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Role of Stress in the Onset of Diabetes Mellitus in Mice.

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Role of Stress in the Onset of Diabetes Mellitus in Mice

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Brief Description:

Although diabetes is an extremely common disease both in the U.S. and in other countries, much remains to be learned about its relationships with environmental factors, such as stress. This study focused on comparing mice injected with low levels of a diabetes-inducing drug and were subsequently stressed with those that were not in order to study the effects of stress on the onset of diabetes mellitus.

Key Points:

1. Because the drug streptozotocin (STZ) destroys the beta cells of the pancreatic islets, it is commonly used to induce diabetes mellitus type 1 in study animals. While stress is commonly applied to already diabetic animals, in the present study lower levels of streptozotocin were administered and stress was immediately applied to mice even before exhibiting diabetic symptoms.

2. Study animals were monitored through glucose measurements taken three times a week and bodyweight gain and feed measurements taken twice weekly in order to determine if stress advanced or delayed the onset of diabetes mellitus. Cortisol concentrations in the blood were measured at the end of the study.

3. Of the three levels of STZ administered (0, 25, 50 mg/kg bodyweight) only the 50 mg group exhibited diabetic symptoms. In fact, it was the non-stress 50mg STZ group that became diabetic.

4. Statistical analysis of baseline values showed that the animals were randomly assigned to treatment groups and no bias existed before the treatments began.

5. Analysis revealed that STZ, stress, and their interaction became statistically significant in causing the measurable differences among treatment groups.

Key Words:

Diabetes mellitus; stress; restraint stress; streptozotocin

Introduction

Stress is commonly seen as something to be removed from our lives because of its potentially harmful effects on the body. Allostasis is the method the body employs in response to stress. Stress can be categorized into two types: acute and chronic. During acute stress the hormones cortisol and adrenaline are released, causing increased blood pressure and heart rate and heightened immune system and memory, which can be helpful for a short period of time. The allostatic load of chronic stress, however, can be detrimental. Stomachaches occur due to increased appetite, and therefore weight, and a weakened immune system results.[17] Blood pressure, heart rate, appetite, cholesterol, triglyceride, and blood sugar levels are all increased during chronic stress. These are not only risk factors for heart disease, atherosclerosis, stroke and obesity, but also diabetes.[17]

The World Health Organization defined diabetes mellitus as a metabolic disorder of multiple origins characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both.[1] Diabetes mellitus can cause lasting damage, dysfunction and even failure of a variety of organs. It sometimes presents with representative symptoms such as polydipsia, polyuria, blurry vision, and weight loss. Ketoacidosis or a non-ketotic hyperosmolar state can result from severe forms of diabetes, which can lead to stupor, coma, and even death without effective treatment. Frequently symptoms are not severe or even absent. As a result, hyperglycemia severe enough to cause pathological and functional changes may be present for a substantial amount time before it is discovered that the patient is suffering from diabetes mellitus. This disease has multiple long-term effects including the progressive development of the specific complications of retinopathy with potential blindness, nephropathy that possibly results in renal failure, and/or neuropathy

with risk of foot ulcers, amputation, Charcot joints, and features of autonomic dysfunction. An increased risk of cardiovascular, peripheral vascular, and cerebrovascular disease may also be expected. The development of diabetes involves several disease-causing processes, including processes which destroy the beta cells of the pancreatic islets, causing insulin deficiency, and others that cause resistance to the function of insulin. Deficient insulin function on target tissues because of insensitivity or lack of insulin causes the abnormalities of carbohydrate, fat and protein metabolism.[1]

Although forms of insulin problems always exist in diabetes mellitus, there are several types of diabetes that have been classified based on the underlying problem which can be identified specifically, such as genetic defects of beta-cell function, genetic defects in insulin action, diseases of the exocrine pancreas, endocrinopathies, drug- or chemical-induced, infections, uncommon forms of immune-mediated diabetes, and other genetic syndromes sometimes associated with diabetes. In the other more publicly well-known classifications of diabetes, type 1 and type 2, the specific causes for the development of abnormalities remain as mysteries.[1] Diabetes type1was previously known as insulin dependent diabetes mellitus, IDDM[1,5] and type 2 was known as non-insulin dependent diabetes mellitus (NIDDM).[1] These names were dropped in favor of type 1 and 2 in order to ensure patients were classified based on pathogenesis rather than treatment. Diabetes type 2 is characterized by the malfunction of insulin action or secretion, either of which may be more prevalent, but both are usually seen at the time of diagnosis. The category of type 1 includes cases of diabetes occurring as a result of the destruction of the beta cells of the pancreatic islets not caused by an underlying condition, such as cystic fibrosis. People suffering from diabetes type1are prone to ketoacidosis and can be diagnosed at an early stage of the disease, hence its former name juvenile onset diabetes

mellitus.[1, 5] Insulin is a required treatment to prevent ketoacidosis and an impending coma. Type 1 diabetic patients have anti-glutamic acid decarboxylase (GAD), which is characteristic of this form of diabetes. Anti-GAD are islet cell or insulin antibodies that identify the autoimmune processes that lead to beta cell destruction.[1] Diabetes type 1is classified as a chronic autoimmune disease. The insulin-producing beta cells, which make up the majority of the pancreatic islets but only 2% of the entire pancreas, are destroyed by T cell-mediated autoimmune destruction in subjects with a genetic predisposition to this disease.[5, 24] The resulting insulin deficiency can lead to a death which is similar to accelerated starvation. While administration of insulin has prevented the majority of these types of deaths, it results in severe hypoglycemia being a constant risk for type 1 diabetics.[5]

Affecting nearly 170 million individuals worldwide, diabetes is already a huge global issue and is expected to continue growing. At least 366 million individuals are anticipated to receive a diagnosis of diabetes within a future span of 25 years. These are also most likely underestimates as they presume obesity levels will remain constant. Although diabetes mellitus type 1 only accounts for 5-10 percent of all diabetics, it represents a noteworthy health concern, as this disorder begins early in life and leads to continuing health complications.[16] Streptozotocin-treated mice are commonly used to model diabetes type1 due to the ability of streptozotocin (STZ) to destroy the insulin-producing beta cells of the pancreatic islets of Langerhans.[16, 23] Many studies have been completed on the effects of stress on STZ diabetic mice and rats. It is known that STZ-induced diabetic rats have greater sensitivity to stress, as seen through increased plasma levels of corticosterone. Some mechanisms between diabetes and stress have also been found to be similar: beta-amyloid toxicity is made more potent in the hippocampus of rats treated with STZ, HNE protein conjugation, proposed to mediate beta-

amyloid toxicity, is increased in the hippocampus of diabetic rats subjected to stress, [23] and the stimulation of morphological changes in the hippocampus have been associated with chronic stress.[11]

Cortisol levels, known to rise in response to stress, are increased in type 1 diabetic patients. It has also been found that stress enhances the production of immunosuppressive cytokines. Also, psychological stress (restraint stress) has been linked with an increased occurrence of infectious disease, which demonstrates that the immunosuppressive actions of stress translate into significant adverse health effects.[6] Changes in STZ-induced diabetic stressed rats were attributed to glucocorticoid impairment.[23] Most research examining the relationship of restraint stress and STZ-induced diabetes has been performed on already diabetic animal models. In the present study, normal, healthy Swiss ICR mice were exposed to restraint stress and lower doses of STZ than normally used to induce diabetes mellitus in mice. Due to the fact that cortisol levels rise in response to stress and increase blood glucose levels, we hypothesized that stress would accelerate the onset of diabetes mellitus.

However, evidence that stress may delay the onset of diabetes also exists. In one study, it was found that light repeated emotional stress hampered development of obesity and diabetes type 2 in mice with the Agouti yellow mutation.[3] In a study using Zucker diabetic fatty rats it was found that intermittent restraint and its adaptations delayed hyperglycemia and improved glucose control, which may be explained by restraint-induced lowering of food intake and lower overall corticosterone exposure with repeated restraint. Ironically, these investigations suggest some types of occasional stress may limit development of diabetes.[2] These, however, are examples of stress delaying the onset of diabetes type 2 in obese mice and rats.

Materials and Methods

Experimental procedure

Sixty-seven 8-week-old mice were housed individually for three days to acclimatize. Mice were weighed and the extra mice were excluded on the basis of weight.

The mice were divided into two groups: Group A and Group B. Group A included mice numbered 1-30; group B included mice numbered 31-60. The experiment began with Group A and ended 18 days later. Experimentation on Group B began one day after Group A had been started. Both groups of mice received injections the first three days of the experiment. The mice received treatment as shown in Table 1 according to two factors: stress and streptozotocin (STZ).

Table 1. Treatment Groups

Factor 1	Factor 2	
Streptozotocin	**No Stress**	**Stress**
0 STZ	#1-5, 31-35	#16-20, 46- 50
25 STZ	#6-10, 36-40	#21-25, 51-55
50 STZ	# 11-15, 41-45	#26-30, 56-60

Baseline animal weight, feed weight, and glucose levels were taken on D_0 for all mice and STZ or buffer injections were given.

Glucose measurements were taken three times a week beginning on D_0 for both groups using blood glucose meters (FreeStyle Freedom Lite, Catalog number 70914, NDC 99073-0709-14, Distributed by: Abbott Diabetes Care Inc) and appropriate test strips (Freestyle Lite Blood Glucose Test Strips).

Mice were weighed twice each week. Feed weight was taken two times per week. Feed was refilled and reweighed as needed to calculate food consumption.

Stressed mice were placed in 50 ml well-ventilated centrifuge tubes packed lightly with approved nesting material for 6 hours beginning at approximately the same time each day during which time the control mice were free in their cages but without access to feed and water. After the 6 hour-period, the treated mice were returned to their cages with access to feed and water and the control mice were again given access to feed and water.

Tissue Collection

At the end of the 18 days of treatment, mice were euthanized and blood was collected.

Corticosterone Assay

The Corticosterone assay was performed according to the method outlined in assay designs© Corticosterone Enzyme Immunoassay Kit (Catalog No. 900-097). First, the reagents were prepared. Assay Buffer 15 was prepared using a 9 to 1 ratio of distilled water to supplied Assay Buffer 15, respectively. Wash buffer was prepared in a similar manner using a 19 to 1 ratio of distilled water to supplied wash buffer. Separate Corticosterone standards were prepared for each plate. The Corticosterone standards were prepared as follows: 900 µL of standard diluent (prepared Assay buffer 15) was added to tube #1; 800 µL of standard diluent was added to tubes #2 - #5. To tube #1, 100 µL of the provided 200,000 pg/mL standard was added, generating standard 1. To tube #2, 200 µL of standard 1 was added, generating standard 2. To tube #3, 200 µL of standard 2 was added, generating standard 3. To tube #4, 200 µL of standard 3 was added, generating standard 4. To tube #5, 200 µL of standard 4 was added, generating

standard 5. The concentrations of standards 1 through 5 in pg/mL are 20,000, 4,000, 800, 160, and 32, respectively.

Standards were run in duplicate while samples were run in triplicate. Reagents were brought to room temperature and 100 µL of Assay Buffer 15 was pipetted into wells designated NSB and Bo. Then 100 µL of Standards 1 through 5 were pipetted into the correct wells. 100 µL of samples were pipetted into their designated wells. Into the NSB wells 50 µL of Assay Buffer 15 was pipetted. Excluding wells designated Total Activity and Blank, 50 µL of blue Conjugate was pipetted into each well. Excluding wells designated Blank, Total Activity, and NSB, 50 µL of yellow Antibody was pipetted into each well. At this time, NSB wells were blue in color, Blank and Total Activity had no color, and every other well used was green.

The plates were covered with the provided plate sealer and incubated at room temperature on a plate shaker for 2 hours. The contents of the wells were dumped and washed using wash solution a total of three times. Following the final wash, the plates were tapped firmly on a lint free paper towel to remove remaining wash buffer. Next 5 µL of blue Conjugate was added to the Total Activity wells. Finally every well received 200 µL of Stop Solution and the plates were read.

Results

Glucose, bodyweight, and feed consumption analysis using two-way ANOVA and ANCOVA

Two-way ANOVA, also known as the two-way analysis of variance, is necessary in this case because there are two factors: STZ and Stress. The experiment was a factorial one in which the response is observed at all factor-level combinations of the independent variables. If the factors were expressed as their sum, the sums would be too large because each would have to

include the overall mean. Instead, the two-way ANOVA model splits the total variability into four sources of variability, which in this case are: the main effects of STZ, the main effects of stress, the possible interaction between STZ and stress, and the unexplained variability from all sources not accounted for by the main effects and interaction, known as error.[22]

Total variability is the variability in the response variable among the 60 mice and is represented by

$$\sum_{i=1}^{3}\sum_{j=1}^{2}\sum_{k=1}^{10}(y_{ijk}-\bar{y})^2 \qquad (1.1)$$

In the present study, the variables in the two-way ANOVA model

$$Y_{ijk} = u+\alpha_i+\beta_j+\gamma_{ij}+e_{ijk} \qquad (1.2)$$

are: Y: measurement (ex. glucose level on day 3), U: mean, α: effect of STZ, β: effect of stress, γ: interaction between them, and e: error, where the subscript $_i$ denotes STZ, $_j$ denotes stress, and $_k$ denotes the individual mouse. ANOVA answers the question: At each point, what is the effect of the treatment? In order to compare changes among individuals, baseline values were taken: measurements taken before any treatments were applied. Baseline measurements were important not only to compare changes among individuals, but also to ensure that the independence assumption of the two-way ANOVA model was not violated. This was done by checking for randomization.[8]

ANCOVA is another statistical tool that can be used alongside ANOVA in order to better control for outside variance. ANCOVA is ANOVA with one or more covariates. One of its important uses is to increase precision in randomized experiments. It does this by removing variability not due to the experimental treatments themselves. Variables other than those of the main scientific interest can be measured and the variability due to them can be partitioned in order to better assess the effects due to the variable of scientific interest.[25] In this case, initial

glucose level of the animal is used as a covariate when studying the difference in glucose between treatments. In this manner, one could determine if the baseline glucose level is an important factor. ANCOVA is an elegant way of accounting for the effect of baseline glucose level when analyzing glucose level for one specific time. The model is similar to 2 Way ANOVA with an extra term which is the baseline glucose.

$$Y_{ijk}=\mu + \alpha_i + \beta_j + \gamma_{ij} + b*\text{baseline glucose} + e_{ijk} \qquad (1.3)$$

ANOVA and ANCOVA GLUCOSE RESULTS

As seen in the two-way ANOVA of the baseline glucose values, no difference existed among the groups. Therefore the mice were randomly assigned to treatments and no bias existed at the beginning of the experiment.

Table 2. Glucose day 0: Raw values

Two-way ANOVA: glucd0 versus STZ, Stress

Source	DF	SS	MS	F	P
STZ	2	1015.2	507.617	1.03	0.363
Stress	1	2.4	2.400	0.00	0.945
Interaction	2	162.9	81.450	0.17	0.848
Error	54	26516.4	491.044		
Total	59	27696.9			

S = 22.16 R-Sq = 4.26% R-Sq(adj) = 0.00%

On the 4th day there was still no difference when the raw scores were analyzed (Table 3), nor ANCOVA of the 4th day with glucose day 0 as a covariate (Table 4).

Table 3. Glucose day 4: Raw scores
Two-way ANOVA: gluc4 versus STZ, Stress

Source	DF	SS	MS	F	P
STZ	2	4158	2078.87	1.08	0.345
Stress	1	2802	2801.67	1.46	0.232
Interaction	2	2241	1120.47	0.58	0.561
Error	54	103483	1916.36		
Total	59	112684			

S = 43.78 R-Sq = 8.16% R-Sq(adj) = 0.00%

Table 4. ANCOVA Glucose day 4 with Glucose day 0 as a covariate

General Linear Model: gluc4 versus STZ, Stress

```
Analysis of Variance for gluc4, using Adjusted SS for Tests

Source      DF  Seq SS  Adj SS  Adj MS     F      P
glucd0       1    3432    4922    4922  2.65  0.110
STZ          2    5873    5833    2916  1.57  0.218
Stress       1    2730    2731    2731  1.47  0.231
STZ*Stress   2    2087    2087    1044  0.56  0.574
Error       53   98561   98561    1860
Total       59  112684

S = 43.1235   R-Sq = 12.53%   R-Sq(adj) = 2.63%
```

On the seventh day the difference among the different levels of STZ began to become significant, although there was no difference yet between stressed and non-stressed mice. By day 7 STZ became significant with a *P*-value of less than 0.05 meaning that because of the STZ, the different groups of mice no longer had the same glucose levels. STZ was also significant on day 7 using ANCOVA with glucose day 0 as a covariate.

Table 5. Glucose day 7: Raw scores
Two-way ANOVA: gluc7 versus STZ, Stress

```
Source       DF       SS       MS     F      P
STZ           2  12734.8  6367.40  4.67  0.013
Stress        1   1706.7  1706.67  1.25  0.268
Interaction   2   1917.7   958.87  0.70  0.499
Error        54  73604.4  1363.04
Total        59  89963.6

S = 36.92   R-Sq = 18.18%   R-Sq(adj) = 10.61%
```

Table 6. ANCOVA Glucose day 7 with Glucose day 0 as a covariate
General Linear Model: gluc7 versus STZ, Stress

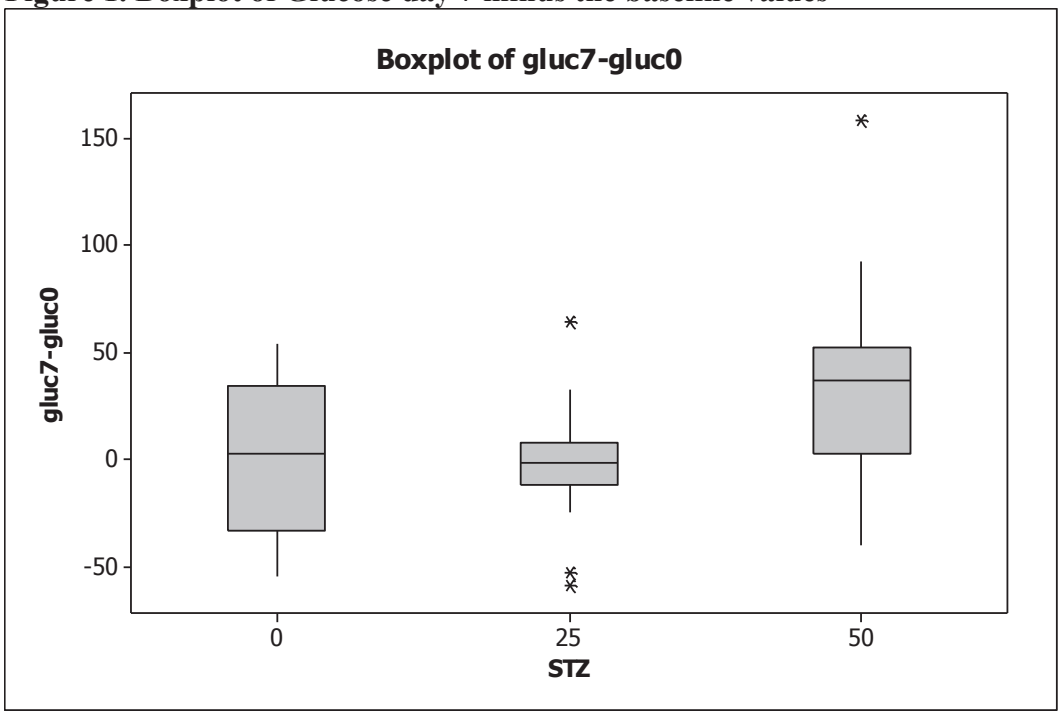
```
Analysis of Variance for gluc7, using Adjusted SS for Tests

Source      DF  Seq SS  Adj SS  Adj MS     F      P
glucd0       1    6654    8150    8150  6.60  0.013
STZ          2   14362   14352    7176  5.81  0.005
Stress       1    1636    1636    1636  1.32  0.255
STZ*Stress   2    1857    1857     929  0.75  0.476
Error       53   65454   65454    1235
Total       59   89964

S = 35.1424   R-Sq = 27.24%   R-Sq(adj) = 19.01%
```

The difference was clearer when using ANCOVA with glucose day 0 as a covariate because baseline variability was negated. The boxplot below indicates that there was not a large difference between the STZ=0 and STZ=25 groups; the group of STZ=50 was the one that appeared different with regard to the change in glucose with respect to the baseline values.

Figure 1. Boxplot of Glucose day 7 minus the baseline values



**** In order to do analysis on days 9, 11, and 14, a glucose level of 501 was assigned to mouse 42; the glucose meter read "HI" which denoted a glucose level above 500.

On day 9 the difference among the different STZ groups was also statistically significant:

Table 7. Glucose day 9: Raw scores

Two-way ANOVA: gluc9 versus STZ, Stress

Source	DF	SS	MS	F	P
STZ	2	41876	20937.9	7.02	0.002
Stress	1	3760	3760.4	1.26	0.266
Interaction	2	18277	9138.7	3.07	0.055
Error	54	160960	2980.7		
Total	59	224874			

S = 54.60 R-Sq = 28.42% R-Sq(adj) = 21.79%

Table 8. ANCOVA Glucose day 9 with Glucose day 0 as a covariate

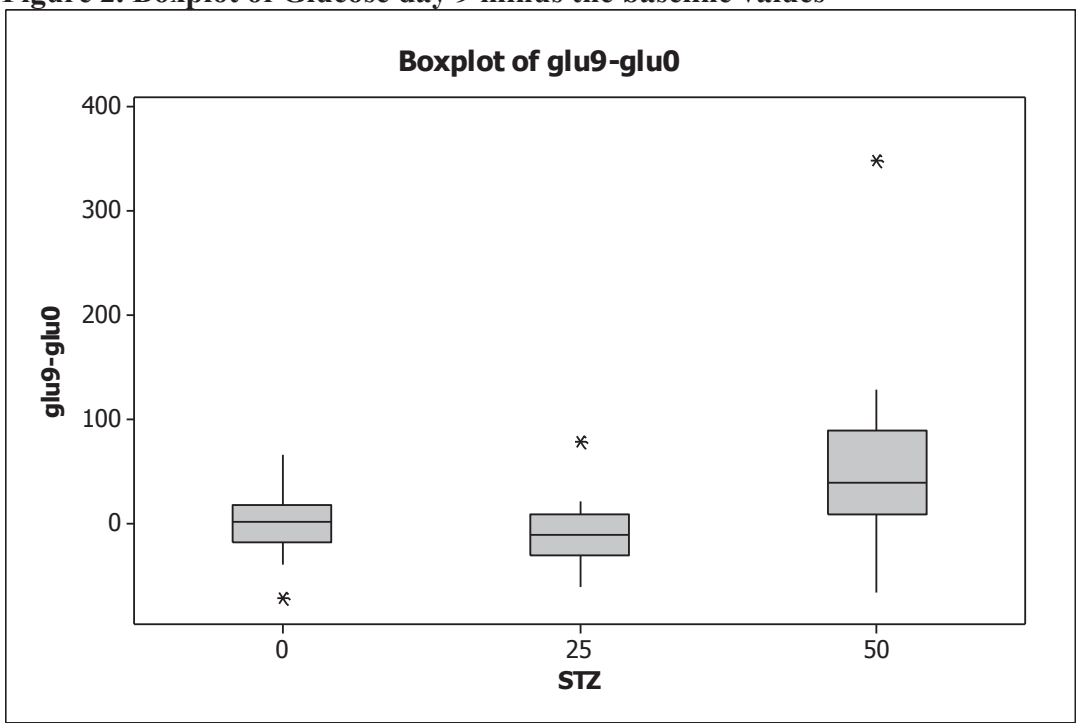
General Linear Model: gluc9 versus STZ, Stress

```
Analysis of Variance for gluc9, using Adjusted SS for Tests

Source      DF  Seq SS  Adj SS  Adj MS     F      P
glucd0       1   11045   14494   14494  5.24  0.026
STZ          2   47435   47017   23509  8.51  0.001
Stress       1    3612    3621    3621  1.31  0.257
STZ*Stress   2   16316   16316    8158  2.95  0.061
Error       53  146466  146466    2764
Total       59  224874
S = 52.5691   R-Sq = 34.87%   R-Sq(adj) = 27.49%
```

Through day 9, the ANCOVA models have shown that baseline glucose seemed to be important in days 7 and 9 but not in days 2 and 4.

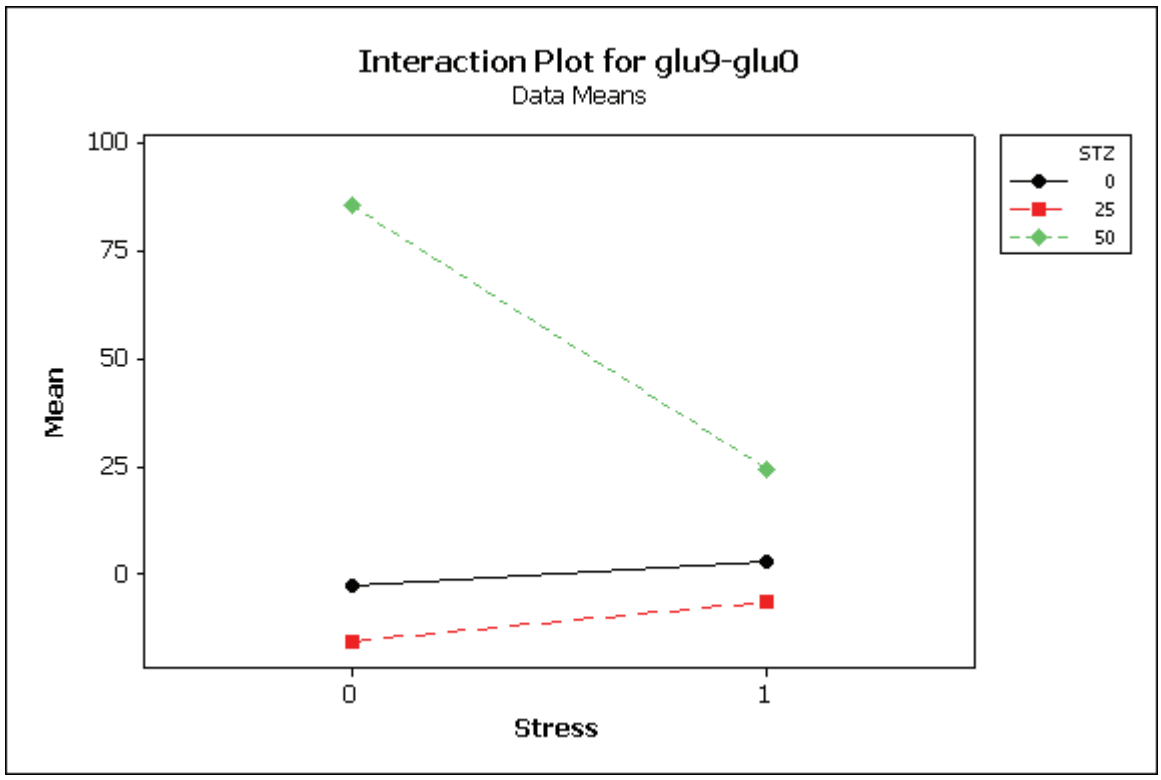
Figure 2. Boxplot of Glucose day 9 minus the baseline values



The interaction between STZ and Stress was not significant at the 0.05 level but it was low enough as to suggest that there was some mild interaction, something that was confirmed by the interaction plot. To show the interaction, the treatments were plotted in a single display called an interaction plot (Figure 3). This plot showed the average of the observations at each

level of one factor broken up by the levels of the other factor.[8] On day 9, the stressed mice of both the 0 and 25mg STZ groups had higher glucose levels than their non-stressed counterparts. The opposite is true with the 50 STZ groups.

Figure 3. Interaction Plot of Glucose day 9 minus baseline values



On the eleventh day the interaction and the effects of stress and STZ all became significant with *P*-values below 0.005. An interaction was noted when the effects of one factor change for different levels of another factor. The interaction plot indicates it is the STZ =50 group which was the one that had a much higher mean for glucose change from the baseline values. However, the STZ affected the mice differently depending if they were stressed or not. Surprisingly the stressed mice showed lower levels of glucose. One possible explanation for this

is that while the mice were being stressed, they were working to escape, which was a form of exercise.[14]

Table 9. Glucose day 11: Raw values
Two-way ANOVA: gluc11 versus STZ, Stress

Source	DF	SS	MS	F	P
STZ	2	101456	50727.8	19.89	0.000
Stress	1	18166	18165.6	7.12	0.010
Interaction	2	33983	16991.5	6.66	0.003
Error	54	137691	2549.8		
Total	59	291295			

S = 50.50 R-Sq = 52.73% R-Sq(adj) = 48.35%

Table 10. ANCOVA Glucose day 11 with Glucose day 0 as a covariate
General Linear Model: gluc11 versus STZ, Stress

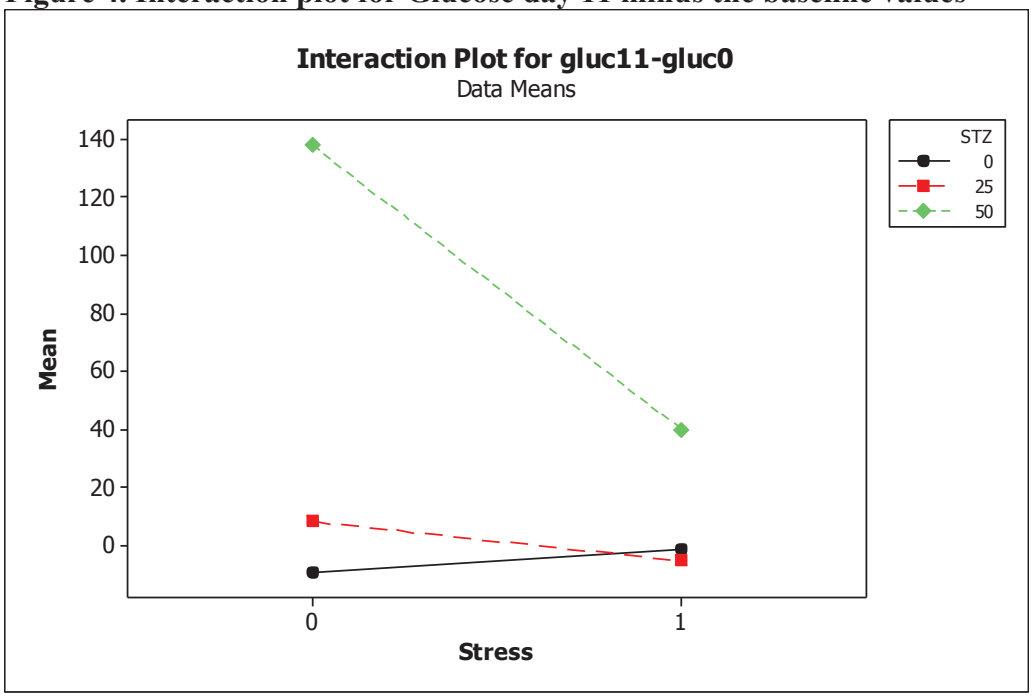
Analysis of Variance for gluc11, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
glucd0	1	3515	5481	5481	2.20	0.144
STZ	2	104967	104477	52238	20.94	0.000
Stress	1	17954	17975	17975	7.21	0.010
STZ*Stress	2	32650	32650	16325	6.54	0.003
Error	53	132209	132209	2495		
Total	59	291295				

S = 49.9451 R-Sq = 54.61% R-Sq(adj) = 49.48%

As seen in the interaction plot in Figure 4, the stressed mice that received 0 STZ had a higher glucose level than their non-stressed counterparts by day 11. Those in the 25 STZ group that were stressed had lower glucose levels than their non-stressed counterparts on day 11, which has changed from day 9.

Figure 4. Interaction plot for Glucose day 11 minus the baseline values



A similar behavior was observed in days 14 and 16. Stress *P*-value was increased by day 14.

Table 11. Glucose day 14: Raw values
Two-way ANOVA: gluc14 versus STZ, Stress

Source	DF	SS	MS	F	P
STZ	2	116397	58198.6	14.18	0.000
Stress	1	11704	11704.1	2.85	0.097
Interaction	2	55412	27705.8	6.75	0.002
Error	54	221709	4105.7		
Total	59	405221			

S = 64.08 R-Sq = 45.29% R-Sq(adj) = 40.22%

Table 12. ANCOVA Glucose day 14 using Glucose day 0 as a covariate
General Linear Model: gluc14 versus STZ, Stress

Analysis of Variance for glug14, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
glucd0	1	7438	10373	10373	2.60	0.113
STZ	2	121918	121237	60618	15.20	0.000
Stress	1	11473	11494	11494	2.88	0.095
STZ*Stress	2	53057	53057	26529	6.65	0.003
Error	53	211336	211336	3987		
Total	59	405221				

S = 63.1464 R-Sq = 47.85% R-Sq(adj) = 41.94%

Figure 5. Interaction Plot for Glucose day 14 minus the baseline values

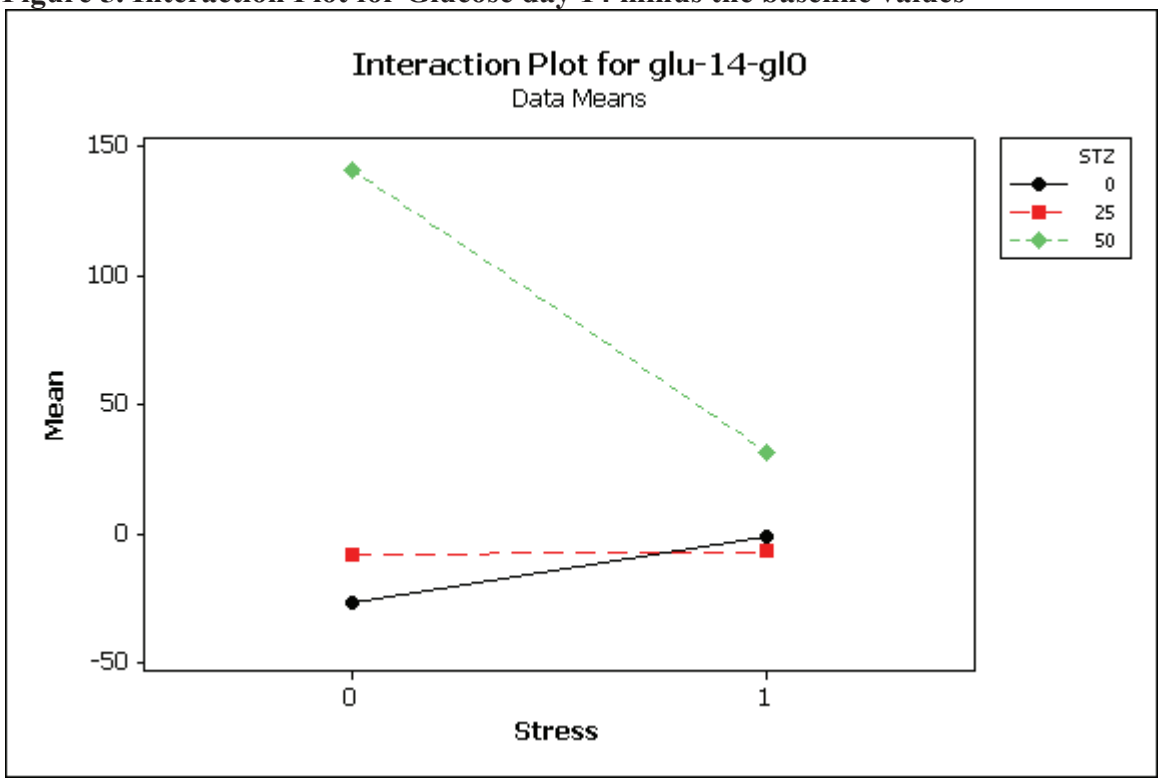


Table 13. Glucose day 16: Raw values

Two-way ANOVA: gluc16 versus STZ, Stress

Source	DF	SS	MS	F	P
STZ	2	185722	92861.2	28.22	0.000
Stress	1	14199	14198.8	4.32	0.043
Interaction	2	44065	22032.5	6.70	0.003
Error	54	177679	3290.4		
Total	59	421665			

S = 57.36 R-Sq = 57.86% R-Sq(adj) = 53.96%

Table 14. ANCOVA Glucose day 16 using Glucose day 0 as a covariate

General Linear Model: gluc16 versus STZ, Stress

Analysis of Variance for gluc16, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
glucd0	1	3625	6955	6955	2.16	0.148
STZ	2	191535	190506	95253	29.57	0.000
Stress	1	13981	14009	14009	4.35	0.042
STZ*Stress	2	41800	41800	20900	6.49	0.003
Error	53	170724	170724	3221		
Total	59	421665				

S = 56.7557 R-Sq = 59.51% R-Sq(adj) = 54.93%

The output of ANCOVA again showed what was already known: at day 16 STZ was significant, stress was significant, and there was an interaction between STZ and stress. The not so small *P*-value (0.148) for baseline glucose indicated that at day 16 the baseline glucose did not really have an effect on the glucose at day 16 but that the treatments caused the difference. However, even when non-significant baseline glucose explained a little bit of the differences among mice, and the R-square (both regular and adjusted) was a little better for ANCOVA than for ANOVA without the covariate. See the output for ANOVA without the covariate.

By day 16, the interaction plot revealed that the non-stressed mice in both the 0 and 25 STZ groups had the lowest mean glucose levels. Also, the stressed 25 STZ group had a slightly higher mean glucose level than the stressed 0 STZ group.

Figure 5. Interaction plot for Glucose day 16 minus baseline values

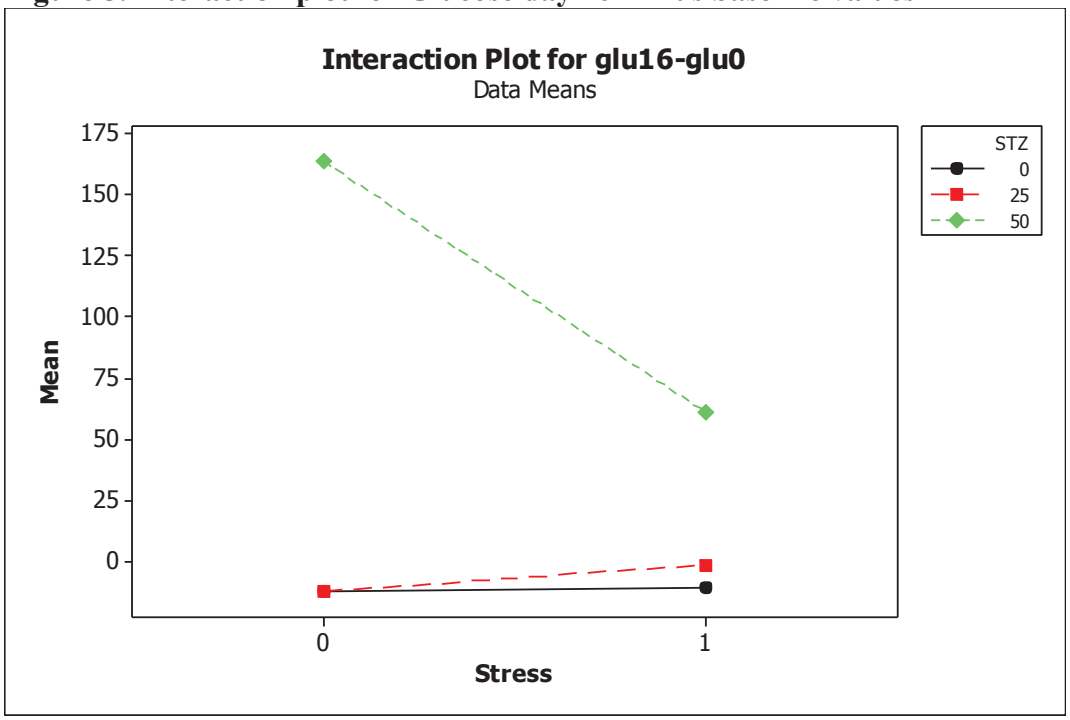


Table 15. Glucose day 18

Two-way ANOVA: gluc18 versus STZ, Stress

```
Source       DF      SS      MS      F      P
STZ           2  252472  126236  34.30  0.000
Stress        1   41082   41082  11.16  0.002
Interaction   2   84933   42467  11.54  0.000
Error        54  198727    3680
Total        59  577214

S = 60.66   R-Sq = 65.57%   R-Sq(adj) = 62.38%
```

Table 16. Glucose day 18 using Glucose day 0 as a covariate

General Linear Model: gluc18 versus STZ, Stress

```
Analysis of Variance for gluc18, using Adjusted SS for Tests

Source      DF  Seq SS  Adj SS  Adj MS      F      P
glucd0       1    4280    9980    9980   2.80  0.100
STZ          2  261177  259795  129898  36.47  0.000
Stress       1   40642   40687   40687  11.42  0.001
STZ*Stress   2   82358   82358   41179  11.56  0.000
Error       53  188750  188750    3561
Total       59  577207

S = 59.6768   R-Sq = 67.30%   R-Sq(adj) = 63.60%
```

The interaction plot below indicates that for the mice who received 0 or 25mg/kg STZ there was not a major difference in mean glucose between the stressed and non-stressed mice, but there was a big difference for those who received 50mg/kg STZ. Actually the non-stressed mice had higher mean glucose.

Figure 6. Interaction of STZ and Stress on Glucose

*** Due to lack of glucose testing strips, glucose measurements were not taken for mouse 48 on day 18. In order to run ANOVA the average glucose value (147) was used for mouse 48.

ANOVA BODYWEIGHT RESULTS

The baseline bodyweight also showed randomization, as the *P*-values for STZ, stress, and their interaction were insignificant. By day 18, STZ and stress were significant factors in the differences of bodyweight among the groups.

Table 17. Bodyweight day 0: Raw values
Two-way ANOVA: bwgd0 versus STZ, Stress

Source	DF	SS	MS	F	P
STZ	2	3.133	1.56650	0.59	0.558
Stress	1	0.793	0.79350	0.30	0.587
Interaction	2	1.677	0.83850	0.32	0.731
Error	54	143.513	2.65765		
Total	59	149.117			

S = 1.630 R-Sq = 3.76% R-Sq(adj) = 0.00%

Table 18. Bodyweight day 4: Raw values
Two-way ANOVA: bwgd4 versus STZ, Stress

Source	DF	SS	MS	F	P
STZ	2	33.334	16.6672	4.90	0.011
Stress	1	28.843	28.8427	8.49	0.005
Interaction	2	1.090	0.5452	0.16	0.852
Error	54	183.526	3.3986		
Total	59	246.793			

S = 1.844 R-Sq = 25.64% R-Sq(adj) = 18.75%

Table 19. Bodyweight day 8: Raw values
Two-way ANOVA: bwgd8 versus STZ, Stress

Source	DF	SS	MS	F	P
STZ	2	34.137	17.0685	4.07	0.023
Stress	1	58.214	58.2135	13.88	0.000
Interaction	2	1.267	0.6335	0.15	0.860
Error	54	226.399	4.1926		
Total	59	320.017			

S = 2.048 R-Sq = 29.25% R-Sq(adj) = 22.70%

Table 20. Bodyweight day 11: Raw values
Two-way ANOVA: bwgd11 versus STZ, Stress

Source	DF	SS	MS	F	P
STZ	2	39.232	19.6162	4.52	0.015
Stress	1	53.016	53.0160	12.21	0.001
Interaction	2	1.129	0.5645	0.13	0.878
Error	54	234.480	4.3422		
Total	59	327.857			

S = 2.084 R-Sq = 28.48% R-Sq(adj) = 21.86%

Table 21. Bodyweight day 15: Raw values
Two-way ANOVA: bwgd15 versus STZ, Stress

Source	DF	SS	MS	F	P
STZ	2	50.317	25.1585	5.81	0.005
Stress	1	49.141	49.1415	11.35	0.001
Interaction	2	2.899	1.4495	0.33	0.717
Error	54	233.839	4.3304		
Total	59	336.197			

S = 2.081 R-Sq = 30.45% R-Sq(adj) = 24.01%

Table 22. Bodyweight day 18: Raw values
Two-way ANOVA: bwgd18 versus STZ, Stress

Source	DF	SS	MS	F	P
STZ	2	49.948	24.9740	5.43	0.007
Stress	1	84.728	84.7282	18.42	0.000
Interaction	2	3.033	1.5167	0.33	0.721
Error	54	248.447	4.6009		
Total	59	386.156			

S = 2.145 R-Sq = 35.66% R-Sq(adj) = 29.70%

ANOVA FEED WEIGHT CONSUMPTION RESULTS

The *P*-values indicate randomization in the beginning of the experiment. However, STZ, stress, and their interaction never became statistically significant.

Table 23. Feed Consumption day 8: Raw values
Two-way ANOVA: fc8 versus STZ, Stress

Source	DF	SS	MS	F	P
STZ	2	401.6	200.81	0.53	0.591
Stress	1	1311.3	1311.34	3.46	0.068
Interaction	2	436.7	218.34	0.58	0.565
Error	54	20441.6	378.55		
Total	59	22591.2			

S = 19.46 R-Sq = 9.52% R-Sq(adj) = 1.14%

Table 24: Feed Consumption day 18: Raw values
Two-way ANOVA: fc18 versus STZ, Stress

Source	DF	SS	MS	F	P
STZ	2	3134	1567.18	0.88	0.422
Stress	1	4333	4333.30	2.42	0.125
Interaction	2	2215	1107.67	0.62	0.542
Error	54	96573	1788.39		
Total	59	106256			

S = 42.29 R-Sq = 9.11% R-Sq(adj) = 0.70%

Table 25: Feed Consumption day 18 minus baseline values
Two-way ANOVA: fc18-8 versus STZ, Stress

Source	DF	SS	MS	F	P
STZ	2	1591.4	795.687	1.21	0.307
Stress	1	877.1	877.073	1.33	0.254
Interaction	2	757.7	378.862	0.57	0.566
Error	54	35617.7	659.586		
Total	59	38843.8			

S = 25.68 R-Sq = 8.31% R-Sq(adj) = 0.00%

Repeated Measures Analysis

Data sets with multiple measurements of a response variable on the same experimental unit are known as repeated measures. The multiple measurements are usually made over a period of time but can also be over a physical space. A completely randomized experimental design with data collected in a sequence of equally spaced points in time is required in order for repeated measures to be applied. Treatments and time are the two factors and repeated measures experiments have a factorial design. How treatment means change over time and how treatment differences change over time are the main focus questions of repeated measures analysis. The covariance structure of the observed data differentiates the repeated measures model from others. Comparing treatment means or treatment regression curves over time are the aims. There are three general types of statistical analyses often used for repeated measures. The method used in the present study applies methods based on the mixed model with special parametric structure on the covariance matrices. This type is applied in PROC MIXED with SAS.[15] In the present study, stress (yes or no) and STZ (0, 25, or 50) were fixed effects factors because the interest was in those specific levels. Mouse was a random effect because the interest was not in the specific individual mice but only as they pertained to a sample of all mice, which is the reason the procedure is called MIXED (mixture of fixed effects and random effect factors).

The SAS output for the mixed model using the following equation is below:

$$Glucose_{ijkm} = u\text{-}STZ_i + stress_j + STZ*s_{\dot{y}} + day + STZ*day + s*day + STZ*s*day + e_{ijkm} \quad (1.3)$$

Table 26. **PROC MIXED**

Effect	Num DF	Den DF	Chi-Square	F Value	Pr > ChiSq	Pr > F
STZ	2	54	51.00	25.50	<.0001	<.0001
Stress	1	54	7.07	7.07	0.0078	0.0103
STZ*stress	2	54	17.33	8.66	0.0002	0.0005
Day	8	432	81.19	10.15	<.0001	<.0001
STZ*day	16	432	80.25	5.02	<.0001	<.0001
Stress*day	8	432	15.01	1.88	0.0590	0.0620
STZ*stress*day	16	432	35.62	2.23	0.0033	0.0043

Cov Parm	Subject	Estimate
AR(1)	mouse	0.6459

Similar to the ANOVA results, the repeated measures model showed that Stress, STZ, and their interaction were significant factors in causing the differences among the six treatment groups.

Longitudinal Analysis

The method of longitudinal analysis centers on analyzing response profiles that can be applied to data which occurs throughout time when the design is balanced. Although all subjects are measured the same set of times, longitudinal analysis is capable of handling missing data. The data can be condensed by the estimated mean response at each time, stratified by levels of the group factor. The mean response profile is the sequence of means over time at any given level of the group factor. The plots are created when the program (Minitab in the present study) calculates the arithmetic average of the responses at each time, within each treatment group, and

joins adjacent means with a series of line segments. The purpose in analyzing response profiles is to characterize the patterns of change in the mean response over time in the groups and to determine whether the shapes of the mean response profiles are different when comparing different treatment groups. Longitudinal analysis looks at the way the variable changes with time and the way factors affect that change.[9]

In longitudinal studies, the presence of a baseline measurement is critical as it can be assumed not rely on treatment group. One can adjust for baseline depending on the scientific question that is to be answered by the study. When the main goal of the study is to compare groups in terms of their average change over time, the analysis that subtracts baseline response is suitable. This method may be used on observations and randomized trials. The analysis of covariance may also be used on randomized trials and may offer a more effective test of group differences.[9]

When viewing response profile plots, there are three questions to keep in mind: 1) Are the mean response profiles similar in groups, in the sense that they are parallel? 2) If they are parallel, are they constant over time so that the mean response profiles are flat? 3) If they are parallel, are the mean response profiles also overlapping? The main scientific interest is in answering the first question. In fact, the last two questions are only asked if the first question is answered positively. If the response profiles are parallel, then all groups change in the same manner across time, regardless of treatment group.[9]

INDIVIDUAL RESPONSE PROFILES: GLUCOSE

The data was plotted for the individual mice throughout the days and for each treatment group. In both 0 STZ groups (Figure 6 and 7) there was variability among mice and among days for the same mouse but no trend was present.

Figure 6. Individual Response Profile: 0 STZ, non-stress

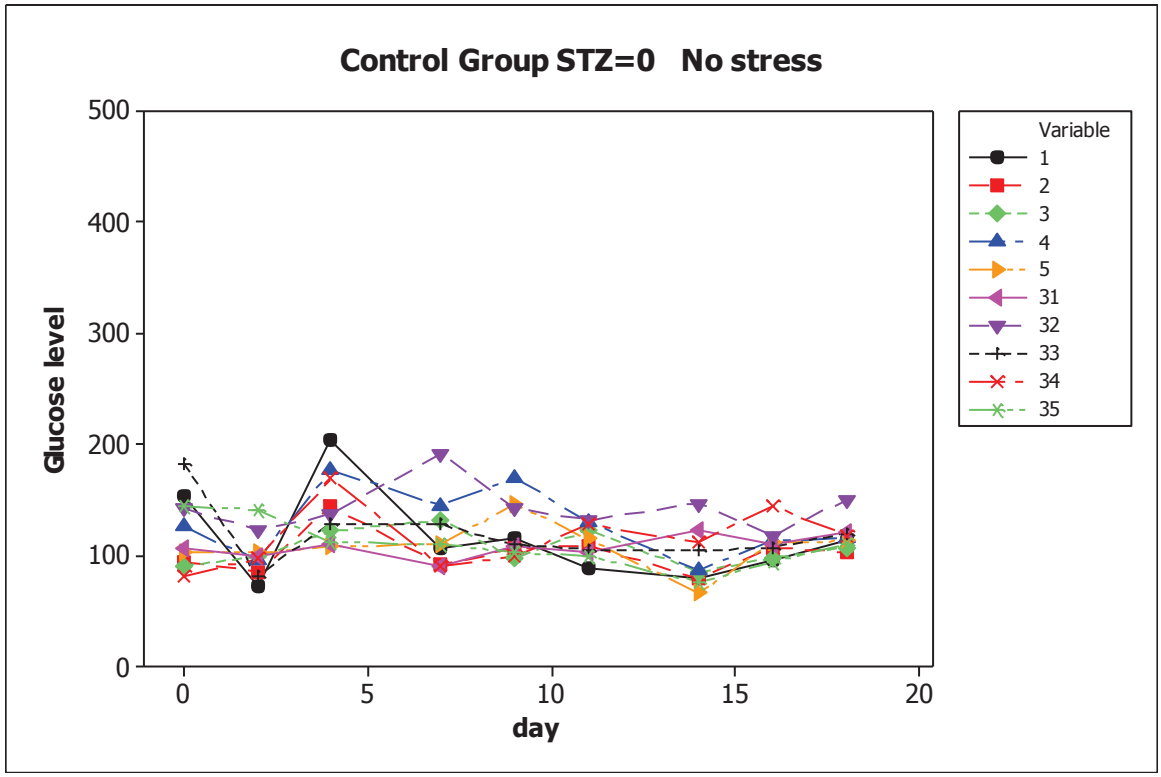
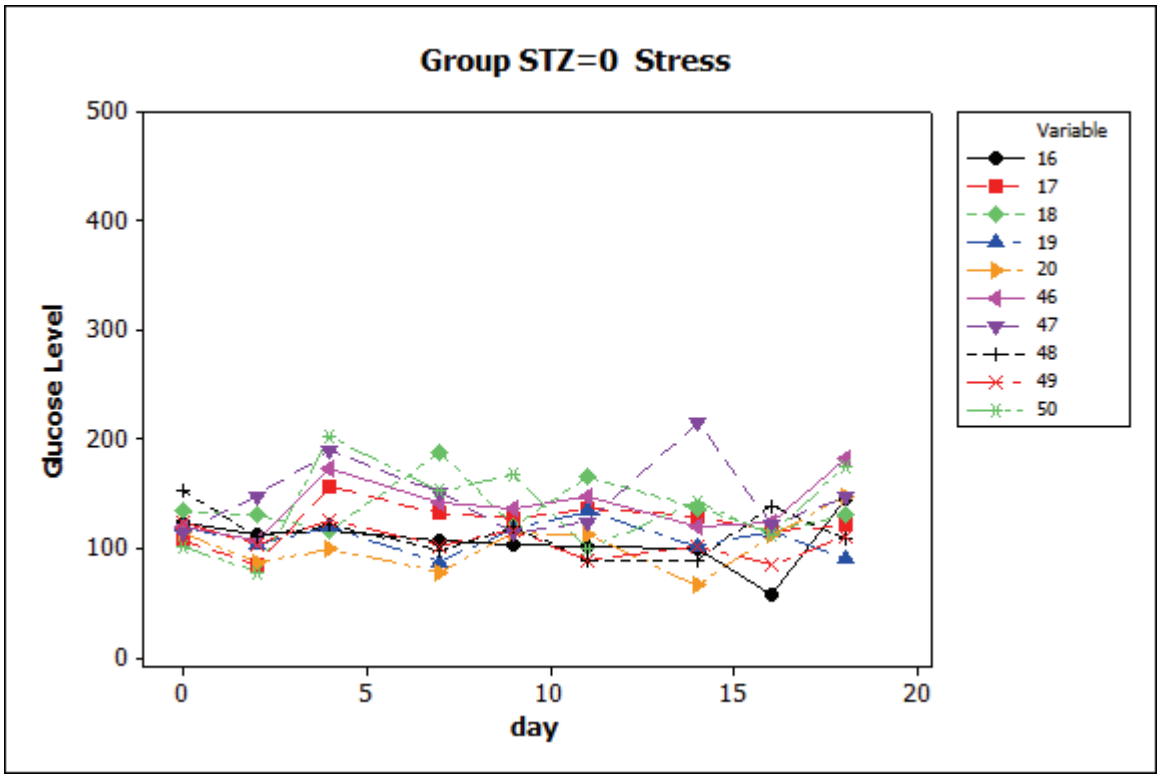


Figure 7. Indifidual Response Profile: 0 STZ, stress



As with the 0 STZ groups, variability was seen among mice and days, but no trend was present.

Figure 8. Individual Response Profile: 25 STZ, Stress

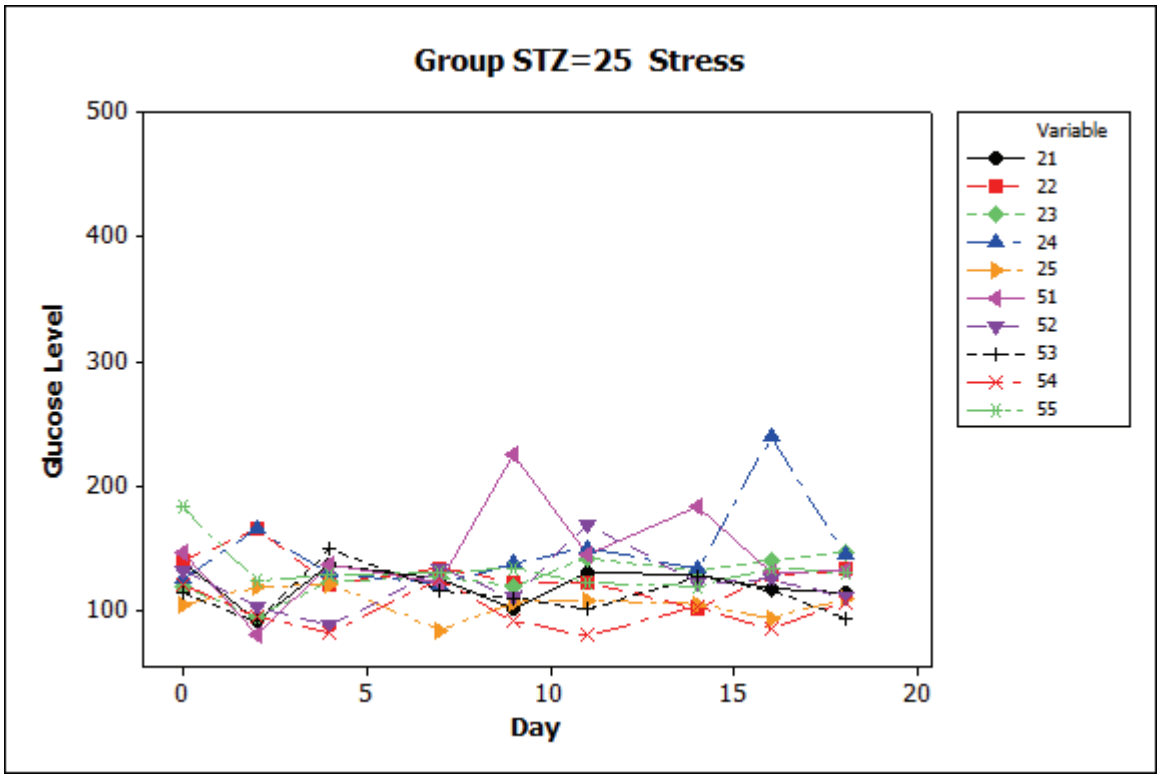
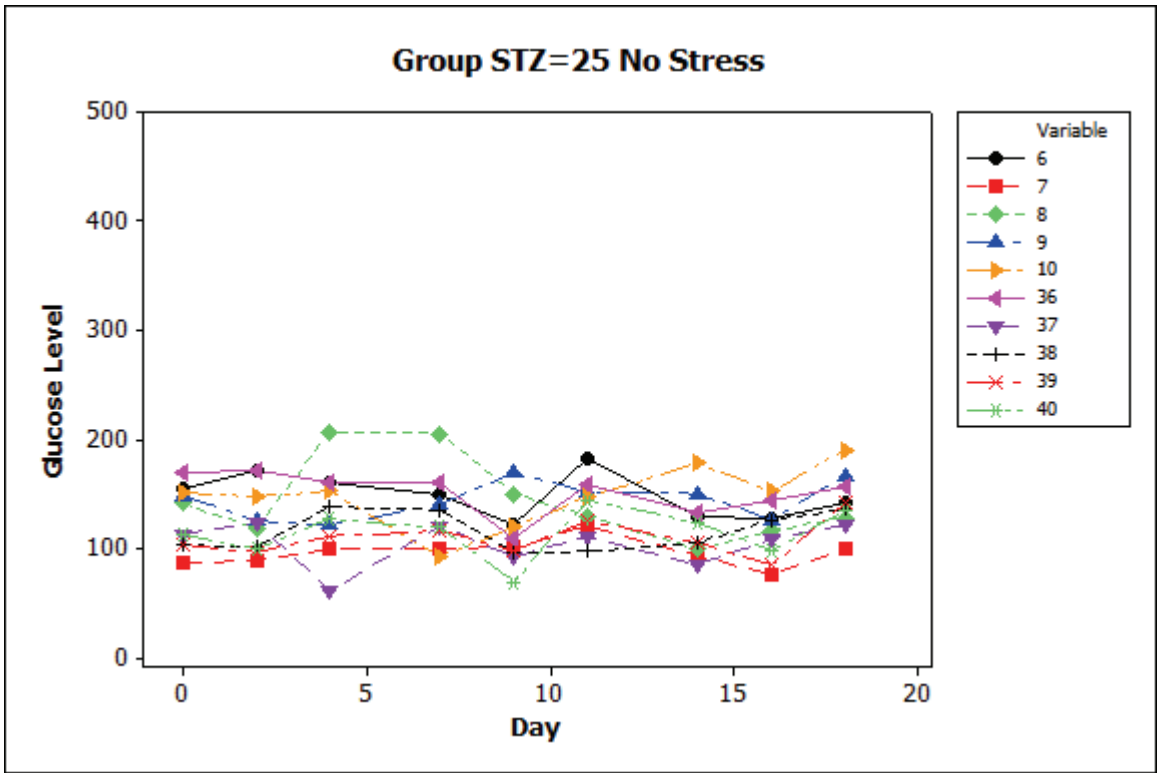
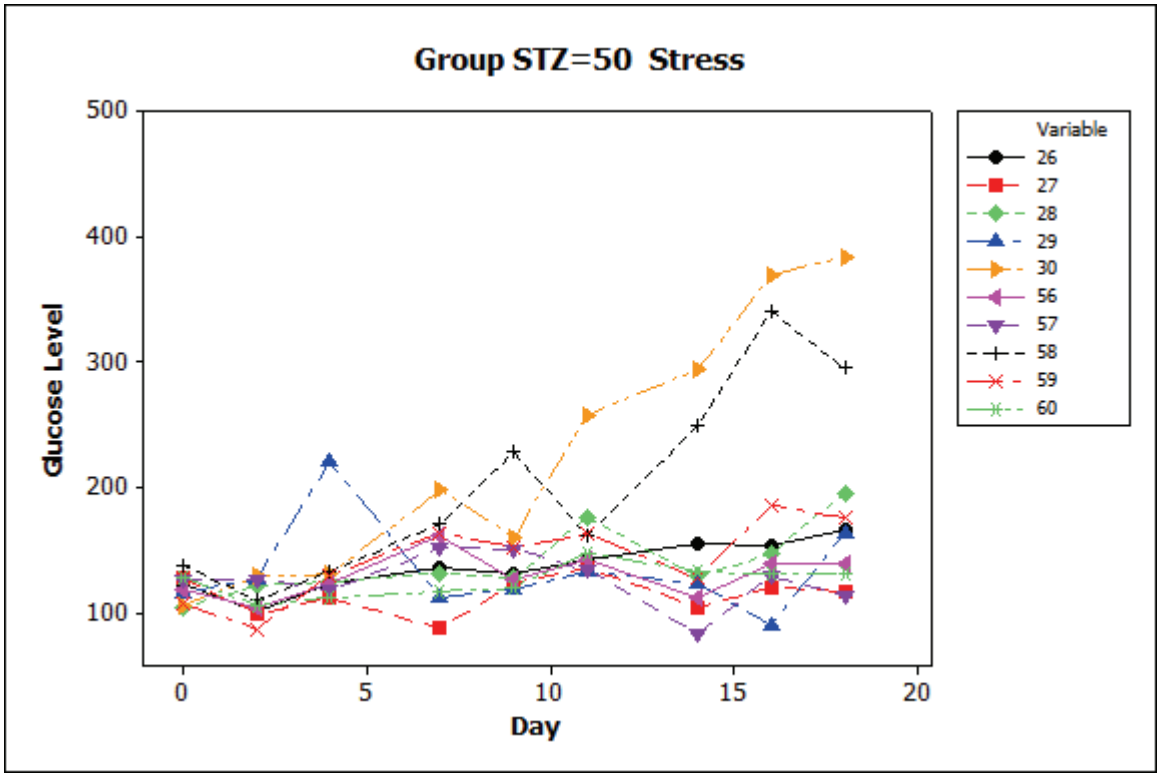


Figure 9. Individual Response Profile: 25 STZ, Non-stress



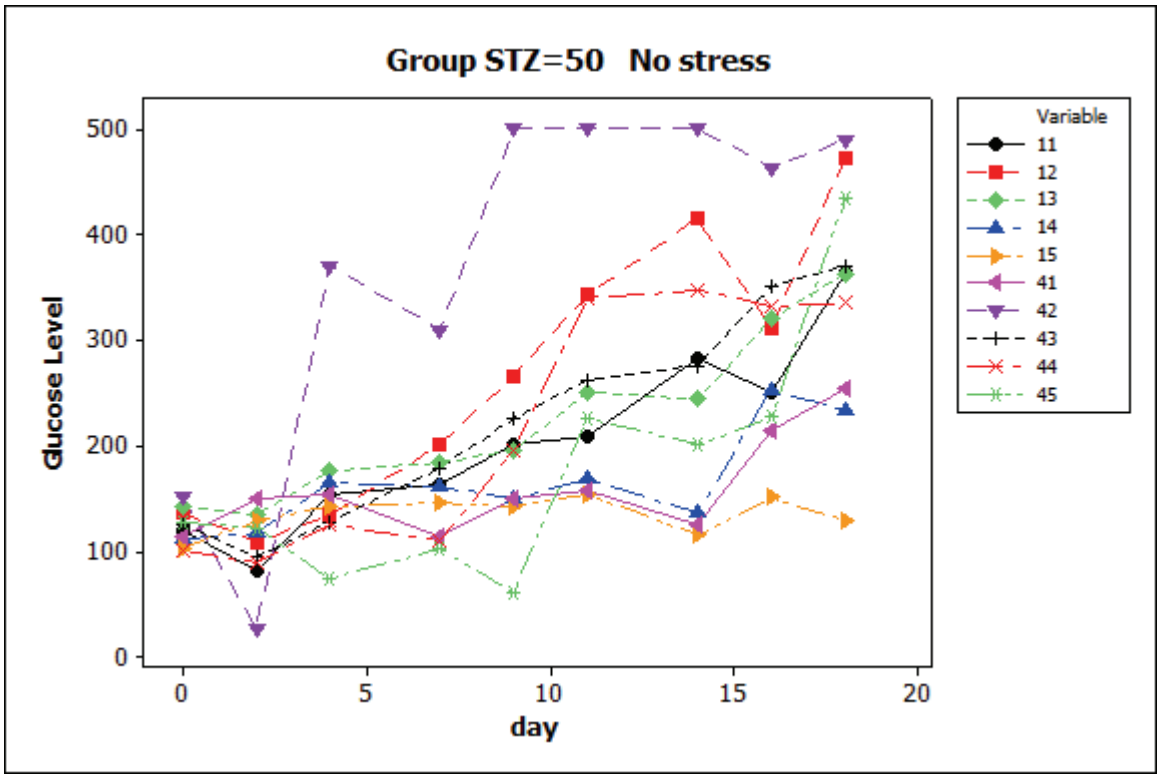
In the 50 STZ stress group, two mice seemed to have an upward trend. Overall, however the plot was flat with slight variability.

Figure 10. Individual Response Profile: 50 STZ, Stress



In the STZ=50 and non-stress groups, a clear upward trend in glucose beginning around the 7th day was present. Comparing different groups, there was no clear difference in the first days; it was around the 7th day that the difference among groups began.

Figure 11. Individual Response Profile: 50 STZ, Non-stress



INDIVIDUAL RESPONSE PROFILES: BODYWEIGHT

As seen in Figures 12- 13 the bodyweight of the non-stressed mice increased steadily overall.

Figure 12. Individual Response Profile: 0 STZ, Non-Stress

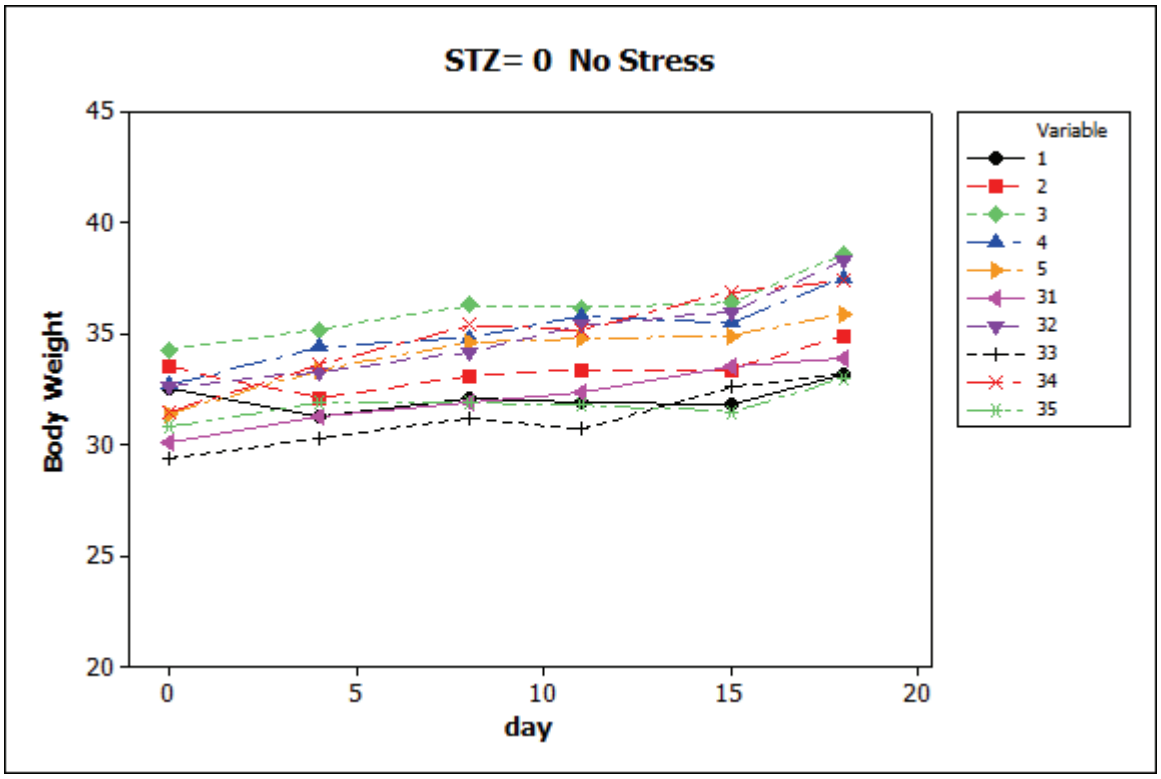


Figure 13. Individual Response Profile: 25 STZ, Non-Stress

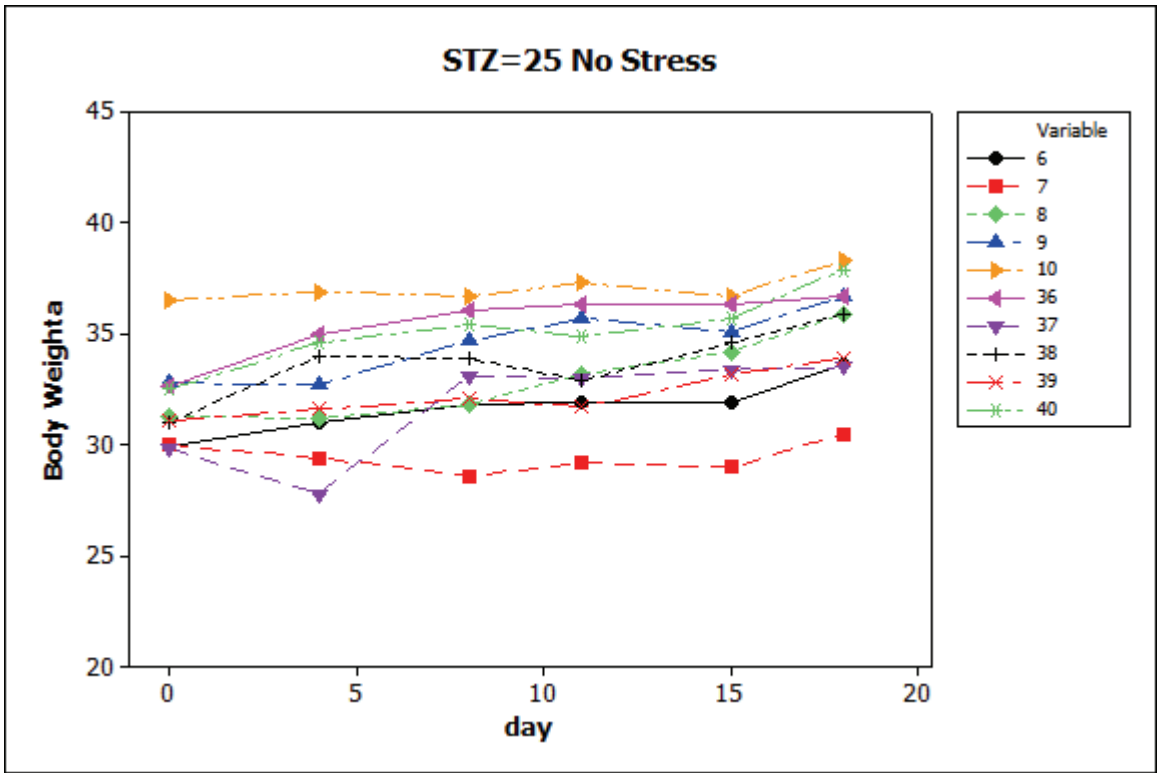
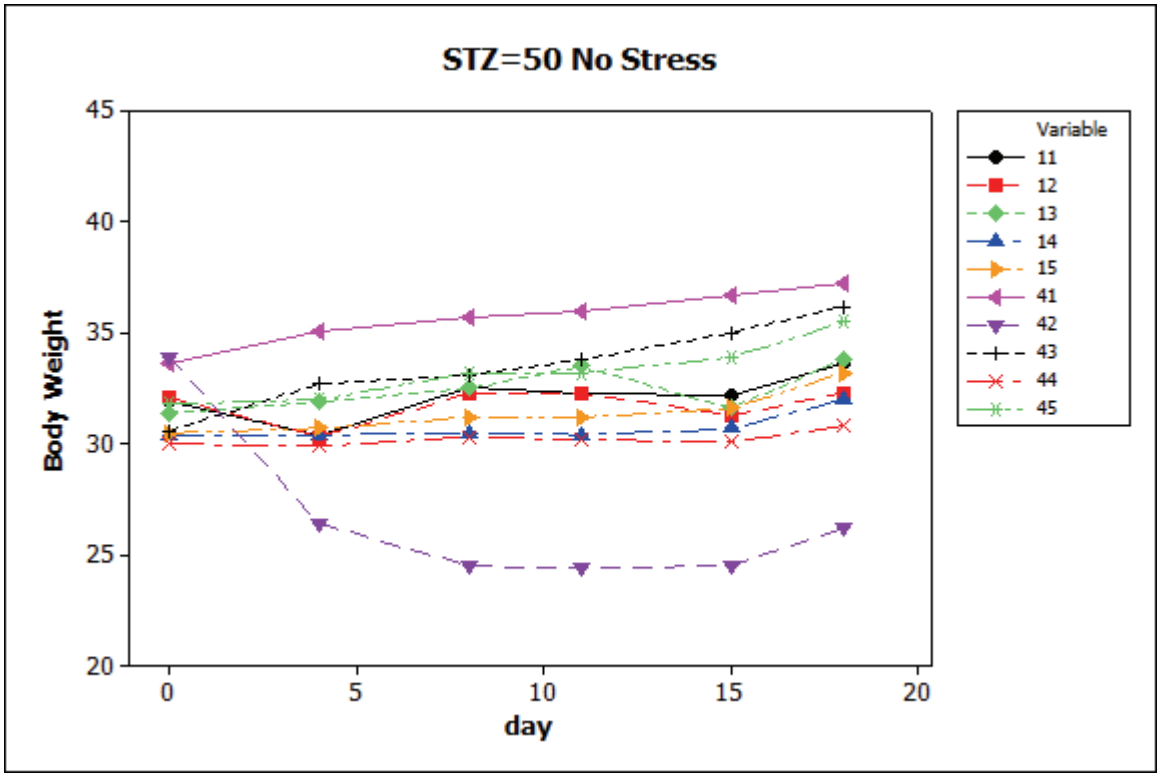
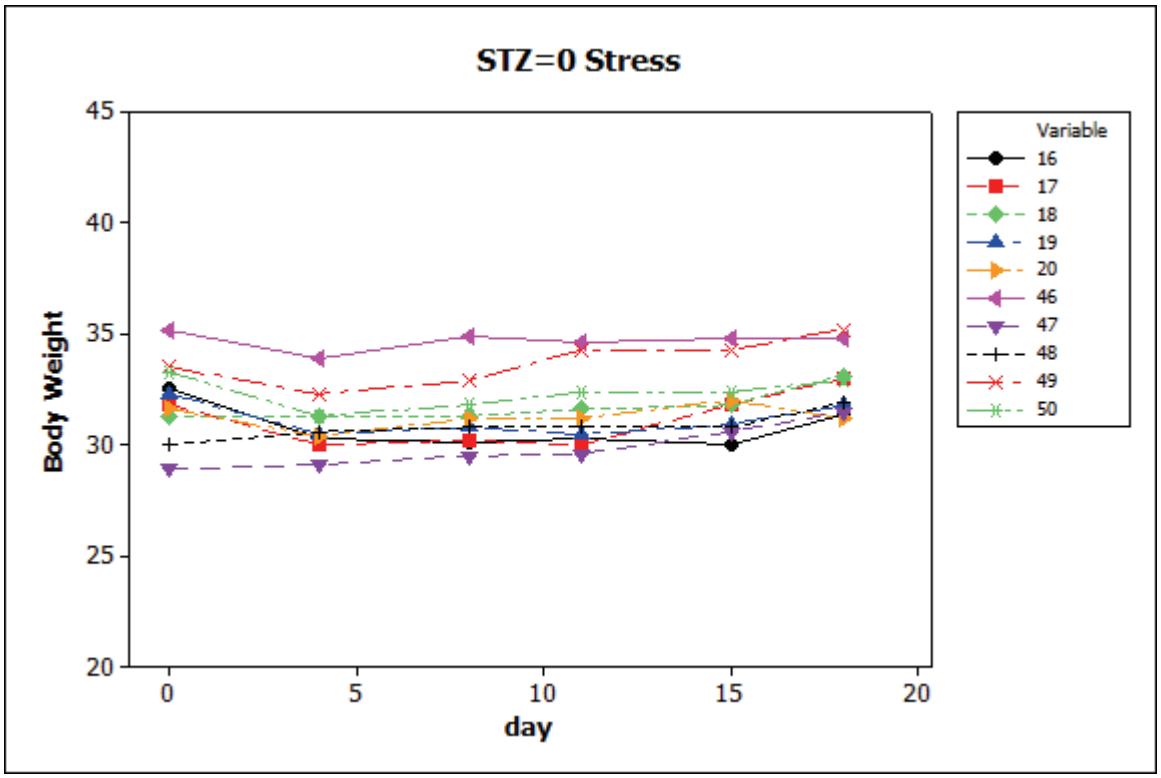


Figure 14. Individual Response Profile: 50 STZ, Non-Stress



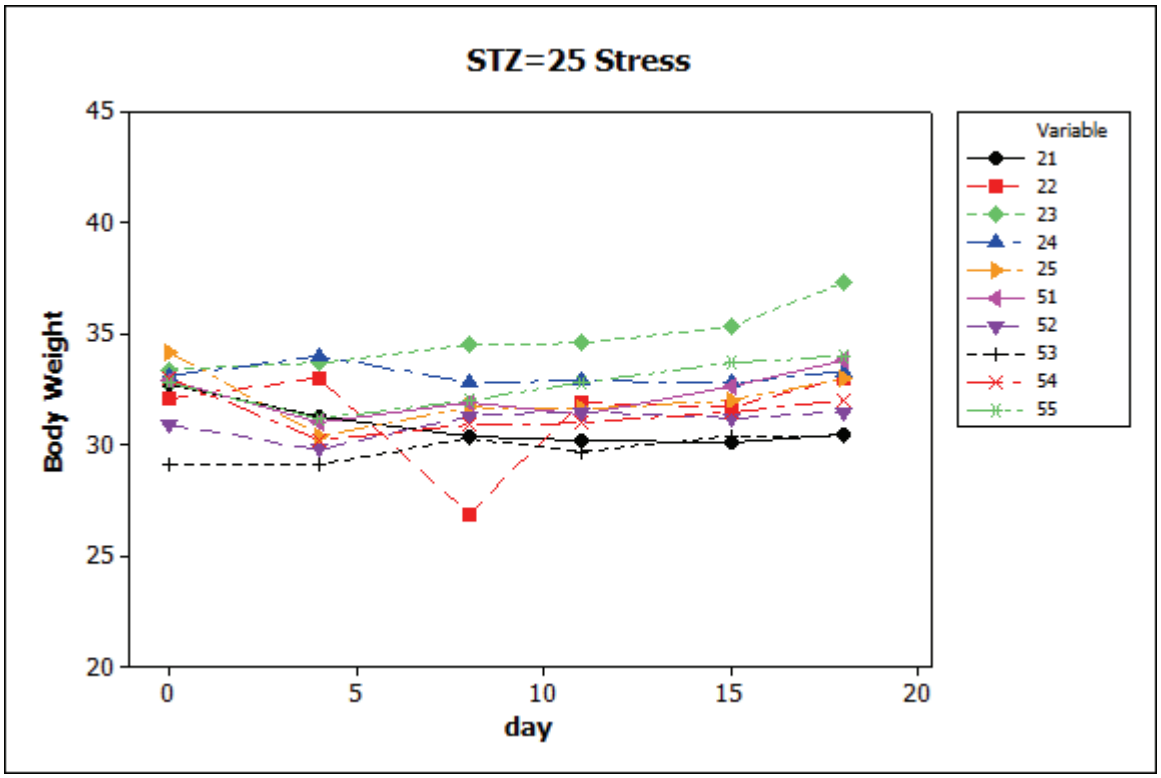
The bodyweight of the 0 STZ stressed group dipped slightly before it began to steady out or slightly increase.

Figure 15. Individual Response Profile: 0 STZ, Stress



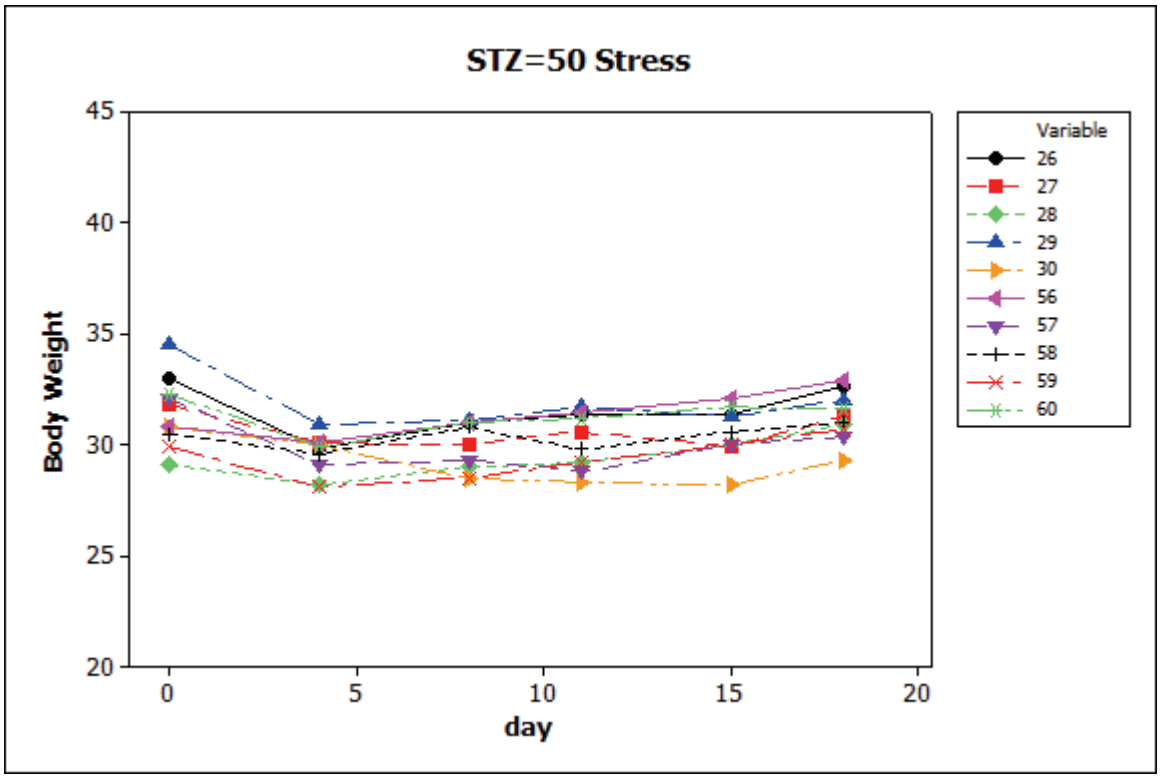
Some mice in the 25 STZ stress group lost weight in the beginning before stabilizing. Other mice seemed to grow from the beginning.

Figure 16. Individual Response Profile: 25 STZ, Stress



All mice in the 50 STZ stress group markedly lost weight in the beginning before stabilizing or increasing. There was very little variability in this group.

Figure 17. Individual Response Profile: 50 STZ, Stress



INDIVIDUAL RESPONSE PROFILES: FEED CONSUMPTION

As seen in the plot of individual response profiles for the 0 STZ non-stressed group in Figure 18 below, feed consumption increased for all mice from the beginning. Other than two individual mice, there was very little variability and the response profiles were parallel. Similarly, in the 0 STZ stressed group the profiles were also increasing and parallel.

The response profiles for both the stressed and non-stressed 25 STZ groups (Figure 20 and 21) increased with very little variability. The 50 STZ stressed group also followed this trend (Figure 22), while the profile for the 50 STZ non-stressed group was more similar to the 0 STZ non-stressed group with three mice which consumed much more than the rest. However, the profiles were still relatively parallel as these mice were consuming more from the beginning.

Figure 18. Feed Consumption Individual Response Profile: 0 STZ, Non-stress

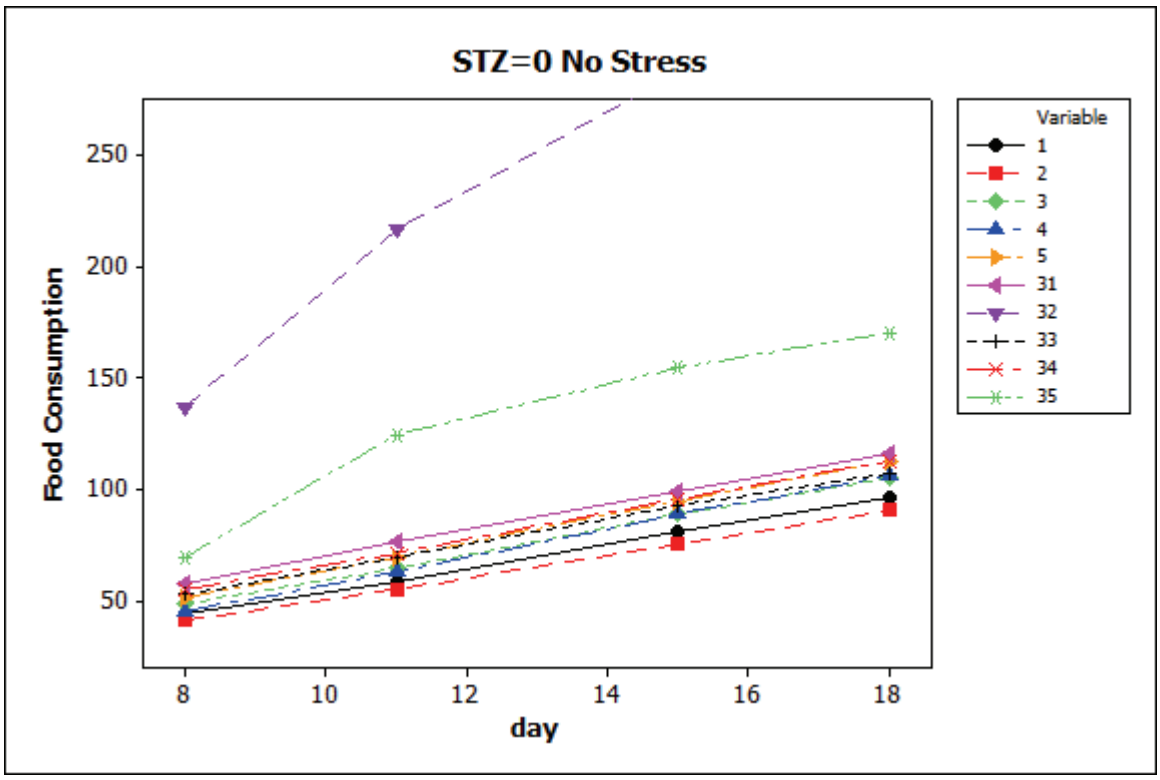


Figure 19. Feed Consumption Individual Response Profile: 0 STZ, Stress

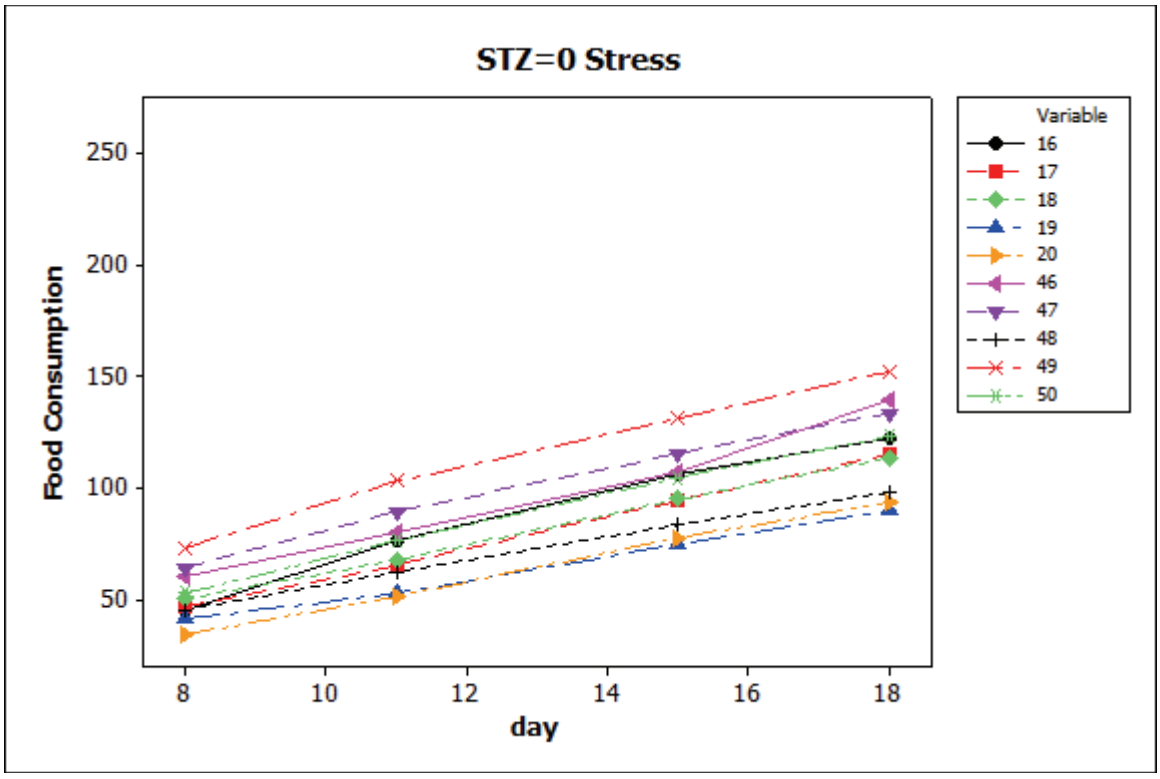


Figure 20. Feed Consumption Individual Response Profile: 25 STZ, Non-stress

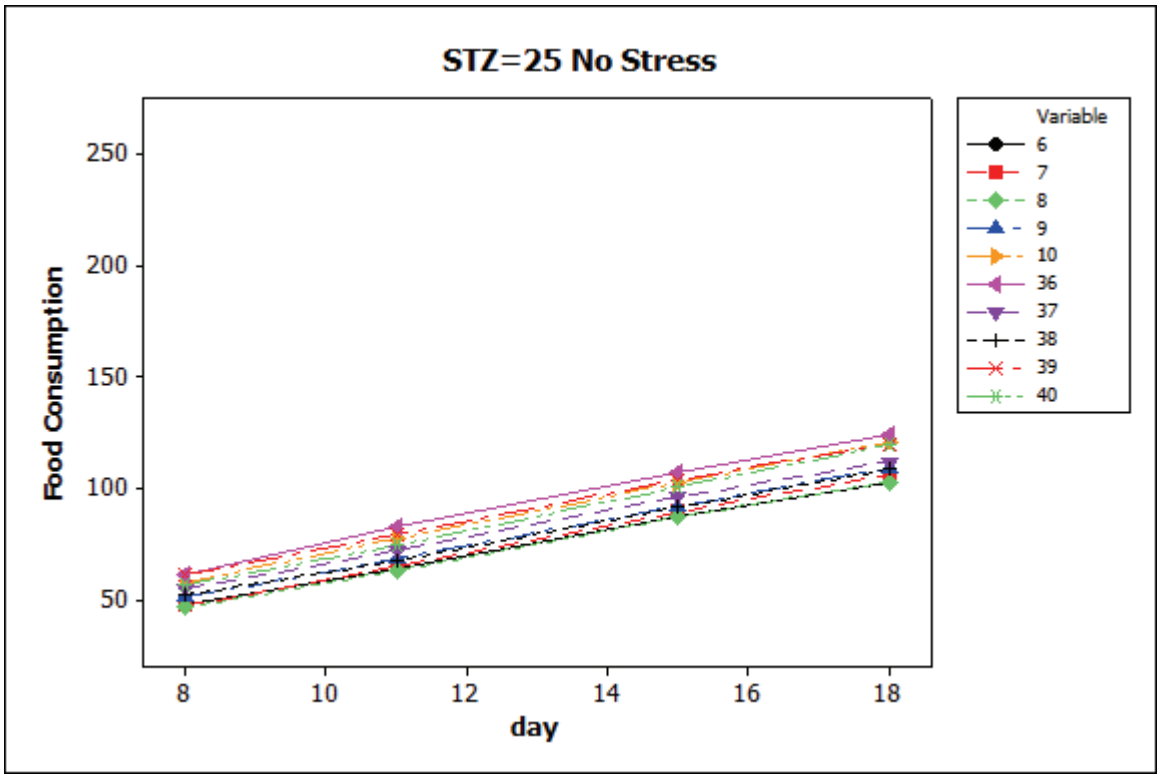


Figure 21. Feed Consumption Individual Response Profile: 25 STZ, Stress

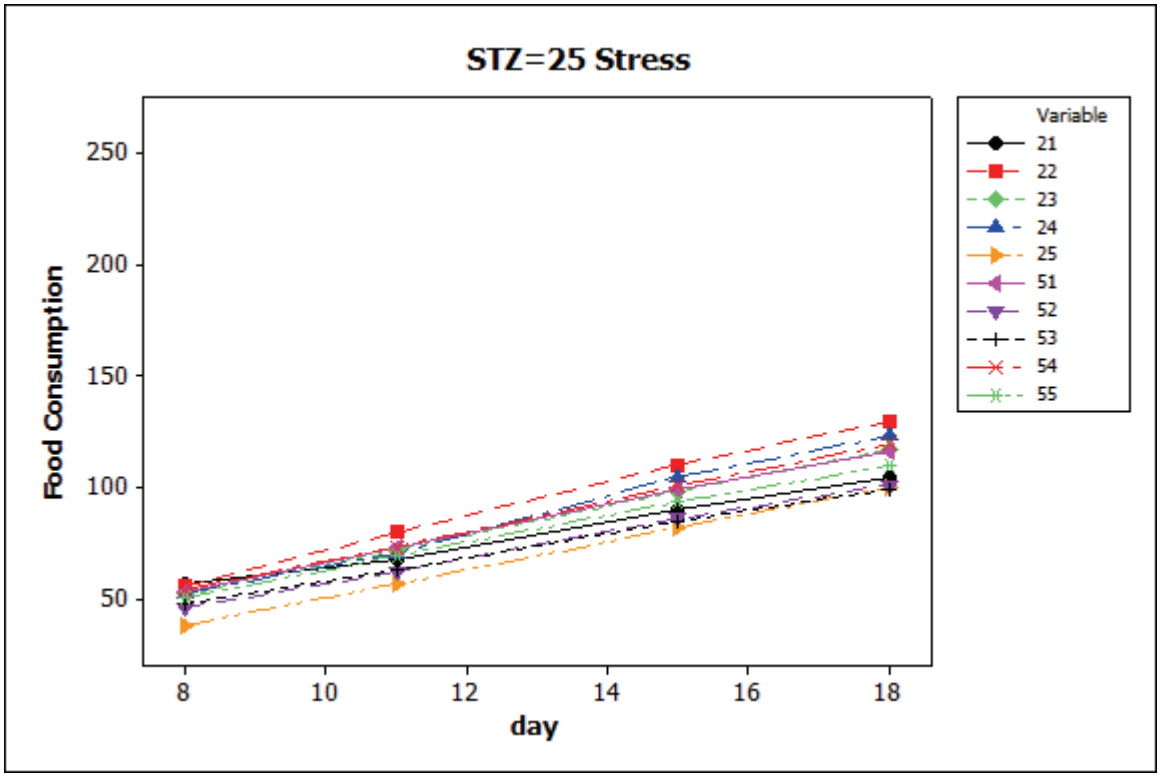


Figure 22. Feed Consumption Individual Response Profile: 50 STZ, Non-stress

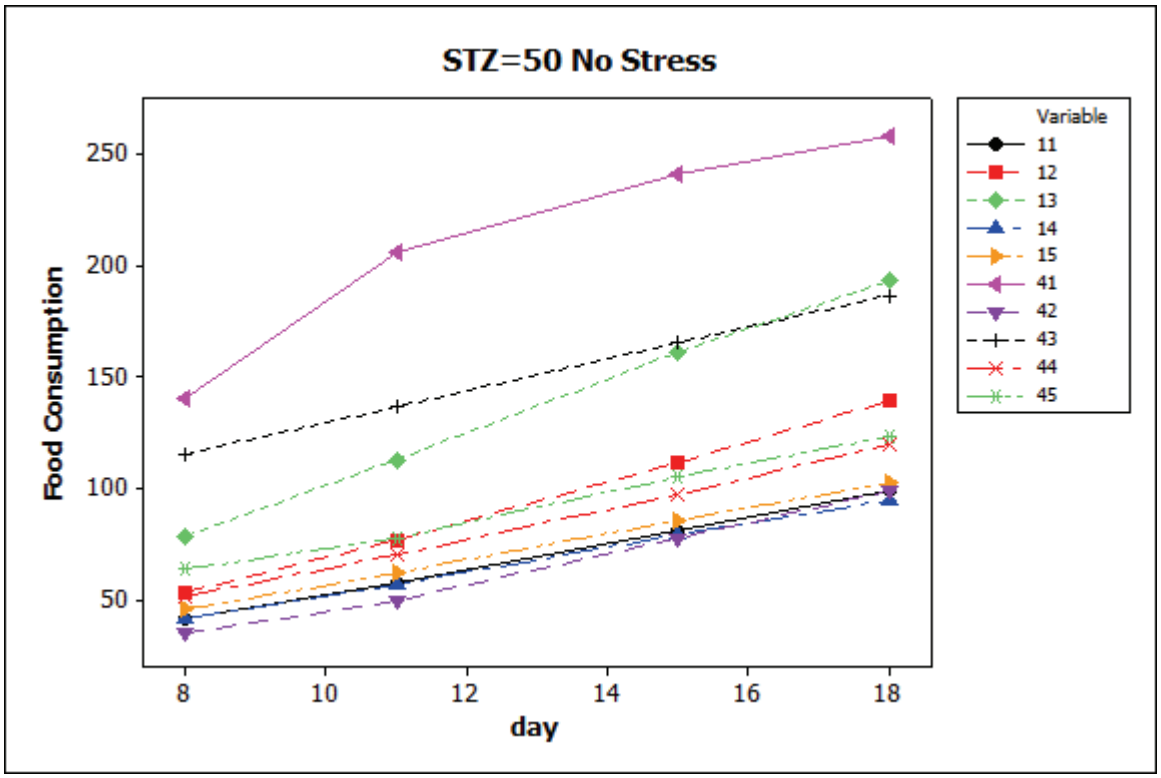
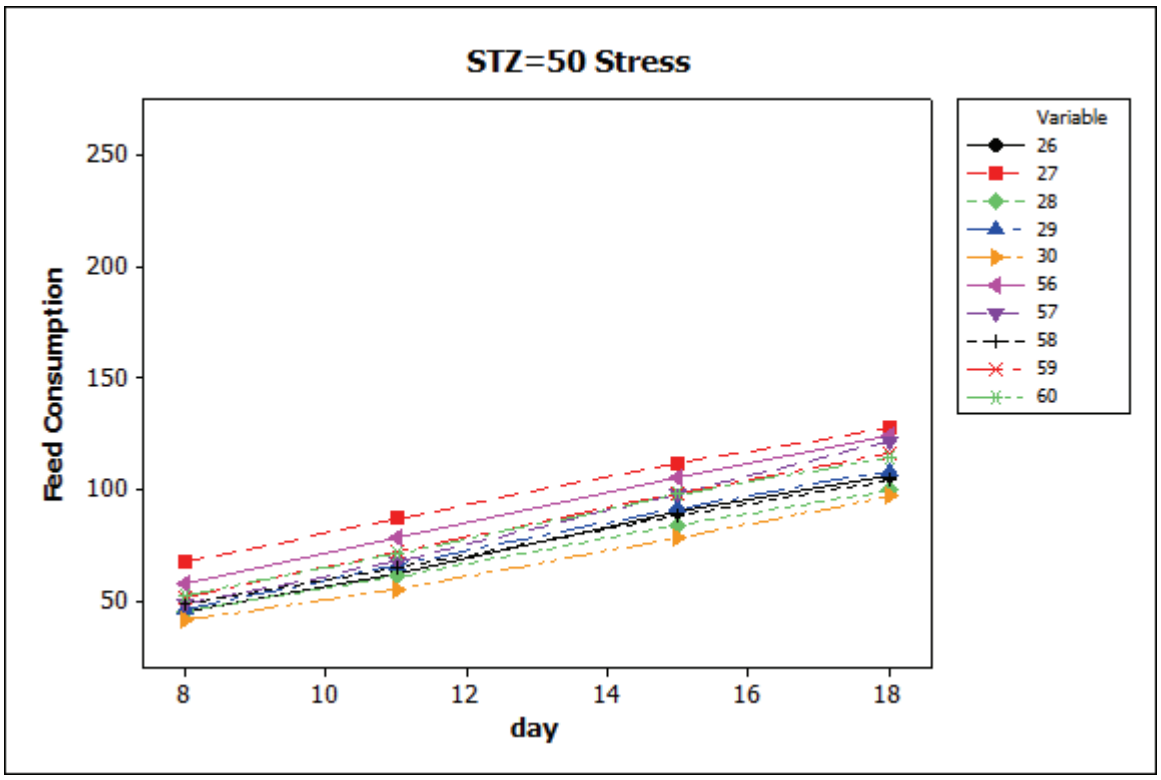


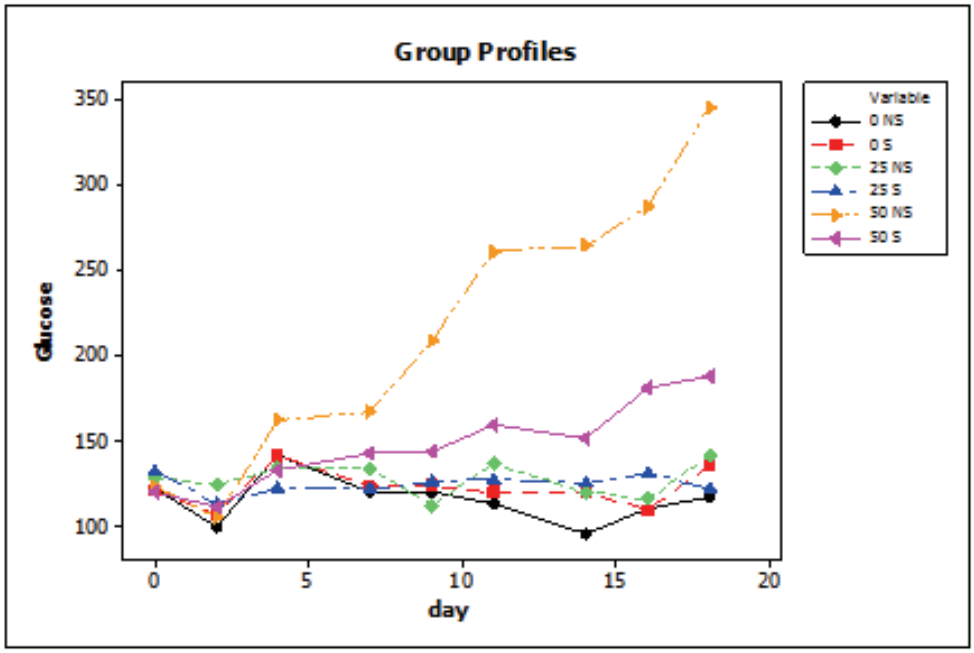
Figure 23. Feed Consumption Individual Response Profile: 50 STZ, Stress



GROUP RESPONSE PROFILES

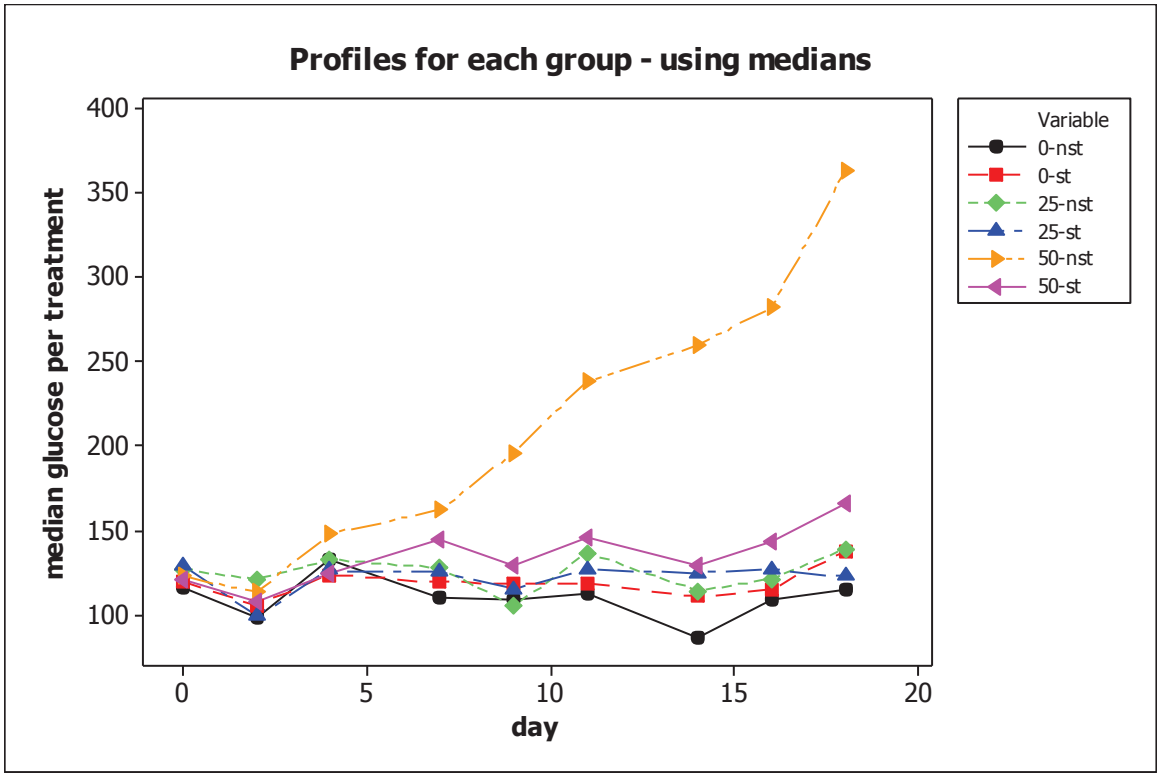
While the other mean glucose levels remained relatively stable, it was evident that the glucose levels of the 50 STZ non-stress group climbed throughout the study. The glucose levels of the 50 STZ stress group increased only slightly.

Figure 24. Group Response Profile: Glucose Means



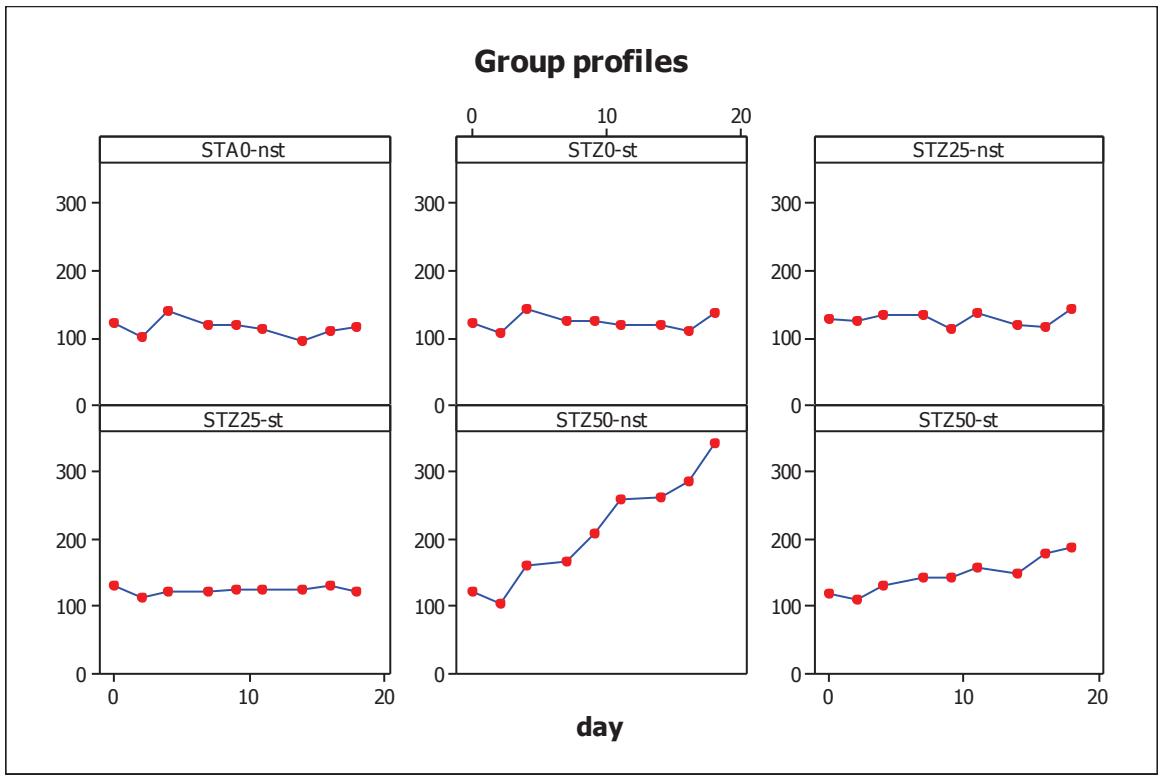
As seen in Figure 24, the response profile using medians was similar to the response profile using means. The 50 STZ non-stress group still had the greatest increase in glucose levels. The 50 STZ stress group still had the second highest levels, although all groups other than 50 STZ non-stress seemed to be parallel.

Figure 25. Group Response Profile: Glucose Medians



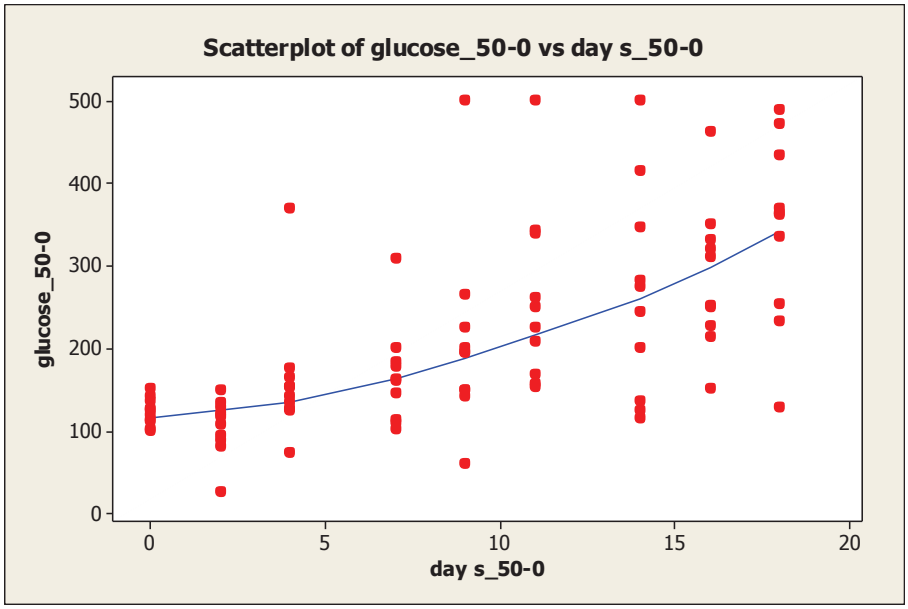
Viewing the group response profiles in separate panels (Figure 25), it was easy to see that the glucose levels increased dramatically in the 50 STZ non-stress group while the glucose levels of other treatment groups remained stable.

Figure 26. Glucose Group Response Profiles: In separate panels



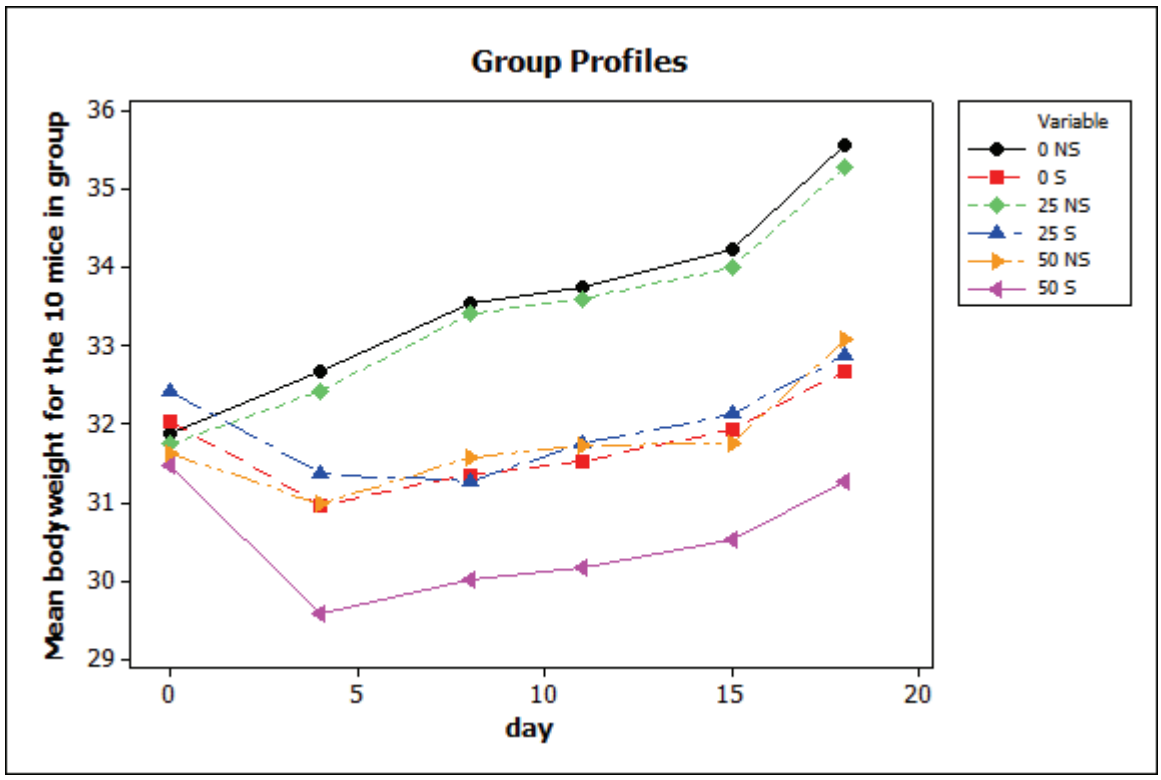
The scatterplot of the 50 STZ non-stress group revealed that the glucose levels increased as time progressed. The variability also increased with time.

Figure 27. Scatterplot of Glucose 50 STZ non-stress group



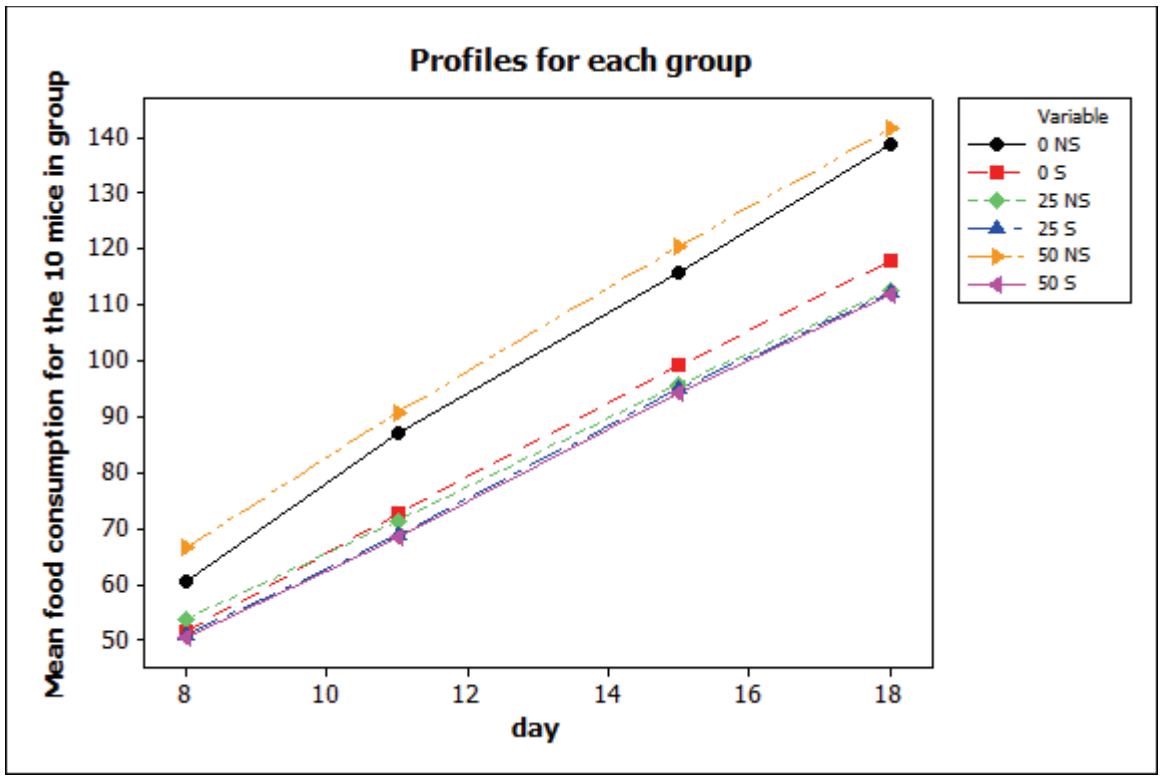
All treatment groups except 0 STZ and 25 STZ non-stress groups lost weight in the beginning before growing. The 50 STZ non-stress group lost the most weight and remained the lightest. It is also important to note that very little variability existed among groups on day 0 compared with day 18.

Figure 28. Group Response Profiles: Bodyweight



In the group response profiles for feed consumption below, it was evident that although there was more variability among groups on day 18 than the beginning, the profiles remained parallel.

Figure 29. Group Response Profiles: Feed Consumption



Principal Components Analysis

Principal components analysis is a standard method of multivariate analysis applied in this case in the context of repeated measurements. The idea is to reduce dimensionality, i.e. the number of variables. The individuals were measured 9 times and there is a lot of variability among individuals in part because they received different treatments. The purpose of principal components analysis is to summarize the 9 measurements taken in the present study into two or three principal components that are functions of the 9 measurements. The principal components are calculated based on the eigenvalues of the correlation matrix. In the case of glucose, the coefficients given to each measurement by the first 3 principal components are:

Table 27. Glucose Principal Components			
Variable	PC1	PC2	PC3
Glucose d0	0.087	-0.694	-0.366
Glucose d2	-0.112	-0.695	0.364
Glucose d4	0.266	0.036	-0.591
Glucose d7	0.347	-0.168	-0.223
Glucose d9	0.395	0.064	-0.231
Glucose d11	0.407	0.024	0.177
Glucose d14	0.409	0.024	0.176
Glucose d16	0.392	-0.023	0.298
Glucose d18	0.381	-0.012	0.361

Principal Component Analysis: glucd0, gluc2, gluc4, gluc7, gluc9, gluc11, gluc18

Eigenanalysis of the Correlation Matrix

Eigenvalue 5.2893 1.2516 1.0913 0.5865 0.3247 0.1674 0.1395 0.0834

Proportion 0.588 0.139 0.121 0.065 0.036 0.019 0.015 0.009

Cumulative 0.588 0.727 0.848 0.913 0.949 0.968 0.983 0.993

Eigenvalue 0.0663

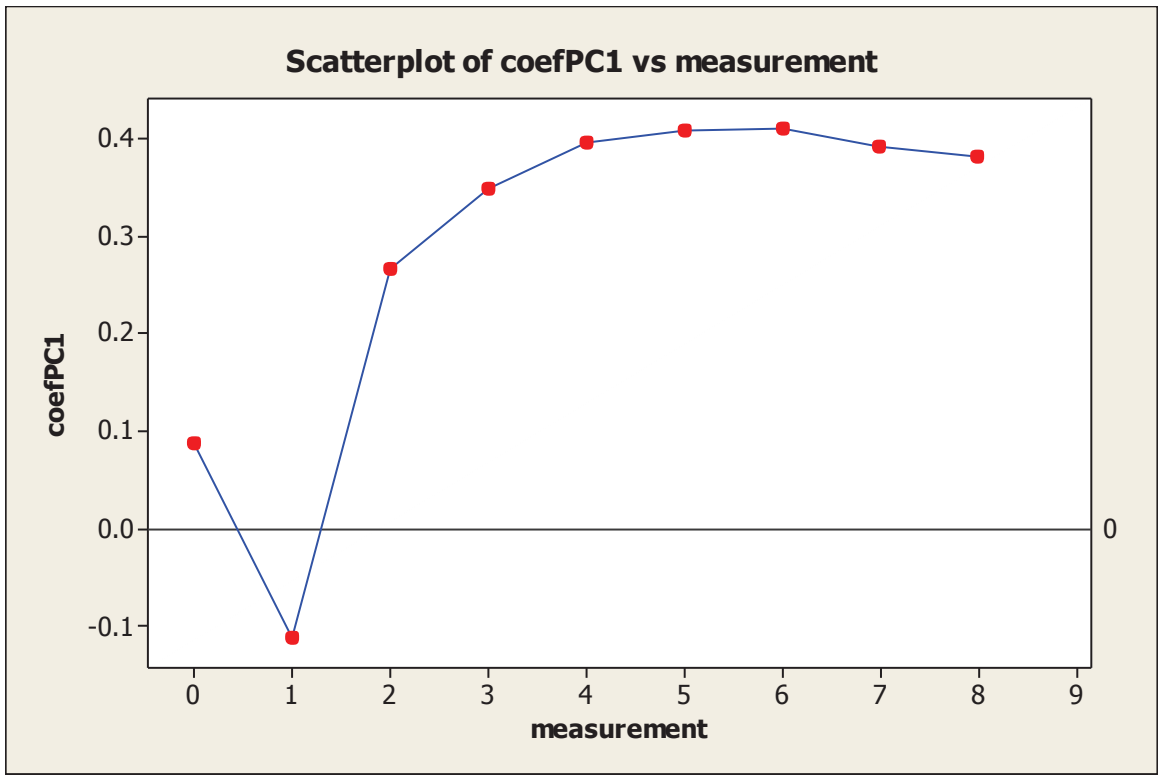
Proportion 0.007

Cumulative 1.000

This analysis reveals that the first principal component captures 58.8% of the variability among individuals, the first 3 principal components capture almost 85%.

Below is a graph of the coefficient for the first principal components. The first principal component makes a weighted average of all the measurements (with more weight starting in the 4th principal component, which is the 7th day) and contrasts that average with the first measurement after the treatments began.

Figure 30. Scatterplot of Coefficient of 1st Principal component



The principal components were calculated for all 60 individual mice and their scatterplots are plotted below. It is evident that the mice that received 50 STZ stand out, especially those that were not stressed. The mice in the other treatment groups are mixed together.

As seen in the plots below, the 50 STZ non-stress group (except for two mice) stands out because of the first principal component, which means that the difference between an average of

the last observations with the first one takes different values for them than for the rest of the groups.

Figure 31. Scatterplot of 3rd Principal Component vs. 1st Principal Component

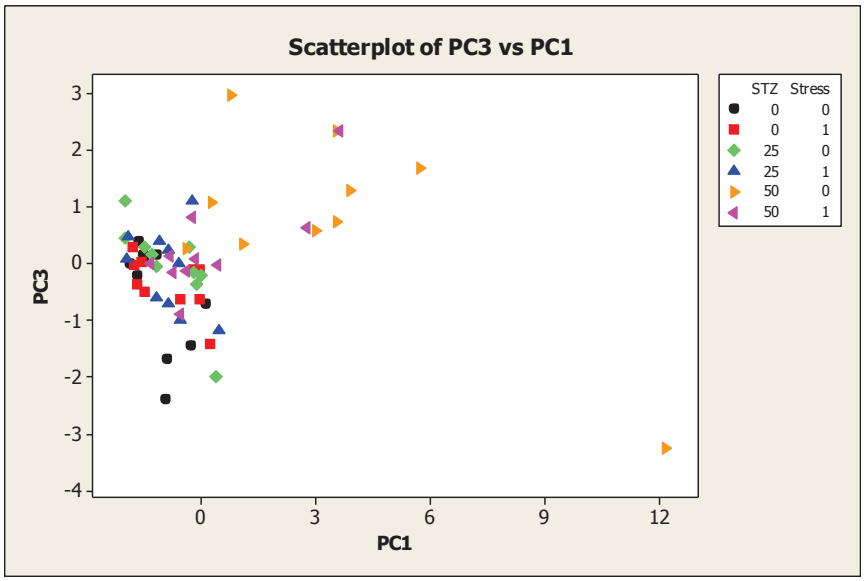
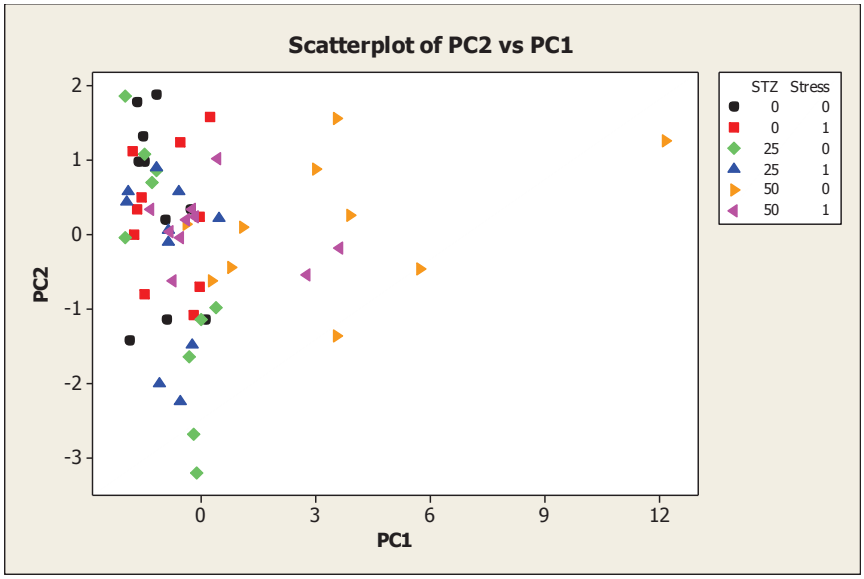


Figure 32. Scatterplot of 2nd Principal Component vs. 1st Principal Component



Correlations: Bodyweight and Glucose

Below are the correlations between bodyweight and glucose on day 18 for the 50 STZ non- stress (Figure 33) and stress (Figure 34) groups. The correlations were not strong, however a negative trend was observed: higher glucose, lower weight.

For the STZ =50 stress group Pearson correlation gluc18 and bwgd18 = -0.593
For the STZ=50 non stress group Pearson correlation gluc18 and bwgd18 = -0.326

Figure 33. Bodyweight and Glucose Correlations: Day 18 50 STZ No stress

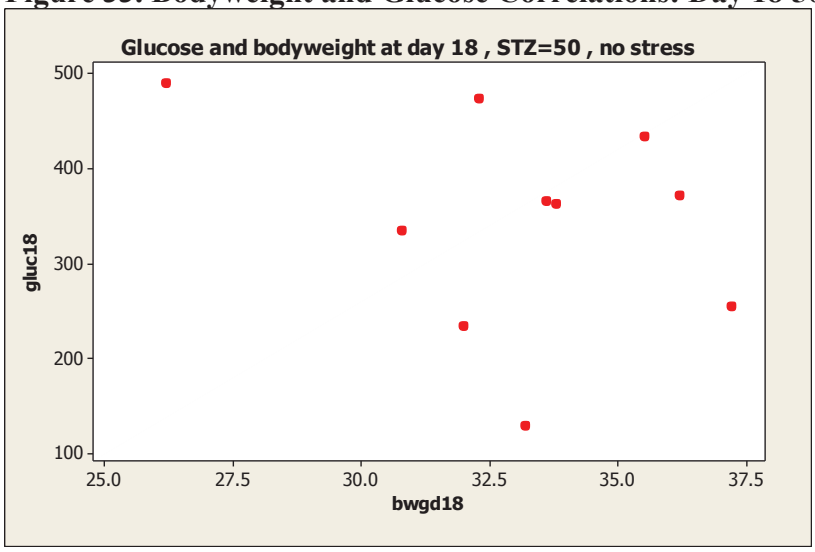
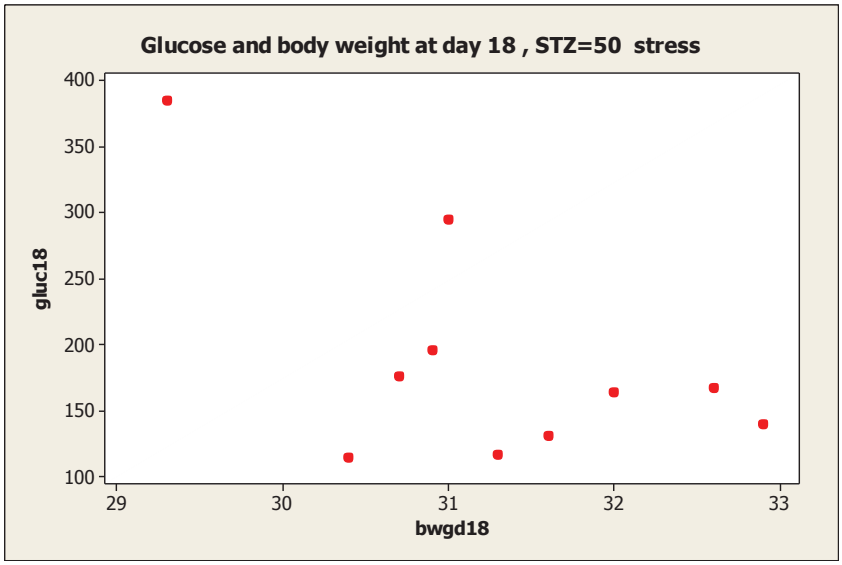


Figure 34. Bodyweight and Glucose Correlations: Day 18 50 STZ Stress



Corticosterone Results

ANOVA

The two-way ANOVA indicated that STZ was what made a difference, and that there seemed to be no effect of stress or interaction between STZ and stress. The low R^2 indicated that only a low portion (18%) of the variability among mice was explained by STZ and stress. However, it is known that ANOVA is sensitive to the presence of outliers which were present in this experiment. Mouse 42 for example was the far right point on the dotplot below.

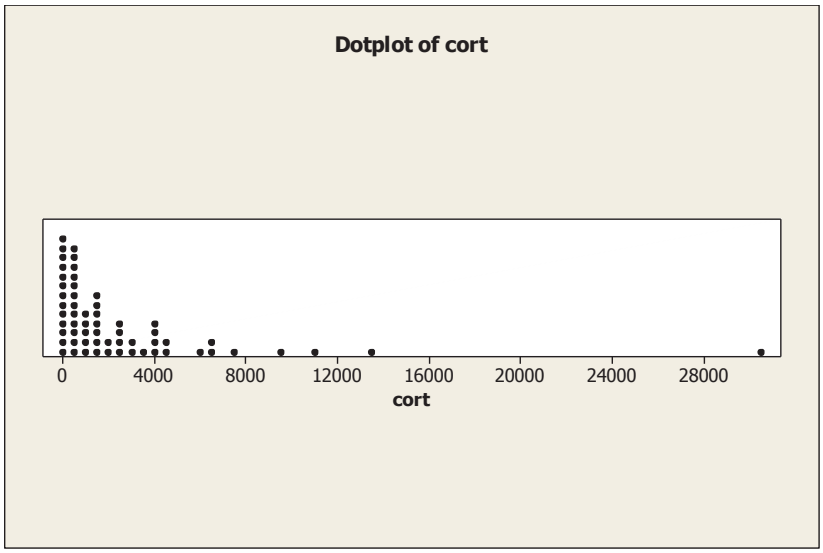
Table 28. Cortisol vs. STZ, Stress

Two-way ANOVA: cortisol versus STZ, Stress

Source	DF	SS	MS	F	P
STZ	2	162764224	81382112	4.28	0.019
Stress	1	39771171	39771171	2.09	0.154
Interaction	2	22613815	11306908	0.59	0.555
Error	54	1026465712	19008624		
Total	59	1251614922			

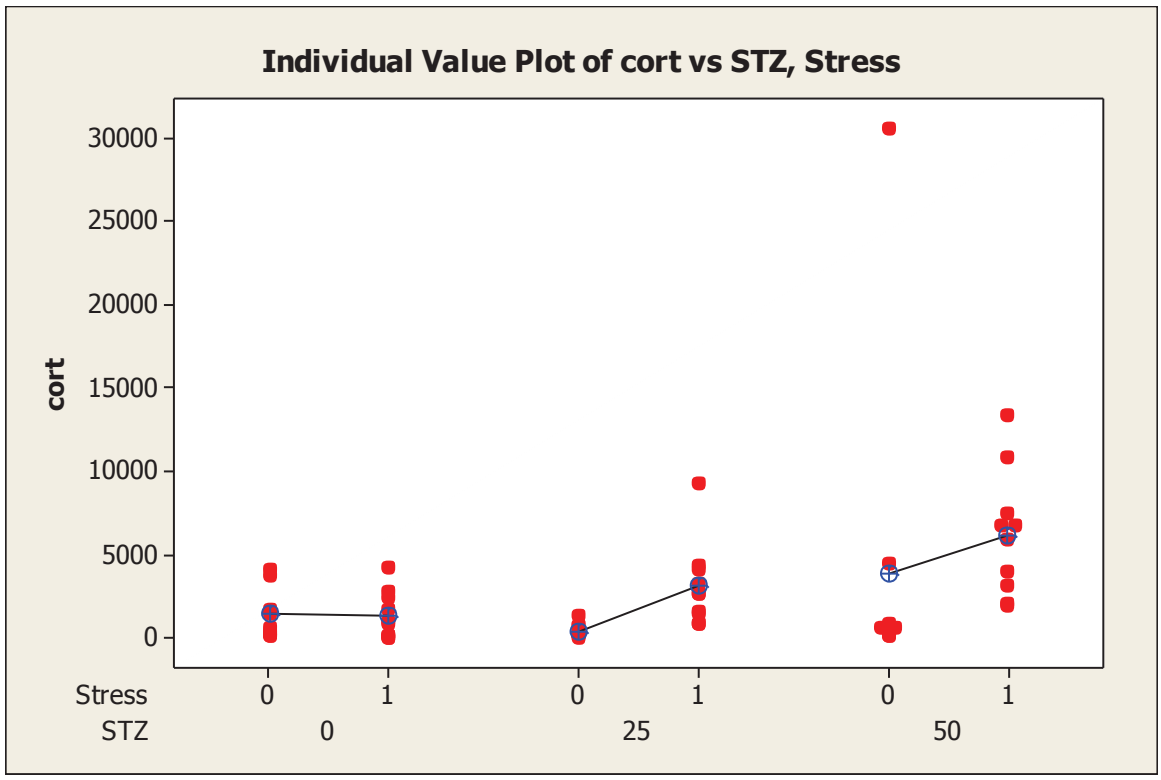
S = 4360 R-Sq = 17.99% R-Sq(adj) = 10.40%

Figure 35. Corticosterone Dotplot



There was a lot of variability among the individuals but stress and STZ together explain only 18% of that variability. The individual value plot below indicates that there was no difference in corticosterone levels between the 0 STZ stress and non-stress groups. The difference was due to STZ (as seen in the ANOVA results).

Figure 36. Individual Value Plot: Corticosterone concentration vs. STZ and Stress



Discussion

Diabetes type 1 can be particularly devastating due to the fact that it results in life-long health complications. These complications occur throughout the body and involve the cardiovascular, renal, and nervous systems. Diseases of the nervous system can prove to be more devastating as they affect sensitive cognitive regions of the brain, such as the hippocampus that

modulates memory function, resulting in significant functional impairment and dementia.[16] Approximately 40% of DM type 1 disease susceptibility is considered to be a result of genetic elements, as revealed through studies in twins, where less than half of identical twins both develop the disease. The short arm of chromosome 6 and regions of chromosome 11 contain the genetic associations with DM type 1, specifically in the region of human leukocyte antigen (HLA) molecules, known as IDDM1. However, some of the regions that have been identified exert only a small influence and the precise genes remain unknown in humans.[20]

In some studies it has been suggested that the incidence of diabetes may vary depending upon environmental factors such as stress.[10] In the study by Fitzpatrick and others, serum glucocorticoid concentrations in basal and stress conditions were measured in non-obese diabetic mice and C57BL/6 control mice. It was found that the diabetic mice generally exhibited a higher corticosterone response than the controls.[10] In the present study, STZ was found to be the source of the difference in corticosterone levels among the different groups, as evidenced by the two-way ANOVA results. The R^2 value indicated that only 18% of variability among mice was explained by STZ and stress. However, ANOVA is sensitive to the presence of outliers, which existed in the present experiment as seen in the dot plot and individual value plot of corticosterone versus STZ and stress (Figure 35 and 36). These observations are in agreement with previous findings that STZ-induced diabetes elevates levels of serum corticosterone.[21, 19] Another study that also observed high resting levels of plasma corticosterone in diabetic rats took these observations to suggest that diabetic rats were in a chronic stress condition.[7] Interestingly, in the present study the non-stressed 25 and 50 STZ mice had higher glucose levels but the stressed 25 and 50 STZ mice on the individual value plot have higher corticosterone levels.

Many studies have researched the effects of stress on already diabetic mice and rats.[2, 3, 10, 12, 13, 18, 23] Reagan and others[23] examined the neurological changes induced by 7 days of restraint stress in STZ diabetic rats and found that the hippocampus of diabetic rats was extremely susceptible to stress. This research group reported that diabetic rats showed dendritic atrophy of pyramidal neurons, increased GLUT3 mRNA and protein expression in the hippocampus, and stress additionally caused an increase of the IGF (insulin-like growth factor) receptor in the hippocampus.[23] In a study by Korolkiewicz and others,[13] using rats made diabetic by a single 70mg/kg STZ injection 5 weeks prior to the experiment, it was found that stressful stimuli such as food deprivation and cold challenge contributed to the elevated susceptibility of diabetic gastric mucosa to damage.[13] Bazhan and others[3] found that light repeated emotional stress decreased the development of obesity and diabetes type 2 in mice with the Agouti yellow mutation.[3] Using borderline, overt, or severe diabetic mice induced by STZ, Meehan and others[18] studied glycemic responses of mice to the stress of a resident-intruder encounter and stress of blood drawing from the retro-orbital sinus. They found that plasma glucose elevation in overtly and severely diabetic mice is not as specific to behavior as in nondiabetic mice.[18] Bates and others[2] found that intermittent restraint and its adaptations delayed hyperglycemia and improved glucose control in Zucker diabetic fatty rats, which may be explained in part by the finding that repeated stress lowered overall corticosterone exposure. This investigation concluded that these findings suggest some types of occasional stress may limit development of diabetes.[2] These findings are similar to what was observed in the present study. In the present study mice were injected with low levels of a diabetes-inducing drug, streptozotocin (STZ). Half of the mice were then stressed to determine if stress accelerates the onset of diabetes mellitus. In order to subject mice to chronic stress, in the present study, mice were subjected to restraint stress for 6 hours per

day for 17 days. This was in line with the study of Gao and others[11] who considered restraint stress for 6 hours per day for 21 days to be chronic stress while one time 6-hour restraint was considered acute stress.

In the present study, the baseline values for glucose versus STZ, stress, and their interaction were not significant as evidenced by the two-way ANOVA results (Table 2). This was an important foundation as it meant that there was no bias among treatment groups before treatments began. Glucose day 4 values using ANOVA and ANCOVA values with glucose day 0 as a covariate contained no significant *P*-values. By day 7, both the ANOVA and ANCOVA *P*-values for STZ became significant: the treatment groups had different glucose concentrations due to the STZ. This observation is inline with previous research, [19] in which one week after STZ injection defined mice as diabetic when they exhibited plasma glucose greater than 300mg/dl.[19] The boxplot of glucose day 7 minus the baseline values (Figure 1) showed that the mean glucose levels were almost the same for the 0 and 25 STZ groups while the mean for the 50 STZ group was higher. The 25 STZ group had the least variation for most of the mice but had three outliers. As seen in the ANOVA and ANCOVA results of glucose day 9, the interaction between STZ and Stress was not significant at the $P<0.05$ level. However, it was low enough as to suggest that there was some mild interaction, which was confirmed by the interaction plot (Figure 3). This plot shows the average of the observations at each level of one factor broken up by the levels of the other factor.[8] On day 9, the stressed mice of both the 0 and 25 STZ groups had higher glucose levels than their non-stressed counterparts. The opposite is true with the 50 STZ groups. Surprisingly the 50 STZ stressed mice exhibited lower levels of glucose. One possible explanation for this is that while the mice were being stressed, they were working to escape, which was a form of exercise. Previous research by Kosovskii and others[14] comparing types of

stress and the development of diabetic syndrome found that mice stressed through cavitary operation exhibited the signs of diabetes while those stressed through suspension by nape of neck did not. They suggested that the differences could be attributed to the fact that cavitary operation resulted in limited mobility while mice stressed by suspension had increased movement while trying to escape.[14] By day 11 STZ, stress, and their interaction were significant in ANOVA and ANCOVA (Table 9 and 10). This was not extremely atypical in comparison with one study which found that using a low-dose STZ regimen of 50 mg/kg STZ injected intraperitoneally for 5 consecutive days in fasted mice produces hyperglycemia within 2 weeks of the low-dose STZ regimen.[4] In the interaction plot for glucose day 11 minus baseline values (Figure 4) the non-stressed 50 STZ mice still have drastically higher glucose levels than the 50 STZ stressed mice. The stressed mice that received 0 STZ had a subtly higher glucose level than their non-stressed counterparts. The 25 STZ stressed mice had subtly lower glucose levels than their non-stressed counterparts, which was a change from day 9. Possibly, the 25 STZ non-stress group had a higher mean glucose for the same reason as the 50 STZ non-stress group. It is logical that the 0 STZ stressed group had a higher mean glucose level than the 0 STZ non-stressed group as stress is known to increase glucose levels in the blood.[17] Perhaps without the interaction of STZ, the 0 STZ groups show the default reaction of the body to stress, which is an increase in corticosterone levels that in turn increase blood glucose levels. In ANOVA and ANCOVA values for day 14 (Table 11 and 12) STZ remained significant while the *P*-values for stress returned to non-significant. By day 16, both STZ and stress once again were significant. Their interaction was also significant with a *P*-value of 0.04 (Table 13 and14). The interaction plot for glucose day 16 minus the baseline values show that the 0 and 25 STZ non-stressed groups had the lowest mean glucose values and were basically the same mean, while the 25 STZ stressed group had a slightly

higher mean glucose level than the 0 STZ stressed group. Stress, STZ, and their interaction were again significant in the ANOVA and ANCOVA results for glucose day 18 (Table 15 and16). Again for the interaction plot for glucose day 18 there was not a huge difference between the mean glucose levels in the 0 and 25 STZ groups: they were both relatively low. The 25 STZ non-stressed group had higher mean glucose levels than the 0 STZ non-stressed group, while it was the opposite between the stressed groups: the 0 STZ group had a higher mean glucose than the 25 STZ group. The markedly different 50 STZ groups remained the same: the non-stressed group had the highest mean glucose levels while those of the stressed group were much lower. It is interesting to note that previous research has found that as the course of streptozotocin-induced diabetes progressed, blood sugar levels became increasingly responsive to the process of obtaining the blood samples, i.e., animals sampled later in a given time period had higher blood glucose levels.[12]

Similar to the ANOVA results, the repeated measures model showed that STZ, stress, and their interaction were significant factors in causing the differences among the six treatment groups. The ANOVA analysis studies the effect of STZ, stress and their interaction one day at the time. For some of the days (starting at day 7) there were significant differences and interactions contrary to what happened during the first days of the experiment. The repeated measures models is more global and analyzes the data for all the days and therefore finds out that the effect of STZ, stress and their interaction are significant. In the glucose individual response profiles, variability was present among mice and days, but no clear trend was present in the stress and non-stress 0 and 25 STZ groups. In the 50 STZ stress group two mice seemed to have an upward trend in glucose levels, while in the 50 STZ non-stress group an upward trend was present for practically all of the mice (Figure 11). This trend was also evident in the glucose

group response profiles using means and medians (Figure 24, 25). The 50 STZ non-stress group still had the greatest increase in glucose levels. The 50 STZ stress group still had the second highest levels, although all groups other than 50 STZ non-stress seemed to be parallel. This difference among groups was also verified when using principal components. The 50 STZ non-stress group stood out because of the first principal component. The first principal component captured 58.8% of the variability among individuals; the first 3 principal components captured almost 85%.

The baseline two-way ANOVA body weight results showed randomization, as the *P*-values for STZ, stress, and their interaction were insignificant. STZ and stress became significant as early as day 4 and remained so through day 18. The interaction of STZ and stress never became significant. As seen in the body weight group response profile (Figure 28), stress seemed to have made all of the animals lose weight initially, as only the 0 STZ and 25 STZ non-stress groups did not lose weight up to day 4. Previous research has observed that body weight significantly decreases as the diabetic state develops in STZ-injected mice.[19] The interaction of STZ and stress was insignificant because the bodyweight group profiles of the 0 and 25 STZ stress group and the 50 STZ non-stress group were parallel and overlapping. The higher dose of STZ caused a decrease in bodyweight as did stress for the lower dose or absence of STZ. The individual response profiles for bodyweight basically followed the same pattern. Most of the 0 and 25 STZ non-stress mice gained weight from the beginning. There was slightly less variability between mice in the 0 STZ group than the 25 STZ non-stress group. Most of the mice in the 50 STZ non-stress group appeared not to lose or gain weight initially except for one mouse that drastically lost weight until day 16. This mouse also had the highest blood glucose levels throughout the study. Many of the mice in the 0 and 25 STZ stress groups lost weight at the

beginning. All of the mice in the 50 STZ stress group markedly lost weight in the first 4 days. This group exhibited the least variability as the response profiles were parallel and tightly stacked. The correlations between bodyweight and glucose on day 18 for the 50 STZ non- stress (Figure 33) and stress (Figure 34) groups were not strong (-0.593, -.0326), however, a negative trend was observed: higher glucose, lower weight. This could be due to the findings that a symptom of diabetes is weight loss.[23]

The insignificant *P*-values for feed consumption in the beginning of the study indicate randomization of groups. However, STZ, stress, and their interaction never became statistically significant. Knowing that there were differences in bodyweight, it was interesting that there were no differences in feed consumption. The individual response profiles showed an increase in feed consumption throughout the 18 days for all groups. Overall, the stress groups tended to have less variability, with the greatest variability arising in the 0 and 50 STZ non-stress groups. The fact that feed consumption did not vary across groups was seen even more clearly through the group response profile. Feed consumption increased for all groups in the same way as the profiles were parallel.

Finally, although stress has been shown to suppress the immune system in some instances in mouse models and decrease resistance against diseases such as herpes simplex, polio, Coxsackie B, and polyoma virus infection, [12] the present study suggests that some types of stress may actually attenuate the onset of diabetes mellitus type 1 in mice. This is in agreement with observations of Huang and others who stressed STZ injected mice through shock stimulation and found that none of the 10 mice stimulated beginning 1 h after STZ injection developed diabetes mellitus type 1. However, the 9 nonstimulated mice developed hyperglycemia between 6 and 8 weeks after STZ injection and all had become diabetic by the end of the experiment.[12]

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