STA 440 Case 2

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Background

Mitochondria play a central role in cellular energy metabolism, and alterations in their molecular structures can translate into measurable physiological phenotypes. Characterizing how mitochondrial efficiency changes under different conditions provides important insights into metabolic function and disease mechanisms.

To this end, researchers often measure mitochondrial respiration under a variety of experimental settings. Multiplexed metabolic assay platforms allow simultaneous evaluation of several aspects of mitochondrial functions across substrates, redox conditions, and energetic states. One critical readout is oxygen flux (JO2), which serves as a direct indicator of mitochondrial respiratory activity. Comparing oxygen flux between experimental groups such as non transgenic and transgenic mice provides a way to evaluate whether genetic differences are associated with altered mitochondrial functions.

Our analysis focuses on modeling and testing for genotype effects on oxygen flux, while accounting for experimental design factors such as substrate type. The aim is to determine whether mitochondrial efficiency differs by genotype, and whether such effects depend on substrate choice or dose.

Data

The data for this study was collected from skeletal muscle mitochondria that are isolated from either non-transgenic or transgenic mice, measured during the mitochondrial energy transduction process. For our model we took into account two main factors: substrate type and dose. The primary factor we are interested in is genotype (non transgenic vs transgenic). Substrate and dose serve as design factors, and their interactions with genotype are of central interest in assessing whether genetic differences in mitochondrial function vary across energetic states or substrate conditions.

The data we were given originally was in a wide format, so we transformed it into a tidy long format for analysis. We also dropped the basal values since they represent the idle state. Some values were missing so we dropped them.

The distribution of raw JO values was right-skewed, with a long tail toward higher flux (Figure 1). A log-transformation produced a more symmetric, approximately normal distribution (Figure 2), suggesting potential benefits for variance stabilization. However, since model assumptions were reasonably satisfied on the raw scale, the log transformation was used only for exploratory visualization and not in the final modeling.

Trajectories of JO across ΔGATP levels revealed consistent dose–response relationships (Figure 3): respiration increased as workload rose, and transgenic (Tg) mice generally exhibited higher flux than

non-transgenic (NT) controls. Group-level means with standard errors reinforced these patterns (Figure 4), showing the strongest Tg–NT separation for carbohydrate-linked substrates (GM, PM), moderate differences for mixed fuels (PMPc, PMOc), and smaller effects for fatty acid substrates (OcM, PcM).

Barplots comparing basal and low-dose conditions confirmed that Tg mitochondria already displayed higher respiration at early workloads (Figure 5). Boxplots of subject-specific JO –dose slopes highlighted that Tg mice exhibited steeper workload sensitivity, particularly under carbohydrate conditions (Figure 6). Finally, a heatmap of mean JO across substrates and doses illustrated the overall pattern of higher respiration in Tg mice, with the largest differences observed for carbohydrate fuels (Figure 7).

Together, these exploratory findings highlighted the importance of genotype, substrate, and dose — and their interactions — as key factors for formal modeling. They also emphasized the need to account for repeated measures within subjects, since while overall patterns were consistent, baseline levels varied by mouse.

Model Rationale

We employed a sequence of models to evaluate the effects of genotype, substrate, and workload (ΔGATP) on oxygen flux (JO). Each model was chosen to address a specific aspect of the experimental design and to progressively incorporate complexity and biological realism.

- **Baseline model (lm(JO2 ~ Substrate * Dose)**

  This model considers only substrate and dose effects, ignoring genotype. It serves as a sanity check to establish whether fuel type and workload influence JO as expected.

- **Genotype-only model (lm(JO2 ~ Genotype)**

  This specification provides a crude comparison of JO between NT and Tg mice by collapsing across all experimental conditions. It is useful as an initial test for an overall genotype effect, but it oversimplifies by ignoring the role of substrate and workload.

- **Full factorial model (lm(JO2 ~ Genotype * Substrate * Dose)**

  This model incorporates genotype, substrate, dose, and all interactions. It allows us to examine whether genotype effects vary across substrates or workload levels. The advantage of this approach is that it captures the complexity of the biology, including higher-order interactions. However, it treats each observation as independent and does not account for repeated measures on the same subject, which violates design assumptions.

- **Mixed-effects model (lmer(JO2 ~ Genotype * Substrate * Dose + (1 | Subject)**

  The mixed model includes genotype, substrate, and dose as fixed effects, while adding a random intercept for the subject. This structure accounts for repeated measures within the same mouse and preserves independence across subjects. It balances interpretability with statistical validity, enabling us to test population-level effects while controlling for subject-level variability. Its main limitation is reduced power to detect high-order interactions, given the modest sample size (6 NT, 6 Tg).

While simpler models provided useful descriptive insights, they failed to fully respect the experimental design. The baseline and genotype-only models ignored key sources of variation, while the

full factorial model captured interaction effects but treated repeated measures from the same subject as independent, inflating the risk of false positives. The mixed-effects model addressed these shortcomings by incorporating subject-level random effects, thereby accounting for within-mouse correlation while still testing the joint effects of genotype, substrate, and workload at the population level. This framework provides the most statistically rigorous and biologically meaningful analysis of our dataset, balancing model complexity with the need to respect the hierarchical structure of the data.

Model Implementation and Evaluation

We reshaped the raw worksheet into a tidy long format, and restricted analysis to active respiration by removing Basal and the lowest dose (-12.95). We converted dose to a numeric ΔGATP scale for modeling and dropped missing values. Initial checks of distribution and scale (see Figure Figure 1 and Figure 2) informed the decision to fit models on raw JO2 while using log-transformed views only for EDA.

We then fit a progression of regression models to quantify effects and interactions: - Baseline linear model without genotype: `JO2 ~ Substrate * Dose` (for design checks). - Genotype-only model: `JO2 ~ Genotype` (coarse group difference). - Full linear model: `JO2 ~ Genotype * Substrate * Dose` (all interactions). - Mixed-effects model with subject intercepts: `JO2 ~ Genotype * Substrate * Dose + (1 | Subject)`.

For the linear models, we report coefficients and ANOVA tables (Table 1, Table 2, Table 3). For the mixed model, we summarize fixed effects (Table 4) and compare overall model fit across candidates using AIC/BIC and log-likelihood (Table 5). Model adequacy was assessed via residual–fitted and normal Q–Q diagnostics (Figure 10 and Figure 11). To aid interpretation, we visualized fitted curves with 95% CIs from the mixed model (Figure 8) and computed estimated marginal means and Tg–NT contrasts across Substrate × Dose cells (Figure 9; Table 6). Supporting exploratory figures show raw trajectories and group means over dose (Figure 3 and Figure 4), per-subject dose–response slopes (Figure 6), and mean JO2 heatmaps (Figure 7).

Results

Across substrates, JO2 exhibits a clear dose-dependent response and substantial substrate-to-substrate variability (see trajectories and means in Figure 3 and Figure 4, and the mean heatmap in Figure 7). The baseline linear model confirms strong effects of Substrate and Dose and their interaction (Table 2). Incorporating Genotype and its interactions in the full linear model indicates that genotype differences are not uniform; they depend on the substrate and/or dose context (Table 3).

Accounting for repeated measures with a subject-level random intercept, the mixed-effects model provides the primary inference. Fixed-effect estimates in Table 4 quantify the average genotype difference at the reference levels and how that difference varies with substrate and dose via interaction terms. The fitted curves from Figure 8 visualize these patterns: separation between Tg and NT varies by substrate and across ΔGATP, with uncertainty bands reflecting the modest sample size. The EMM contrast heatmap and table (Figure 9; Table 6) pinpoint specific Substrate × Dose combinations where Tg differs from NT and summarize effect sizes with standard errors and p-values.

Taken together, the results support dose- and substrate-dependent mitochondrial respiration with

evidence of genotype effects that are context-specific. Where significant contrasts appear in Table 6, they align with the visual separation seen in Figure 8, while diagnostics in Figure 10 and Figure 11 do not indicate major violations of linear model assumptions. Model comparison in Table 5 favors the mixed-effects specification, balancing improved fit with appropriate handling of within-subject correlation.

Limitations

There are several limitations of this analysis that should be considered when interpreting the results. First, the analysis focused exclusively on the dose-response slopes of the mitochondrial oxygen flux (JO2) after dropping basal condition. Additionally, the analysis is limited to mitochondrial oxygen flux and does not consider other relevant outcomes like reactive oxygen species, membrane potential, and redox state. Also, although the model accounted for some heteroscedasticity, the increasing variance of the JO2 with higher mean values was only partially addressed and may still affect estimates.

The study was also limited by sample size (6 non-transgenic mice and 6 transgenic mice) which reduces the statistical power, particularly for detecting complex interactions or subtle nonlinearities. To account for this, the analysis assumed a linear relationship between the dose and JO2 across the studied range. While the assumption appeared reasonable within the available data, nonlinear responses outside of the observed range cannot be ruled out. Finally, the mixed effects modeling approach included random intercepts to capture subject level variability but did not incorporate random slopes, potentially underestimating within subject differences in dose response patterns. However, residuals were approximately normal after accounting for fixed effects, and the assumption of independence across subjects was reasonable given the experimental design.

Conclusions

Our analysis evaluated genotype, substrate, and energetic workload effects on skeletal-muscle mitochondrial respiration (JO), ultimately selecting a mixed-effects model to account for experimental factors and within-subject correlation.

We observed strong dose- and substrate-dependent variation, with genotype effects that depended on energetic context rather than being uniform across conditions. The mixed-effects model provided the most biologically and statistically relevant inference, and significant contrasts highlighted settings where Tg differed from NT. Interpretation should consider the small sample size, possible heteroscedasticity, and our focus on JO alone.

Overall, considering substrate and dose alongside genotype reveals context-dependent mitochondrial function and motivates future studies with larger cohorts, additional mitochondrial outcomes, and more flexible dose–response modeling.

Appendix

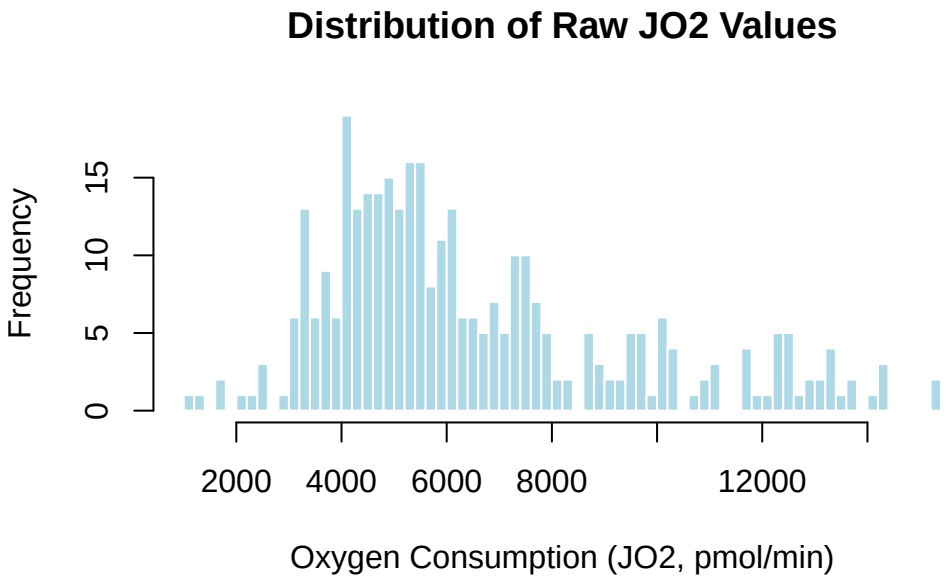

Figure 1: Distribution of raw JO2 values

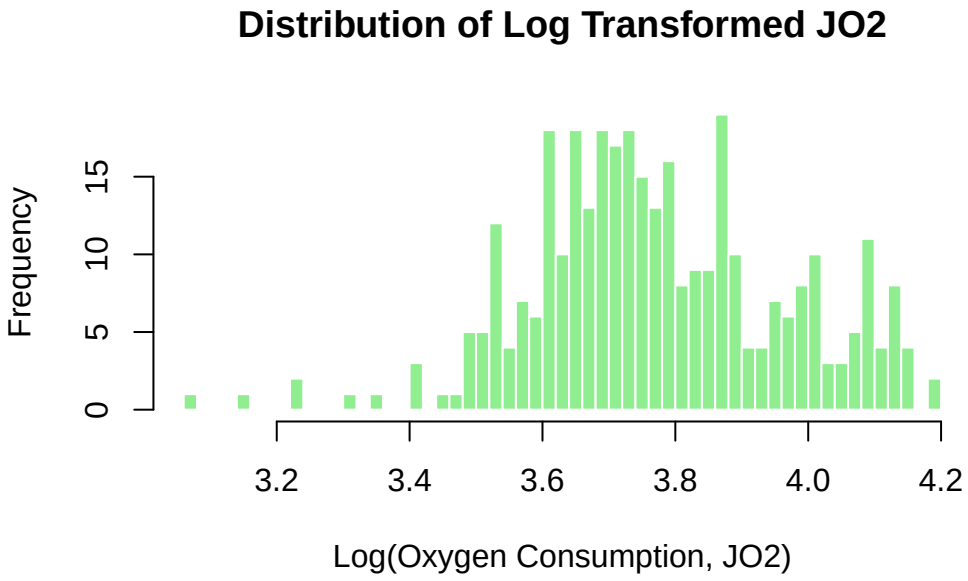

Figure 2: Distribution of log10-transformed JO2 values

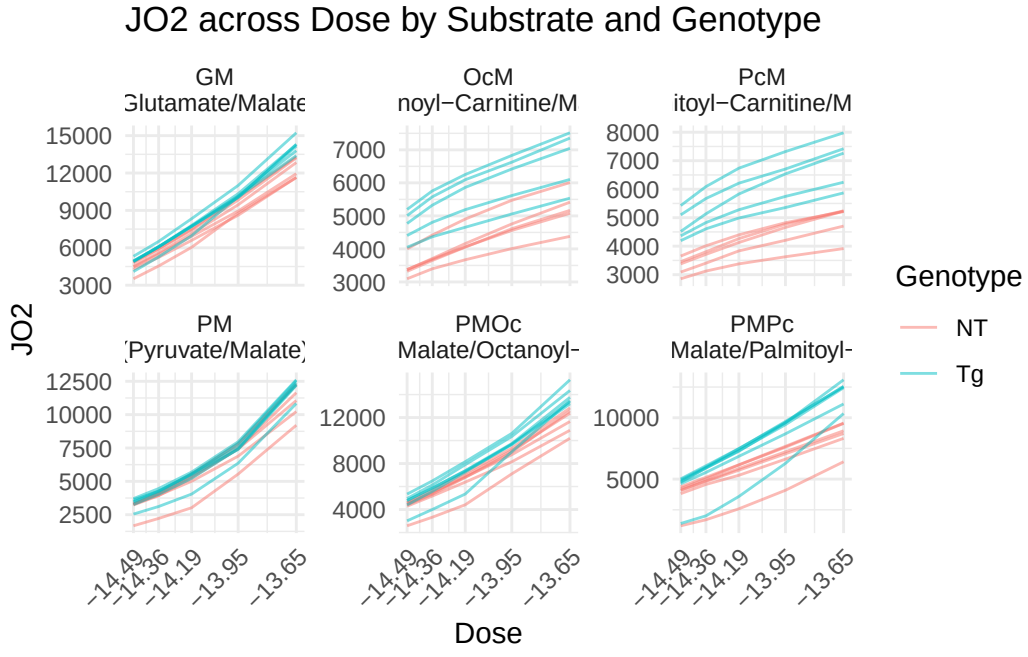

Figure 3: JO2 across dose by substrate and genotype

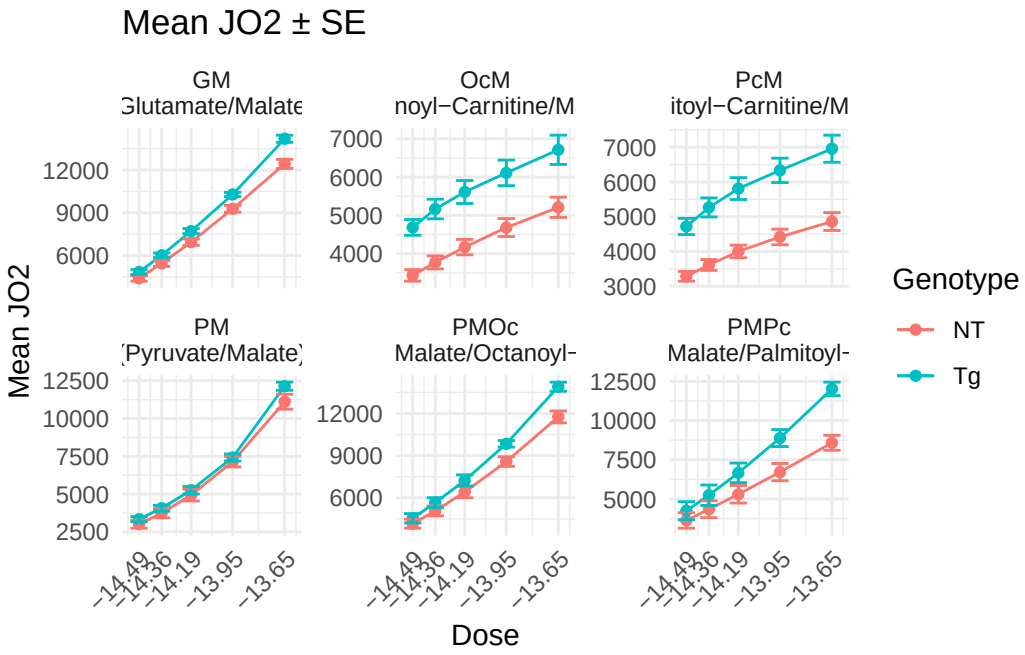

Figure 4: Mean JO2 with ±SE by genotype and substrate

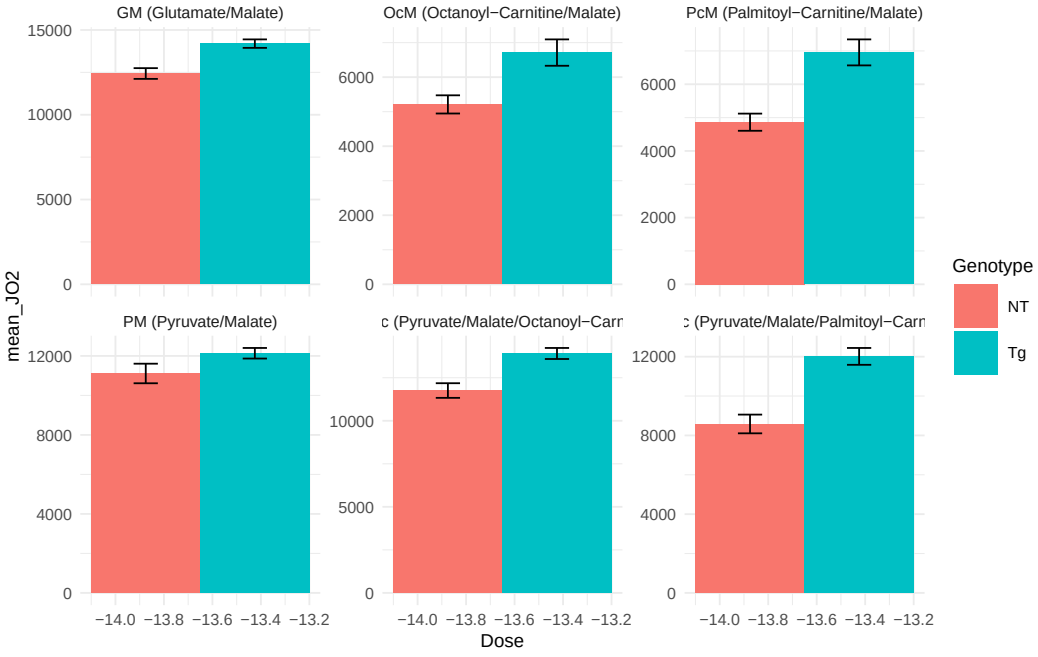

Figure 5: Mean JO2 at Basal and low dose (-13.65)

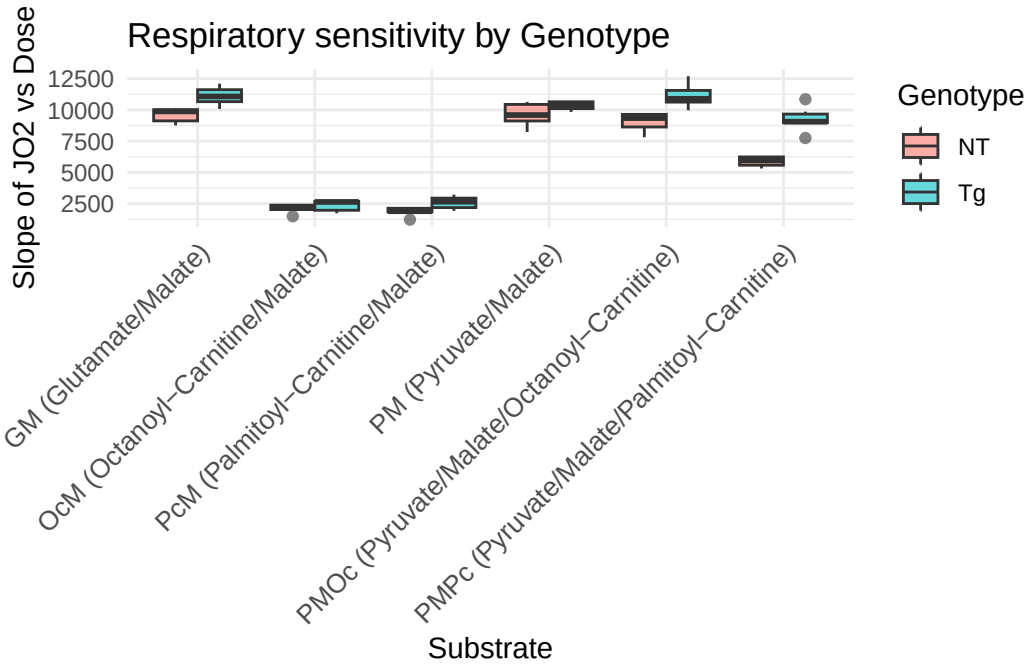

Figure 6: Distribution of JO2–dose slopes by genotype and substrate

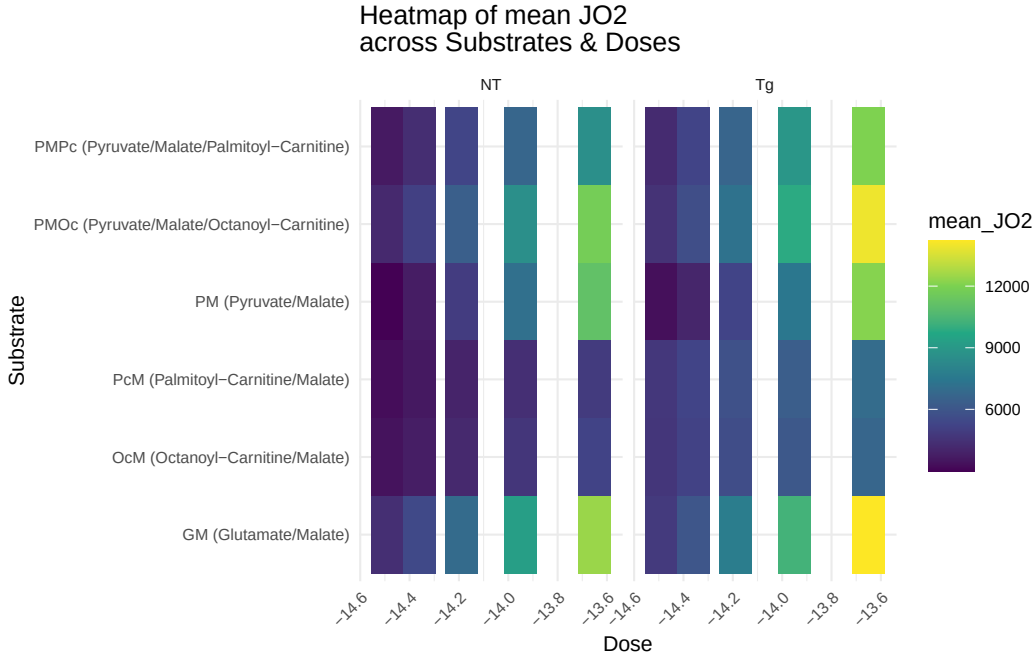

Figure 7: Heatmap of mean JO2 across substrates and doses

Model Tables

Table 1: Linear model (JO2 ~ Substrate * Dose) coefficients

term	estimate	std.error	statistic	p.value
(Intercept)	154427.081	6618.435	23.333	0.000
SubstrateOcM (Octanoyl-Carnitine/Malate)	-117987.489	9816.726	-12.019	0.000
SubstratePcM (Palmitoyl-Carnitine/Malate)	-118080.399	9816.726	-12.028	0.000
SubstratePM (Pyruvate/Malate)	-7154.046	9359.881	-0.764	0.445
SubstratePMOc (Pyruvate/Malate/Octanoyl-Carnitine)	-4072.084	9359.881	-0.435	0.664
SubstratePMPc (Pyruvate/Malate/Palmitoyl-Carnitine)	-40862.833	9359.881	-4.366	0.000
Dose	10353.285	468.357	22.106	0.000
SubstrateOcM (Octanoyl-Carnitine/Malate):Dose	-8124.780	694.686	-11.696	0.000
SubstratePcM (Palmitoyl-Carnitine/Malate):Dose	-8129.319	694.686	-11.702	0.000
SubstratePM (Pyruvate/Malate):Dose	-369.061	662.357	-0.557	0.578
SubstratePMOc (Pyruvate/Malate/Octanoyl-Carnitine):Dose	-257.125	662.357	-0.388	0.698
SubstratePMPc (Pyruvate/Malate/Palmitoyl-Carnitine):Dose	-2779.600	662.357	-4.197	0.000

Table 2: Type II ANOVA for JO2 ~ Substrate * Dose

	Term	Sum Sq	Df	F value	Pr(>F)
Substrate	Substrate	501456649	5	84.879	0
Dose	Dose	1654342978	1	1400.103	0
Substrate:Dose	Substrate:Dose	362539320	5	61.365	0
Residuals	Residuals	387560330	328	NA	NA

Table 3: Type III ANOVA for JO2 ~ Genotype * Substrate * Dose

	Term	Sum Sq	Df	F value	Pr(>F)
(Intercept)	(Intercept)	276876709	1	392.648	0.000
Genotype	Genotype	3352741	1	4.755	0.030
Substrate	Substrate	170806542	5	48.445	0.000
Dose	Dose	248030403	1	351.740	0.000
Genotype:Substrate	Genotype:Substrate	8560933	5	2.428	0.035
Genotype:Dose	Genotype:Dose	3085222	1	4.375	0.037
Substrate:Dose	Substrate:Dose	162166791	5	45.995	0.000
Genotype:Substrate:Dose	Genotype:Substrate:Dose	8399558	5	2.382	0.038
Residuals	Residuals	222828039	316	NA	NA

Table 4: Mixed model fixed effects (lmer)

	Term	Estimate	Std. Error	df	t value	p-value
(Intercept)	(Intercept)	143278.423	4551.721	308.850	31.478	0.000
GenotypeTg	GenotypeTg	22297.316	6437.105	308.850	3.464	0.001
SubstrateOcM (Octanoyl-Carnitine/Malate)	SubstrateOcM (Octanoyl-Carnitine/Malate)	109528.826	- 6734.222	305.904	- 16.265	0.000
SubstratePcM (Palmitoyl-Carnitine/Malate)	SubstratePcM (Palmitoyl-Carnitine/Malate)	113298.516	- 6734.222	305.904	- 16.824	0.000
SubstratePM (Pyruvate/Malate)	SubstratePM (Pyruvate/Malate)	1342.392	- 6420.797	305.904	- 0.209	0.835
SubstratePMOc (Pyruvate/Malate/Octanoyl-Carnitine)	SubstratePMOc (Pyruvate/Malate/Octanoyl-Carnitine)	8026.608	- 6420.797	305.904	- 1.250	0.212
SubstratePMPc (Pyruvate/Malate/Palmitoyl-Carnitine)	SubstratePMPc (Pyruvate/Malate/Palmitoyl-Carnitine)	54350.934	- 6420.797	305.904	- 8.465	0.000
Dose	Dose	9596.474	321.289	305.904	29.869	0.000
GenotypeTg:SubstrateOcM (Octanoyl-Carnitine/Malate)	GenotypeTg:SubstrateOcM (Octanoyl-Carnitine/Malate)	17511.829	- 9523.628	305.903	- 1.839	0.067
GenotypeTg:SubstratePcM (Palmitoyl-Carnitine/Malate)	GenotypeTg:SubstratePcM (Palmitoyl-Carnitine/Malate)	10158.270	- 9523.628	305.903	- 1.067	0.287
GenotypeTg:SubstratePM (Pyruvate/Malate)	GenotypeTg:SubstratePM (Pyruvate/Malate)	11623.308	- 9080.378	305.903	- 1.280	0.201
GenotypeTg:SubstratePMOc (Pyruvate/Malate/Octanoyl-Carnitine)	GenotypeTg:SubstratePMOc (Pyruvate/Malate/Octanoyl-Carnitine)	7909.048	9080.378	305.903	0.871	0.384
GenotypeTg:SubstratePMPc (Pyruvate/Malate/Palmitoyl-Carnitine)	GenotypeTg:SubstratePMPc (Pyruvate/Malate/Palmitoyl-Carnitine)	26976.201	9080.378	305.903	2.971	0.003
GenotypeTg:Dose	GenotypeTg:Dose	1513.622	454.371	305.903	3.331	0.001
SubstrateOcM (Octanoyl-Carnitine/Malate):Dose	SubstrateOcM (Octanoyl-Carnitine/Malate):Dose	7485.712	- 476.548	305.903	- 15.708	0.000
SubstratePcM (Palmitoyl-Carnitine/Malate):Dose	SubstratePcM (Palmitoyl-Carnitine/Malate):Dose	7737.110	- 476.548	305.903	- 16.236	0.000
SubstratePM (Pyruvate/Malate):Dose	SubstratePM (Pyruvate/Malate):Dose	26.091	454.371	305.904	0.057	0.954
SubstratePMOc (Pyruvate/Malate/Octanoyl-Carnitine):Dose	SubstratePMOc (Pyruvate/Malate/Octanoyl-Carnitine):Dose	532.464	- 454.371	305.904	- 1.172	0.242
SubstratePMPc (Pyruvate/Malate/Palmitoyl-Carnitine):Dose	SubstratePMPc (Pyruvate/Malate/Palmitoyl-Carnitine):Dose	3706.611	- 454.371	305.904	- 8.158	0.000
GenotypeTg:SubstrateOcM (Octanoyl-Carnitine/Malate):Dose	GenotypeTg:SubstrateOcM (Octanoyl-Carnitine/Malate):Dose	1278.135	- 673.941	305.903	- 1.897	0.059
GenotypeTg:SubstratePcM (Palmitoyl-Carnitine/Malate):Dose	GenotypeTg:SubstratePcM (Palmitoyl-Carnitine/Malate):Dose	784.418	- 673.941	305.903	- 1.164	0.245
GenotypeTg:SubstratePM (Pyruvate/Malate):Dose	GenotypeTg:SubstratePM (Pyruvate/Malate):Dose	790.304	- 642.578	305.903	- 1.230	0.220
GenotypeTg:SubstratePMOc (Pyruvate/Malate/Octanoyl-Carnitine):Dose	GenotypeTg:SubstratePMOc (Pyruvate/Malate/Octanoyl-Carnitine):Dose	550.678	642.578	305.903	0.857	0.392
GenotypeTg:SubstratePMPc (Pyruvate/Malate/Palmitoyl-Carnitine):Dose	GenotypeTg:SubstratePMPc (Pyruvate/Malate/Palmitoyl-Carnitine):Dose	1854.022	642.578	305.903	2.885	0.004

Table 5: Model fit statistics

Model	AIC	BIC	logLik	DF_Residual	N
lm_marginal	6383.189	6394.676	-3188.594	338	340
lm_raw	5732.667	5782.443	-2853.333	328	340
lm_multi	5568.486	5664.210	-2759.243	316	340
lmm	5002.741	5102.293	-2475.370	NA	340

Results

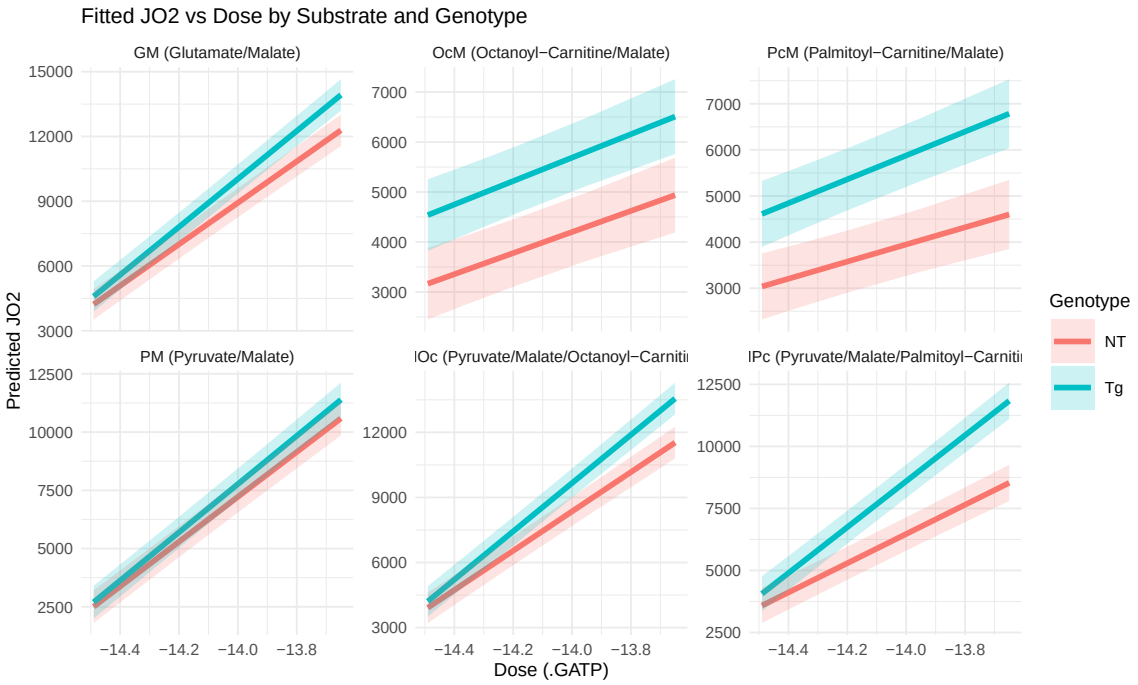

Figure 8: Predicted JO2 vs dose by substrate and genotype from mixed model

Table 6: Estimated genotype differences (Tg – NT) by substrate and dose

Substrate	Dose	Estimate	SE	df	t	p
GM (Glutamate/Malate)	-14.128	912.865	477.733	11.470	1.911	0.081
OcM (Octanoyl-Carnitine/Malate)	-14.128	1458.532	482.546	11.934	3.023	0.011
PcM (Palmitoyl-Carnitine/Malate)	-14.128	1836.859	482.546	11.934	3.807	0.003
PM (Pyruvate/Malate)	-14.128	454.965	477.733	11.470	0.952	0.361
PMOc (Pyruvate/Malate/Octanoyl-Carnitine)	-14.128	1041.937	477.733	11.470	2.181	0.051
PMPc (Pyruvate/Malate/Palmitoyl-Carnitine)	-14.128	1695.440	477.733	11.470	3.549	0.004

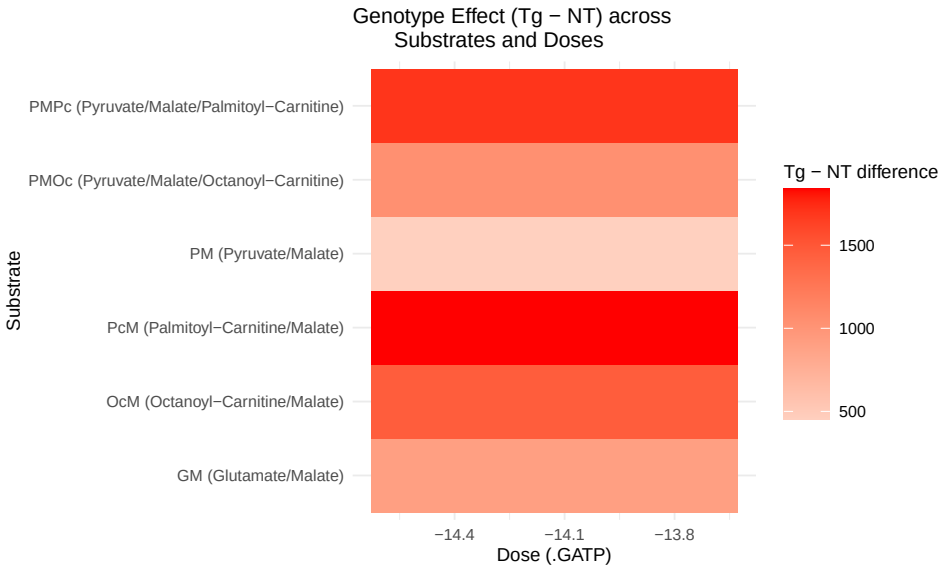

Figure 9: Genotype effect (Tg – NT) across substrates and doses

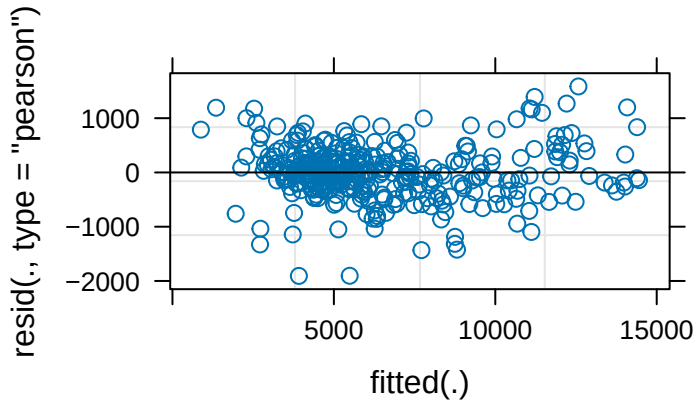

Figure 10: Residuals versus fitted values for mixed model

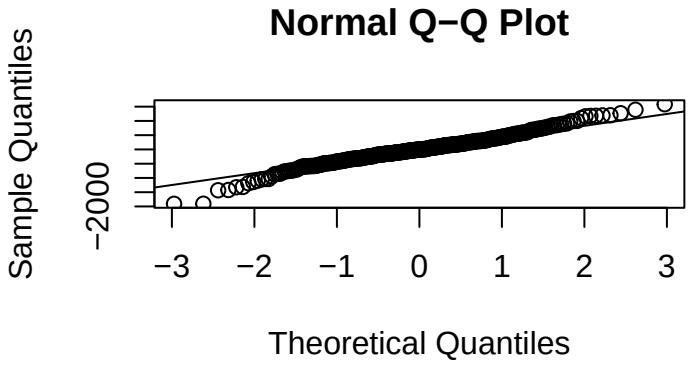

Figure 11: QQ plot of mixed model residuals