high affinity of CL to inner mitochondrial membrane proteins [3,6-7].

CLs play multiple key roles in the regulation of mitochondrial metabolism [2,3,8-10]. These functions include maintenance of proper mitochondrial quaternary structure, regulation of essential enzymatic activities involved in electron transport and oxidative phosphorylation, and assembly of respiratory supercomplexes. In particular, CL is required for the proper functioning of mitochondrial respiratory complexes I, III, IV (cytochrome oxidase), ATP synthase [6, 11-19], cardiolipin synthase [20]; and several transporters in the inner mitochondrial membrane, e.g., the ADP/ATP translocator [10,18,21], phosphate transporter [22], pyruvate transporter [23], carnitine/acylcarnitine carrier [24]. In the intermembrane space, CL serves as a binding receptor for creatine kinase [24] and electrostatically anchors cytochrome c to the inner mitochondrial membrane [26]. CL has been proposed to participate directly in proton conductance via cytochromes [14] and to prevent osmotic instability and uncoupling at high respiration rates [27]. CL also regulates mitochondrial biogenesis [3,10].

Changes both in absolute CL levels and, to a much lesser extent, in FA composition of the CL pool have been observed (patho)physiologically. Specifically, decreases in overall CL content have been implicated or observed in a range of overt pathological conditions associated with mitochondrial dysfunction, including Barth syndrome [28,29], experimental brain and heart ischemic-reperfusion injury [3,8], heart failure [30], and experimental diabetes [31,32]. During aging in rats, the total CL concentration is reduced in the heart, skin, and liver, and linoleate (18:2) decreases while arachidonate (20:4) increases [33,34]. Analysis of CL in hearts explanted from patients with dilated cardiomyopathy revealed a loss of tetralinoleoyl cardiolipin species [30]. Surprisingly, all physiologically modified scenarios appeared associated with decreased CL levels [3,8,30,31,43,44]. There seemed to be no active regulation of CLs to restore homeostasis or to counteract a biased diet.

Different dietary interventions affect CL acyl chain composition [35-38]. Functionally, thirty days on a diet deficient in linoleic acid (18:2), an essential fatty acid, significantly reduces tetralinoleoyl CL and affects mitochondrial oxygen consumption in the rat heart [39-41]. Conversely, dietary supplementation with linoleic acid restores tetralinoleoyl CL in cultured fibroblasts from Barth syndrome patients and elevates CL level [42]. Notably, the preferential accumulation of 18:2 side chains and the tetralinoleoyl-CL species was, until recently, one of the only recognized levels of CL regulation. These data speak to the need for an essential fatty acid in the diet to provide the raw building blocks of the CL molecule, a finding consistent with stochastic incorporation of fatty acids in the CL pool and the ability of diet to modulate the pool. These findings do not, however, address CL dietary-mediated regulation and/or response to a nutritionally replete diet. The present report addresses these latter issues.

The data discussed above suggest that an in depth study of the CL pool composition is central to probing the roles of CL in mitochondrial biology. Detailed analysis of the CL profile is technically challenging, because it requires either multiple extraction and purification steps or the simultaneous analysis of the intact CL molecule and each of its side chains both qualitatively and quantitatively within the context of the overall mitochondrial lipid pool. We used a newly developed lipidomics platform to examine diet-mediated changes in CL composition in depth, and we focus on a healthy rat model, Fisher 344 x Brown Norway F1 (FBNF1), maintained on low-fat isocaloric

diets differing systematically in primary fat and glycemic index (GI). Our results suggest that CL regulation is unexpectedly active and compensatory, and provide a biological model to probe these levels of CL regulation further.

b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.

The centerpiece of the original study was a focus on the identification of sera markers that reflect the interaction between diet, mitochondrial dysfunction, and long-term chronic disease risk. As noted, the study presented comes out of the analysis of one component of this study.

Animals are needed (e.g., vs cell cultures) because we cannot currently otherwise model the overall effects of diet on the organism. Rats were chosen as they are well-characterized animal models of both diet and mitochondrial function, and they are the phylogenetically the lowest animal species that provided sufficient material to complete the planned experiments. The final experiments involve crossing the data to human plasma analysis. We have existing data in the laboratory that this approach is fruitful for other, related lines of research. The main study examining diet/mitochondrial/disease interactions remains in progress.

INTRODUCTION: Objectives

4 Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.

The effects noted on cardiolipins were not the initial target of the study. Rather the study was targeted at identifying sera markers that reflect mitochondrial abnormalities (original abstract below). As a component added to the original study, we conducted a lipidomics profiling experiment of liver mitochondria drawn from one cohort of animals from this study. The analysis of this data forms the centerpiece of the current report.

Links between diet and human disease, and between reactive species and disease, are so commonly considered as to lie in the realm of textbooks and the popular press.

Links between mitochondria and energy production are generally appreciated by junior high school.

Links between mitochondria and calcium (including signaling), free radicals, or cell death may be less known to the general public, but each has in excess of 10,000 PubMed citations.

However, despite broad and strong theoretical considerations supporting causal connections between diet effects on mitochondria and diet effects on disease – and some specific experimental support – there are, to our knowledge, no systematic studies that bridge this fundamental gap.

Bridging this gap is central to understanding environment-gene interactions, as suboptimal dietary macronutrient choices are arguably the major environmental stressor in individuals living in Western societies. We therefore propose to bridge this gap using

an interdisciplinary, product-development approach to discover and confirm innovative plasma metabolomic and proteomic biomarkers for dietary intake of subclasses of fats and carbohydrates, and for their effects on mitochondrial (dys)function. We will then validate these markers by using them to test the hypothesis that diet-associated effects on mitochondria are linked to diet-associated changes in disease risk. Five Aims are proposed.

Aim 1	To determine the effects of dietary changes in fatty acid and carbohydrate composition on mitochondrial physiology
Aim 2	To determine the effects of dietary changes in fatty acid and carbohydrate composition on the plasma metabolome and proteome
Aims 3, 4:	To determine the extent to which adherence to/presence of each diet, dietary constituent, and mitochondrial property predict type II diabetes (Aim 3) and breast cancer (Aim 4) in previously profiled case control studies nested within the Nurses' Health Study
Aim 5:	To provide an electronic archive of the metabolomic and proteomic constituents of the blood of participants that could be repeatedly mined for future testing of new hypotheses.

The proposed studies are directly responsive to the RFA and further general NIH goals of focusing on health and early interventions rather than late stage disease.

METHODS: Ethical statement

5 Indicate the nature of the ethical review permissions, relevant licenses (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.

IACUC approval at Harvard Medical SChool, following The Guide for the Care and Use of Laboratory Animals

METHODS: Study design

6 For each experiment, give brief details of the study design including:

a. The number of experimental and control groups.

There are 24 groups of 8 animals each (6 fats x 4 carbohydrate mixes). The original study planned for three cohorts, one year apart, off-set by 4 month periods. An adaptive approach enabled us to cut one cohort. There are no specific control groups, as each diet is compared to all of the others at the level of fat group, carbohydrate group, or diet group. Dietary information directly follows the ARRIVE writeup. Four diets (#'s 406, 412, 418, 424), each having a 1:1 ratio of w-3/w-6 fats, were later dropped from the study due to apparent partial peroxidation.

b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when).

We studied two diets simultaneously. The diets were chosen in advance of the animals arriving. The animals were ordered from Harlan to be approximately 8 weeks

of age (7-9 weeks) and of similar body weight. The animals were assigned to cages randomly by the husbandry staff at unpacking and then the diets were assigned to each cage (2 animals per cage) randomly. For the key issue in the current study, assessment of cardiolipins was done by an automated method (SIEVE).

c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.

The experimental unit is one animal (or, more specifically, mitochondria isolated from one animal). Animals were always done in pairs, one from each of the two diets currently being used. Dietary information directly follows the ARRIVE writeup (the codes are the last three digits of the diet number). The diet pairings were:

Diets 403 and 416 (completed 04/08/09)
Diets 412 and 420 (completed 04/30/09)
Diets 401 and 417 (completed 05/28/09)
Diets 409 and 422 (completed 07/01/09)
Diets 402 and 418 (completed 07/29/09)
Diets 407 and 423 (completed 09/02/09)
Diets 404 and 415 (completed 09/30/09)
Diets 408 and 424 (completed 10/29/09)
Diets 405 and 413 (completed 11/20/09)
Diets 410 and 421 (completed 12/16/09)
Diets 414 and 406 (completed 01/28/10)
Diets 411 and 419 (completed 03/03/10)

EXPERIMENTAL

7 For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example:

a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s).

No drugs and no specialist equipment were used. Complete dietary information is provided in this appendix.

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b. When (e.g. time of day).

Sacrifice was at ~ 9 am, EST.

c. Where (e.g. home cage, laboratory, water maze).

Animal colony procedure room

d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).

Sacrifice was by decapitation without anesthesia following IACUC approved exemption due to known interference with assays.

EXPERIMENTAL: Experimental animals

8 a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range).

We used FBNF1 male rats. Animals were brought into the colony at approximately 8 weeks of age and then maintained for 8-10 weeks on their assigned diet prior to sacrifice. Some variation in body weight upon delivery from the supplier was noted, especially in the first few sets of rats (overall, the first cohort was not used in the current report).

b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.

The animals were FBNF1 male rats acquired from Harlan Laboratories at approximately 8 weeks of age and of similar body weight. The diets were purchased from Research Diets, Inc. The animals were maintained in a non-barrier animal facility operated by Harvard Medical School.

EXPERIMENTAL: Housing and husbandry

9 Provide details of:

a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions;

The animal facility is a non-barrier animal facility operated by Harvard Medical School. The animals were housed 2 per cage. The cages are standard plastic rat cages with wire lids (to hold food and water) and plastic, filtered tops. The animals live on the solid cage bottom with wood chip bedding (alpha chip). Cages are changed at least twice per week.

b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment).

The light/dark cycle is 12 hour/12 hour. The temperature and humidity are maintained at 69 degrees and 45% respectively. The animals have *ad libitum* access to food and water.

c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.

All work with animals was approved by the IACUC committee at Brigham and Women's Hospital. Harvard Medical School has a full time staff of veterinarians and veterinary technicians that visit the facility on a regular weekly schedule. A veterinarian

is on call during evenings, weekends, and holidays. The husbandry crew is present in the facility every day including weekends and holidays.

The animals on this study were not expected to suffer any ill effects clinically. And as expected, no animal appeared to be anything less than healthy at any time.

EXPERIMENTAL: Sample size

10 a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group.

There are 24 groups of 8 animals each (6 fats x 4 carbohydrate mixes). The original study planned for three cohorts, one year apart, off-set by 4 month periods. The adaptive approach enabled us to cut one cohort. There are no specific control groups, as each diet is compared to all of the others at the level of fat group, carbohydrate group, or diet group. Only the second cohort is reported in this study.

As noted earlier, four diets (#'s 406, 412, 418, 424), each having a 1:1 ratio of w-3/w-6 fats, were later dropped from the study due to apparent partial peroxidation.

b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used.

From experience it was estimated that this N was sufficient for building and validating primary metabolomic serotypes under the conditions tested. This was the primary study end-point related to animals.

c. Indicate the number of independent replications of each experiment, if relevant.

The cohorts were replicated (2x 192 rats), but mitochondrial lipidomics was only conducted on the second cohort.

EXPERIMENTAL: Allocating animals to experimental groups

11 a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done.

As noted above, we studied two diets at a time (16 animals). The diets were chosen in advance of the animals arriving. The animals were randomly assigned to cages, 2 per cage, by the husbandry crew at arrival. The cages were randomly assigned a diet. For the key issue in the current study, assessment of cardiolipins was done by an automated method (SIEVE).

b. Describe the order in which the animals in the different experimental groups were treated and assessed.

The experimental unit is one animal (or, more specifically, mitochondria isolated from one animal). Animals were always done in pairs, one from each of the two diets currently used. Lipidomics analysis was run as a single batch series.

EXPERIMENTAL OUTCOMES

12 Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).

The design is somewhat different in that it seeks to link a series of outcomes, specifically, diet to mitochondrial changes (functional or, here, biochemical) to sera changes. Thus, the cardiolipin, prenol, and related changes observed would all be considered a "primary change" of interest.

STATISTICAL METHODS

13 a. Provide details of the statistical methods used for each analysis.

Initial cardiolipin and respiration values were tested by ANOVA to check if they significantly differed across diet groups. If values were found to be significant, we followed up with a Tukey Honest Significance Difference (HSD) test to ascertain which pair of groups differed significantly. The Tukey HSD incorporates an adjustment for p-values to account for multiple pairwise comparisons.

Two-way hierarchical clustering analysis was performed as described in the methods. Clusters were built based on a distance metric assigned by the Pearson correlation of the median data for each CL within each diet, normalized as percent of total CL per rat. Coloring is by Z-scores of median intensity of CLs by diet.

b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron).

Single animals grouped by diet, by fat, or by carbohydrate, depending on the analysis.

c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.

Tukey HSD is based on an ANOVA analysis of the data. We assumed the following: independence, normality, and equality of variances. Normality was estimated visually by looking at boxplots as the N was considered too small for valid normality testing, and variances of the different groups (Diets) under consideration were close to being equal. We used TukeyHSD for multiple comparison, as it validated our initial test with ANOVA.

As implemented, clustering is assumption free.

RESULTS: Baseline data

14 For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).

See attached graphs. All animals were considered healthy at the time of sacrifice by a laboratory veterinarian (CLP).

NUMBERS ANALYZED

15 a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%) (Schulz et al., 2010). The study evaluated 8 animals per diet; 160 animals in the study as presented; data from all animals were reported. As noted above, four diets (#'s 406, 412, 418, 424), each having a 1:1 ratio of w-3/w-6 fats, were later dropped from the study due to apparent partial peroxidation.

b. If any animals or data were not included in the analysis, explain why. Outcomes and estimation

Data from all animals were reported. As noted above, four diets (#'s 406, 412, 418, 424), each having a 1:1 ratio of w-3/w-6 fats, were later dropped from the study due to apparent partial peroxidation.

16 Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).

See main text for different assays

Adverse events

17 a. Give details of all important adverse events in each experimental group.

None

b. Describe any modifications to the experimental protocols made to reduce adverse events.

NA

DISCUSSION: Interpretation/scientific implications

18 a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature.

These data provide evidence of previously unrecognized levels of regulation of cardiolipins in response to diet type.

b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results (Schulz et al., 2010).

The animal model is unusual in that the strain, FBNF1, is a hybrid. This strain is a healthy and hardy animal that is not prone to specific disease or obesity. These animals were also fed a low fat diet.

The results have the precision to show that the results obtained are not explicable by current knowledge about the regulation of the cardiolipins. Analytical precision on the mass spec is on the order of 4%, well under the biological variation observed.

c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.

None that are obvious.

DISCUSSION: Generalisability/translation

19 Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.

These data provide evidence that the major lipid involved in energy metabolism has a much more complex pattern of regulation than previously assumed. In general, cardiolipin data has generalized broadly across phylogeny, and these data would be expected to be similarly generalisable. The study also provides a model, as intended, of the early consequences of mitochondrial dysfunction. The work provides potential markers and potential targets, albeit this is a very early recognition of such possibilities. Continued analysis of the main work (linking diet, mitochondria, and disease) is in progress and should allow us to evaluate the initial hypothesis without additional use of animals.

FUNDING

20 List all funding sources (including grant number) and the role of the funder(s) in the study.

The animal studies reported were funded by U01-ES16048 (BSK, PI), a part of the NIH Genes and Environment Initiative (GEI) and internal funding from BWH. The authors also thank ThermoFisher for the loan of an Exactive Benchtop orbitrap for demonstration testing and financial support for scientific meeting attendance. The funders have no role in any aspect of the conduct, analysis, or presentation of the study.

ARRIVE CRITERIA Literature

1: Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. J Pharmacol Pharmacother. 2010 Jul;1(2):94-9. PubMed PMID: 21350617; PubMed Central PMCID: PMC3043335.

2: Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG; National Centre for the Replacement, Refinement and Reduction of Amimals in Research. Animal research: reporting in vivo experiments--the ARRIVE guidelines. J Cereb Blood Flow Metab. 2011 Apr;31(4):991-3. Epub 2011 Jan 5. PubMed PMID: 21206507; PubMed Central PMCID: PMC3070981.

3: Kilkenny C, Altman DG. Improving bioscience research reporting: ARRIVE-ing at a solution. Lab Anim. 2010 Oct;44(4):377-8. Epub 2010 Jul 21. PubMed PMID: 20660161.

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5: McGrath JC, Drummond GB, McLachlan EM, Kilkenny C, Wainwright CL. Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol. 2010 Aug;160(7):1573-6. PubMed PMID: 20649560; PubMed Central PMCID: PMC2936829.

6: Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 2010 Jun 29;8(6):e1000412. PubMed PMID: 20613859; PubMed Central PMCID: PMC2893951.

7: Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG; NC3Rs Reporting Guidelines Working Group. Animal research: reporting in vivo experiments: the ARRIVE guidelines. J Gene Med. 2010 Jul;12(7):561-3. PubMed PMID: 20607692.

D10001 and D07102401 - 06

AIN-76A Rodent Diet and Same with Varying Fatty Acid Compositions and 65% Corn Starch

Product #	**D10001**		**D07102401**		**D07102402**		**D07102403**		**D07102404**		**D07102405**		**D07102406**	
	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*
Protein	20	*21*	20	*21*	20	*21*	20	*21*	20	*21*	20	*21*	20	*21*
Carbohydrate	66	*68*	66	*68*	66	*68*	66	*68*	66	*68*	66	*68*	66	*68*
Fat	5	*12*	5	12	5	12	5	12	5	12	5	12	0	*0*
Total		*100.0*		*100.0*		*100.0*		*100.0*		*100.0*		*100.0*		*88.5*
kcal/gm	3.90		3.90		3.90		3.90		3.90		3.90		3.90	
Ingredient	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***
Casein	200	*800*	200	*800*	200	*800*	200	*800*	200	*800*	200	*800*	200	*800*
DL-Methionine	3	*12*	3	*12*	3	*12*	3	*12*	3	*12*	3	*12*	3	*12*
Corn Starch	150	*600*	525	*2100*	525	*2100*	525	*2100*	525	*2100*	525	*2100*	525	*2100*
Maltodextrin 10	0	*0*	125	*500*	125	*500*	125	*500*	125	*500*	125	*500*	125	*500*
Sucrose	500	*2000*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*
Cellulose, BW200	50	*0*	50	*0*	50	*0*	50	*0*	50	*0*	50	*0*	50	*0*
Corn Oil	50	*450*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*
Hydrogenated Coconut Oil	0	*0*	28	*252*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*
Linseed Oil (Flaxseed Oil)	0	*0*	0.6	*5*	0.6	*5*	0.6	*5*	0.6	*5*	1.7	*15*	7.5	*68*
Menhaden Oil	0	*0*	0	*0*	0	*0*	0	*0*	1.3	*12*	8.3	*75*	32.5	*293*
Safflower Oil	0	*0*	21.4	*193*	21.4	*193*	15.9	*143*	48.1	*433*	40	*360*	10	*90*
Safflower Oil, High Oleic	0	*0*	0	*0*	9	*81*	33.5	*302*	0	*0*	0	*0*	0	*0*
Shortening, Tem Cote	0	*0*	0	*0*	19	*171*	0	*0*	0	*0*	0	*0*	0	*0*
Mineral Mix S10001	35	*0*	35	*0*	35	*0*	35	*0*	35	*0*	35	*0*	35	*0*
Vitamin Mix V10001	10	*40*	10	*40*	10	*40*	10	*40*	10	*40*	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*	2	*0*	2	*0*	2	*0*	2	*0*	2	*0*
FD&C Red Dye #40	0	*0*	0.1	*0*	0	*0*	0	*0*	0.05	*0*	0.05	*0*	0	*0*
FD&C Yellow Dye #5	0	*0*	0	*0*	0.1	*0*	0	*0*	0.05	*0*	0	*0*	0.05	*0*
FD&C Blue Dye #1	0	*0*	0	*0*	0	*0*	0.1	*0*	0	*0*	0.05	*0*	0.05	*0*
Total	**1000**	***3902***	**1000.1**	***3902***	**1000.1**	***3902***	**1000.1**	***3902***	**1000.1**	***3902***	**1000.1**	***3902***	**1000.1**	***3902***

D10001 and D07102407 - 12

AIN-76A Rodent Diet and Same with Varying Fatty Acid Compositions and 43% Corn Starch and 22% Sucrose

Product #	**D10001**		**D07102407**		**D07102408**		**D07102409**		**D07102410**		**D07102411**		**D07102412**	
	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*
Protein	20	*21*	20	*21*	20	*21*	20	*21*	20	*21*	20	*21*	20	*21*
Carbohydrate	66	*68*	66	*68*	66	*68*	66	*68*	66	*68*	66	*68*	66	*68*
Fat	5	*12*	5	12	5	12	5	12	5	12	5	12	5	12
Total		*100.0*		*100.0*		*100.0*		*100.0*		*100.0*		*100.0*		*100.0*
kcal/gm	3.90		3.90		3.90		3.90		3.90		3.90		3.90	
Ingredient	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***
Casein	200	*800*	200	*800*	200	*800*	200	*800*	200	*800*	200	*800*	200	*800*
DL-Methionine	3	*12*	3	*12*	3	*12*	3	*12*	3	*12*	3	*12*	3	*12*
Corn Starch	150	*600*	333	*1332*	333	*1332*	333	*1332*	333	*1332*	333	*1332*	333	*1332*
Maltodextrin 10	0	*0*	100	*400*	100	*400*	100	*400*	100	*400*	100	*400*	100	*400*
Sucrose	500	*2000*	217	*868*	217	*868*	217	*868*	217	*868*	217	*868*	217	*868*
Cellulose, BW200	50	*0*	50	*0*	50	*0*	50	*0*	50	*0*	50	*0*	50	*0*
Corn Oil	50	*450*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*
Hydrogenated Coconut Oil	0	*0*	28	*252*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*
Linseed Oil (Flaxseed Oil)	0	*0*	0.6	*5*	0.6	*5*	0.6	*5*	0.6	*5*	1.7	*15*	7.5	*68*
Menhaden Oil	0	*0*	0	*0*	0	*0*	0	*0*	1.3	*12*	8.3	*75*	32.5	*293*
Safflower Oil	0	*0*	21.4	*193*	21.4	*193*	15.9	*143*	48.1	*433*	40	*360*	10	*90*
Safflower Oil, High Oleic	0	*0*	0	*0*	9	*81*	33.5	*302*	0	*0*	0	*0*	0	*0*
Shortening, Tem Cote	0	*0*	0	*0*	19	*171*	0	*0*	0	*0*	0	*0*	0	*0*
Mineral Mix S10001	35	*0*	35	*0*	35	*0*	35	*0*	35	*0*	35	*0*	35	*0*
Vitamin Mix V10001	10	*40*	10	*40*	10	*40*	10	*40*	10	*40*	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*	2	*0*	2	*0*	2	*0*	2	*0*	2	*0*
FD&C Red Dye #40	0	*0*	0.075	*0*	0	*0*	0	*0*	0.0375	*0*	0.0375	*0*	0	*0*
FD&C Yellow Dye #5	0	*0*	0	*0*	0.075	*0*	0	*0*	0.0375	*0*	0	*0*	0.0375	*0*
FD&C Blue Dye #1	0	*0*	0	*0*	0	*0*	0.075	*0*	0	*0*	0.0375	*0*	0.0375	*0*
Total	**1000**	***3902***	**1000.0750**	***3902***	**1000.075**	***3902***	**1000.075**	***3902***	**1000.075**	***3902***	**1000.075**	***3902***	**1000.075**	***3902***

D10001 and D07102413 - 18

AIN-76A Rodent Diet and Same with Varying Fatty Acid Compositions and 22% Corn Starch and 43% Sucrose

Product #	**D10001**		**D07102413**		**D07102414**		**D07102415**		**D07102416**		**D07102417**		**D07102418**	
	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*
Protein	20	*21*	20	*21*	20	*21*	20	*21*	20	*21*	20	*21*	20	*21*
Carbohydrate	66	*68*	66	*68*	66	*68*	66	*68*	66	*68*	66	*68*	66	*68*
Fat	5	*12*	5	12	5	12	5	12	5	12	5	12	5	12
Total		*100.0*		*100.0*		*100.0*		*100.0*		*100.0*		*100.0*		*100.0*
kcal/gm	3.90		3.90		3.90		3.90		3.90		3.90		3.90	
Ingredient	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***
Casein	200	*800*	200	*800*	200	*800*	200	*800*	200	*800*	200	*800*	200	*800*
DL-Methionine	3	*12*	3	*12*	3	*12*	3	*12*	3	*12*	3	*12*	3	*12*
Corn Starch	150	*600*	167	*668*	167	*668*	167	*668*	167	*668*	167	*668*	167	*668*
Maltodextrin 10	0	*0*	50	*200*	50	*200*	50	*200*	50	*200*	50	*200*	50	*200*
Sucrose	500	*2000*	433	*1732*	433	*1732*	433	*1732*	433	*1732*	433	*1732*	433	*1732*
Cellulose, BW200	50	*0*	50	*0*	50	*0*	50	*0*	50	*0*	50	*0*	50	*0*
Corn Oil	50	*450*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*
Hydrogenated Coconut Oil	0	*0*	28	*252*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*
Linseed Oil (Flaxseed Oil)	0	*0*	0.6	*5*	0.6	*5*	0.6	*5*	0.6	*5*	1.7	*15*	7.5	*68*
Menhaden Oil	0	*0*	0	*0*	0	*0*	0	*0*	1.3	*12*	8.3	*75*	32.5	*293*
Safflower Oil	0	*0*	21.4	*193*	21.4	*193*	15.9	*143*	48.1	*433*	40	*360*	10	*90*
Safflower Oil, High Oleic	0	*0*	0	*0*	9	*81*	33.5	*302*	0	*0*	0	*0*	0	*0*
Shortening, Tem Cote	0	*0*	0	*0*	19	*171*	0	*0*	0	*0*	0	*0*	0	*0*
Mineral Mix S10001	35	*0*	35	*0*	35	*0*	35	*0*	35	*0*	35	*0*	35	*0*
Vitamin Mix V10001	10	*40*	10	*40*	10	*40*	10	*40*	10	*40*	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*	2	*0*	2	*0*	2	*0*	2	*0*	2	*0*
FD&C Red Dye #40	0	*0*	0.05	*0*	0	*0*	0	*0*	0.025	*0*	0.025	*0*	0	*0*
FD&C Yellow Dye #5	0	*0*	0	*0*	0.05	*0*	0	*0*	0.025	*0*	0	*0*	0.025	*0*
FD&C Blue Dye #1	0	*0*	0	*0*	0	*0*	0.05	*0*	0	*0*	0.025	*0*	0.025	*0*
Total	**1000**	***3902***	**1000.05**	***3902***	**1000.05**	***3902***	**1000.05**	***3902***	**1000.05**	***3902***	**1000.05**	***3902***	**1000.05**	***3902***

D10001 and D07102419 - 24

AIN-76A Rodent Diet and Same with Varying Fatty Acid Compositions and 65% Sucrose

Product #	**D10001**		**D07102419**		**D07102420**		**D07102421**		**D07102422**		**D07102423**		**D07102424**	
	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*	gm%	*kcal%*
Protein	20	*21*	20	*21*	20	*21*	20	*21*	20	*21*	20	*21*	20	*21*
Carbohydrate	66	*68*	66	*68*	66	*68*	66	*68*	66	*68*	66	*68*	66	*68*
Fat	5	*12*	5	12	5	12	5	12	5	12	5	12	5	12
Total		*100.0*		*100.0*		*100.0*		*100.0*		*100.0*		*100.0*		*100.0*
kcal/gm	3.90		3.90		3.90		3.90		3.90		3.90		3.90	
Ingredient	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***
Casein	200	*800*	200	*800*	200	*800*	200	*800*	200	*800*	200	*800*	200	*800*
DL-Methionine	3	*12*	3	*12*	3	*12*	3	*12*	3	*12*	3	*12*	3	*12*
Corn Starch	150	*600*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*
Maltodextrin 10	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*
Sucrose	500	*2000*	650	*2600*	650	*2600*	650	*2600*	650	*2600*	650	*2600*	650	*2600*
Cellulose, BW200	50	*0*	50	*0*	50	*0*	50	*0*	50	*0*	50	*0*	50	*0*
Corn Oil	50	*450*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*
Hydrogenated Coconut Oil	0	*0*	28	*252*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*
Linseed Oil (Flaxseed Oil)	0	*0*	0.6	*5*	0.6	*5*	0.6	*5*	0.6	*5*	1.7	*15*	7.5	*68*
Menhaden Oil	0	*0*	0	*0*	0	*0*	0	*0*	1.3	*12*	8.3	*75*	32.5	*293*
Safflower Oil	0	*0*	21.4	*193*	21.4	*193*	15.9	*143*	48.1	*433*	40	*360*	10	*90*
Safflower Oil, High Oleic	0	*0*	0	*0*	9	*81*	33.5	*302*	0	*0*	0	*0*	0	*0*
Shortening, Tem Cote	0	*0*	0	*0*	19	*171*	0	*0*	0	*0*	0	*0*	0	*0*
Mineral Mix S10001	35	*0*	35	*0*	35	*0*	35	*0*	35	*0*	35	*0*	35	*0*
Vitamin Mix V10001	10	*40*	10	*40*	10	*40*	10	*40*	10	*40*	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*	2	*0*	2	*0*	2	*0*	2	*0*	2	*0*
FD&C Red Dye #40	0	*0*	0.025	*0*	0	*0*	0	*0*	0.0125	*0*	0.0125	*0*	0	*0*
FD&C Yellow Dye #5	0	*0*	0	*0*	0.025	*0*	0	*0*	0.0125	*0*	0	*0*	0.0125	*0*
FD&C Blue Dye #1	0	*0*	0	*0*	0	*0*	0.025	*0*	0	*0*	0.0125	*0*	0.0125	*0*
Total	**1000**	***3902***	**1000**	***3902***	**1000**	***3902***	**1000**	***3902***	**1000**	***3902***	**1000**	***3902***	**1000**	***3902***

Typical Fatty Acid Profiles of Formulas Based on Data from the Manufacturer of Coconut Oil, Linseed Oil, Menhaden Oil, Safflower Oil, High Oleic Safflower Oil, and Tem Cote Shortening

	Formula 1	Formula 2	Formula 3	Formula 4	Formula 5	Formula 6
	gm	gm	gm	gm	gm	gm
Coconut Oil, 101	28					
Linseed Oil	0.6	0.6	0.6	0.6	1.7	7.5
Menhaden Oil				1.3	8.3	32.5
Safflower Oil	21.4	21.4	15.9	48.1	40	10
Safflower Oil, High Oleic		9	33.5			
Shortening, Tem Cote		19				
Total	50	50	50	50	50	50
C2, Acetic	0.0	0.0	0.0	0.0	0.0	0.0
C4, Butyric	0.0	0.0	0.0	0.0	0.0	0.0
C6, Caproic	0.2	0.0	0.0	0.0	0.0	0.0
C8, Caprylic	2.2	0.0	0.0	0.0	0.0	0.0
C10, Capric	1.7	0.0	0.0	0.0	0.0	0.0
C12, Lauric	13.3	0.0	0.0	0.0	0.0	0.0
C14, Myristic	5.0	0.0	0.0	0.1	0.6	2.2
C14:1, Myristoleic	0.0	0.0	0.0	0.0	0.0	0.0
C15:0	0.0	0.0	0.0	0.0	0.0	0.2
C16, Palmitic	3.8	4.2	2.8	3.3	3.9	5.9
C16:1, Palmitoleic	0.0	0.0	0.0	0.1	0.8	3.2
C16:1, Trans	0.0	0.0	0.0	0.0	0.0	0.0
C16:2	0.0	0.0	0.0	0.0	0.1	0.5
C16:3	0.0	0.0	0.0	0.0	0.1	0.5
C16:4	0.0	0.0	0.0	0.0	0.1	0.5
C17:0	0.0	0.0	0.0	0.0	0.0	0.2
C18, Stearic	3.5	2.1	1.0	1.2	1.2	1.3
C18:1, Oleic	2.9	12.9	28.1	6.0	5.9	5.6
C18:1, Trans	0.0	9.9	0.0	0.0	0.0	0.0
C18:2, Linoleic, n-6	16.9	18.3	17.3	37.8	31.7	9.5
C18:2, Trans	0.0	0.4	0.0	0.0	0.0	0.0
C18:3, Linolenic, n-3	0.4	0.4	0.4	0.4	1.1	4.6
C18:4	0.0	0.0	0.0	0.0	0.3	1.0
C20, Arachidic	0.0	0.1	0.2	0.0	0.0	0.1
C20:1,	0.0	0.0	0.1	0.0	0.1	0.5
C20:2	0.0	0.0	0.0	0.0	0.0	0.1
C20:3, n-6	0.0	0.0	0.0	0.0	0.0	0.1
C20:3, n-3	0.0	0.0	0.0	0.0	0.0	0.0
C20:4, Arachidonic, n-6	0.0	0.0	0.0	0.0	0.2	0.7
C20:5, Eicosapentaenoic	0.0	0.0	0.0	0.2	1.2	4.6
C21:5, n-3	0.0	0.0	0.0	0.0	0.1	0.2
C22, Behenic	0.0	0.1	0.0	0.0	0.0	0.0
C22:1, Erucic	0.0	0.0	0.0	0.0	0.0	0.1
C22:4, Clupanodonic, n-6	0.0	0.0	0.0	0.0	0.0	0.1
C22:5, n-6	0.0	0.0	0.0	0.0	0.2	0.9
C22:6, Docosahexaenoic	0.0	0.0	0.0	0.2	1.0	4.0
C24, Lignoceric	0.0	0.0	0.0	0.0	0.0	0.2
C24:1	0.0	0.0	0.0	0.0	0.0	0.1
Total	49.8	48.5	49.9	49.5	48.9	46.9
Saturated (g)	29.7	6.6	4.0	4.6	5.8	10.1
Monounsaturated (g)	2.9	22.9	28.2	6.2	6.9	9.5
Polyunsaturated (g)	17.2	18.6	17.7	38.8	36.4	28.0
n-6 PUFA (g)	16.9	18.3	17.3	37.9	32.2	11.3
n-3 PUFA (g)	0.4	0.4	0.4	0.8	3.4	13.5
n-6/n-3	47	51	49	49	10	1
ALA (g)	0.4	0.4	0.4	0.4	1.1	4.6
EPA (g)	0.0	0.0	0.0	0.2	1.2	4.6
DHA (g)	0.0	0.0	0.0	0.2	1.0	4.0
Trans (g)	0.0	10.4	0.0	0.0	0.0	0.0
Saturated (%)	60	14	8	10	13	22
Monounsaturated (%)	6	47	57	13	15	20
Polyunsaturated (%)	35	38	35	78	73	60
n-6 PUFA (%)	33.9	37.6	34.7	75.2	61.1	25.9
Alpha-linolenic acid (%)	0.7	0.7	0.7	0.9	3.3	9.9
EPA (%)	0	0	0	1	3.3	9.8
DHA (%)	0	0	0	1	6.2	18.3
n-3 PUFA (%)	0.7	0.7	0.7	2.2	9.7	28.7
Ratio n-6/n-3	47	51	49	34	6	1
Trans (%)	0	21.4	0	0	0	0