Stat 541 Experimental Design Project 2

Ben DeVries, Eric Folsom, Ryan Kardoes, Alex Trauner

Part 1: Experimental Design Plan (9-step checklist)

1. Define the objectives:

    1. The objective of this experiment was to compare how three levels of postbiotic supplementation may attenuate stress, inflammation, and indicators of leaky gut in young horses undergoing an exercise challenge.

2. Identify response and sources of variation.

    1. Treatment factors and levels: One treatment factor (postbiotic supplementation) with 3 levels: 0 mg/kg BW, 46 mg/kg BW, and 92 mg/kg BW.
    2. Response: Prostaglandin E2 PGE2 Repeated measures over time surrounding the SET (submaximal exercise test): PRE (before exercise), h0 (immediately post), h1, h6 post-exercise.
    3. Sources of variation: Individual horse, consumption of full amount of supplement, baseline stress/inflammation levels.
    4. Blocking factor: Sex

3. Choose a rule for assigning the experimental units to trt (design).

    1. We would choose a randomized complete block design.

4. Specify measurements to be made, experimental procedure, and the anticipated difficulties.

    1. Measurements:
        - PGE2.
    2. Procedure: 30 quarter horse yearlings were be stratified by age, body weight, and sex and assigned to one of three treatment groups: a control, not receiving any postbiotic, a low dose group receiving 46 mg/kg of the postbiotic, and a high dose group receiving 92 mg/kg postbiotic product, which was top-dressed on concentrate fed two times daily. Horses also had ad lib (free-choice) access to water and hay.

Horses underwent progressive exercise training for 30 minutes a day, 5 days a week to simulate industry-standard sales preparation protocols. At the end of the study, a submaximal exercise test, or SET, was used to simulate a prolonged intense exercise bout. Horses were balanced by treatment and assigned to one of 4 SET groups which started exercise on 4 consecutive days, with one group per day. Horses arrived at day - 30 to adapt to the new location. At day - 14, baseline samples were collected and at day - 7, basal diet adaptation began. Day 0 marks initiation of experimental treatments via postbiotic supplementation. Sample collection occured at days -14, 0, 11, 45, and pre- and post-SET. Post-SET samples were taken at 0 h post, 1 hour, 6 hours, and 24 hours post exercise. For the purposes of this project, we will focus on samples surrounding the SET. To evaluate stress and inflammation, serum samples will be analyzed by ELISA for cortisol, prostaglandin E2 (PGE2), and superoxide dismutase (SOD). Fecal samples will be analyzed by ELISA for fecal IgA. Altogether, these markers allow for evaluation of inflammatory responses to stress events and gut dysfunction. Samples will be numerically coded and processed randomly to prevent any bias from researchers.

3. Anticipated difficulties:
    1. Working with animals - Sometimes dangerous to obtain samples, leading to missing observations for some animals. Injuries/lameness can prevent animals from participating in exercise. Animals may not always eat the entirety of their diet, leading to differences in supplement dosing - monitored by collecting and weighing refusals. Individual differences in levels of stress and inflammation in any given animal.
    2. Lab work - Difficulty in troubleshooting and optimizing kits, sample preservation and degradation, human error in lab techniques, environmental conditions in lab.
    3. Equipment - For this particular study, there were issues with the hot walker (how we exercise the horses) that prevented the SET from being as intense as it should have been to illicit a true stress response.

5. Pilot experiment:

    1. For the purposes of this project, cortisol values will be used as a "Pilot Study" to determine sample size through a power analysis. A true difference of 20,000 pg/mL serum cortisol will be used (found to be significantly different in a similar study in horses). The standard deviation used is from the current cortisol samples as previously analyzed. The power analysis based on PGE2 values determined that 2 horses per treatment group are needed for a power of 0.90 and significance level of 0.05.

6. Specify the model: $Y_{ijkht} = \mu + \alpha_i + \beta_j + \gamma_k + T_h + \epsilon_{ijkht}$

    Where:

Y_{ijkht} is the response (cortisol, PGE2, SOD, or fecal IgA)

μ is a constant

α_i is main effect of treatment (CON, LOW, HIGH postbiotic supplementation)

β_j is main effect of time

γ_k is the block effect (sex)

T_h is random effect of horse (ID)

ϵ_{ijkht} is the error term

7. Outline the analysis:
    1. Results will be analyzed using ANOVA for mixed effects models with repeated measures of time.
8. Calculate number of observations and time/budget.
    1. 30 horses were used for this study (n = 10 per trt).
    2. The study was completed over a span of 69 days in Fall of 2022.
9. Review and revise. Following the pilot study power analysis, the sample size for this study (n = 10/trt) should be more than sufficient to detect a difference if there truly is one.

Part 2: Report

Introduction

A healthy intestinal barrier helps aid in nutrient absorption and immune defense to pathogens in the gut. When this barrier becomes compromised by things such as illness or stress, pathogens can enter the bloodstream via gaps in cell junctions. This condition is most commonly known as "leaky gut". This leaky gut can challenge the immune system and lead to weight loss, decreased performance, and other health complications for horses. Dietary supplementation with postbiotics may be used to support proper gut function and overall health, as well as mitigating stress responses. Postbiotics are beneficial byproducts that are created as a result of probiotic activity. Supplementing directly with postbiotics can support normal intestinal barrier integrity and decrease stress responses. Little is known about the response of young horses to supplementation with postbiotics when faced with exercise stress, therefore the objective of this study was to investigate the use of dietary Saccharomyces cerevisiae fermentation product in mitigating the impacts of training and exercise on intestinal integrity and stress in the young performance horse.

Experimental units and randomization

Thirty Quarter Horse yearlings (374 ± 25 kg BW; 562 ± 16 d of age; 15 fillies and 15 geldings) were used for this study. Treatment allocations (n = 10 per treatment) were fixed, while experimental units were assigned random numbers and stratified within treatment groups until optimally balanced groups were created. Horses were stratified by BW, age, and sex and randomly assigned to one of three dietary treatments (n = 10/treatment): CON (0), LOW (46), or HIGH (92 mg/kg BW postbiotic). Blood and fecal samples will be obtained at pre-determined time points surrounding the SET and analyzed by ELISA for biological markers indicating stress and inflammation in response to an exercise challenge.

Pilot study and sample size calculations

From a power analysis based on cortisol values with a true difference of 20,000 pg/mL and a standard deviation of 5419 pg/mL, 3 horses per treatment will be required for a power level of 0.90 and an alpha of 0.05. The sample size of n = 10/trt in the present study is sufficient.

Data overview

For our analysis of the effects of postbiotic treatments on prostaglandin E2 (PGE2) levels in quarter horse yearlings, we will control for time and sex while accounting for repeated measures on the horses. Figure 1 displays a panel of plots used to visualize the response against the predictors, and explore the potential for interactions between our covariates. The violin plots in (A) suggest the treatment may have some effect as the observed PGE2 tends to be higher for horses with a larger postbiotic supplement. The violin plots in (B) appear nearly identical, providing little to no evidence for a sex effect. In plot (C), we explore the potential for changing PGE2 over time. There is no clear discernible trend, but there appears to be slightly less variation in PGE2 prior to exercise. This may suggest a potential time effect, as PGE2 measurements were more similar prior to exercise. In plot (D), we see PGE2 measurements for geldings were slightly higher on average then fillies in the control and low probiotic treatments; but the reverse is true for the high treatment group. This hints at a potential interaction, but it's quite possible that patterns may be due to random chance. Both plots (E) and (F) show slightly different patterns in PGE2 measurements over time by treatment and sex respectively. These variations are slight and provide little to no evidence of interactions.

Data analysis and results

After fitting our model for prostaglandin E2, we checked our modeling assumptions via visual diagnostics. We observed a clear violation of the assumption of homoscedasticity in plot (A) of Figure 2 where the spread of residuals tends to increase with predicted values. To remedy

this violation we log transformed our response and refit the model. After fitting our model on the log transformed response, the homeskedacticity assumption appears reasonable. Plot (A) of Figure 3 shows a fairly constant spread of residuals. The Q-Q plot in (B) indicates that our residuals are not perfectly normally distributed, showing a mildly heavy left tail and a slightly light right tail. This suggest that the intervals from the model will be wider than desired, and power below the nominal level. Next, we checked our assumptions of linearity with the effects plot in Figure 4. The distributions of partial residuals are all roughly centered around the marginal means, suggesting that the linearity assumptions have been met. The partial resiudals in for TRT in (A) show a bimodal distribution. We did not see any obvious interaction during our exploratory data analysis that could explain this, suggesting that a potentailly unobserved omitted variable. Finally, we checked tthe assumption of normally distributed random effects with a Q-Q plot displayed in Figure 5. The plot displays a slight left tail, indicating a small violation of normality. Intervals are likely to be slightly wider and our power to be slightly lower than desired.

We proceeded to assess the effect of postbiotic treatments on the log transformed prostaglandin E2 via a type II ANOVA. Under the null hypothesis, the mean effect of low, high, and no probiotics are the same ($\alpha_1 = \alpha_2 = \alpha_3$); once sex, time, and horse are accounted for. Our alternative hypothesis states at least one of these means differ. The test provides very weak evidence of a treatment effect ($\chi^2_2 = 5.4885$, p-value=0.0643). Using the Bonferroni correction, we then constructed contrasts for the treatment levels. The Low probiotic treatment had a mean PEG2 34.8% lower than the control (95% confidence interval for control-low log scale: $[-1.897, 0.1631]$; Exponentiated: $[.15, 1.1772]$). The high probiotic treatment group had a PGE2 that roughly 37.9% lower than the control on average (95% confidence interval for control-high log scale: $[-1.053, 0.0986]$; Exponentiated $[0.3488, 1.103625]$). Finally, the contrast for Low probiotic treatment versus High probiotic treatment indicated that the PGE2 levels for horses which recieved the Higher probiotic treatments were on average, 95% of the PGE2 levels of the horses which recieved the Low probiotic treatment (95% confidence interval log scale: $[-0.648, 0.5512]$; Exponentiated: $[0.523, 1.7353]$). These results do not indicate a clear difference in PGE2 among horses based on postbiotic treatment, but a relationship is cerrtainly possible. Increasing sample size and addressing some of the complications during experimentation might provide stronger evidence for a postbiotic effect on PGE2 levels in horses.

Table 1: Analysis of Deviance Table (Type II Wald Chi-Squared tests)

	Chisq	Df	Pr(>Chisq)
TRT	5.4884520	2	0.0642980
SEX	0.0218626	1	0.8824531
TIME	6.8618731	3	0.0764331

Table 2: Estimated Contrasts: Treatment Effects

contrast	estimate	SE	df	t.ratio	p.value
Control - Low	-0.4290775	0.2261396	22.01900	-1.8974010	0.1630675
Control - High	-0.4772435	0.2291971	21.96227	-2.0822400	0.1167824
Low - High	-0.0481660	0.2385686	21.96682	-0.2018957	0.9778020

Table 3: Confidence Intervals: Treatment Effects

contrast	estimate	SE	df	lower.CL	upper.CL
Control - Low	-0.4290775	0.2261396	22.01900	-0.9971196	0.1389647
Control - High	-0.4772435	0.2291971	21.96227	-1.0530713	0.0985844
Low - High	-0.0481660	0.2385686	21.96682	-0.6475297	0.5511977

Discussion

Results suggest that there is very weak evidence for a true difference in PGE2 levels (a marker of inflammation) due to postbiotic supplementation (dietary treatment) in yearling quarter horses faced with an exercise challenge.

In terms of experimental design and analysis, a number of changes may have improved this study. During this study, equipment difficulties caused the hot walker to be unusable at certain speeds. Due to this, the SET was not as intense as originally planned in order to avoid injury to the horses due to uneven/suboptimal footing. As a result, the SET was likely not a true "exercise stressor" and did not induce the desired level of stress to be able to parse out differences in stress responses by dietary treatments.

As PGE2 differed by treatment group at the PRE timepoint, it may be appropriate to use baseline PGE2 values (PRE values) as a covariate to normalize starting inflammation levels between groups, as individual animal levels of stress/inflammation may influence the analysis of these markers.

Appendix

Power test for approximate delta of serum cortisol found to be significantly different in a similar study, SD from current cortisol samples.

```
round(power.t.test(delta = 6000,
      sd = sd(HorseDat$PGE2), sig.level = 0.05,
      type = "two.sample", power = 0.90,
```

```
      alternative = "two.sided")$n
    )
```

R code for fitting models and diagnostic plots.

```
# No log Transformation

fit.lm.PGE2 <- lmer(PGE2 ~ TRT + SEX + TIME + (1|ID), data= HorseDat)
summary(fit.lm.PGE2)
car::Anova(fit.lm.PGE2)
emm <- emmeans(fit.lm.PGE2, specs = "TRT", adjust = "bonferroni")
pairs(emm)
confint(pairs(emm))

resid_panel(fit.lm.PGE2)
grid.text("(A)", 0.03, 0.95)
grid.text("(C)", 0.03, 0.45)
grid.text("(D)", 0.53, 0.45)
grid.text("(B)", 0.53, 0.95)

eff_list <- allEffects(fit.lm.PGE2, residuals = T)
pfx <- lapply(eff_list, plot)
ggarrange(plotlist = pfx, labels = c("(A)", "(B)", "(C)"), ncol = 2, nrow = 2)

ggplot() +
  geom_qq(aes(sample = ranef(fit.lm.PGE2)$ID$`(Intercept)`)) +
  geom_qq_line(aes(sample = ranef(fit.lm.PGE2)$ID$`(Intercept)`)) +
  labs(title = "Q-Q Plot for Random Effects", x = "Theoretical Quantiles",
       y = "Estimated Random Effects")

# Log Transformation


HorseDat <- HorseDat |> mutate(LogPGE2 = log(PGE2))

fit.lm.PGE2.log <- lmer(LogPGE2 ~ TRT + SEX + TIME + (1|ID), data= HorseDat)

summary(fit.lm.PGE2.log)
car::Anova(fit.lm.PGE2.log)
emm.log <- emmeans(fit.lm.PGE2.log, specs = "TRT", adjust = "bonferroni")
pairs(emm.log)
confint(pairs(emm.log))
```

```r
resid_panel(fit.lm.PGE2.log)
grid.text("(A)", 0.03, 0.95)
grid.text("(C)", 0.03, 0.45)
grid.text("(D)", 0.53, 0.45)
grid.text("(B)", 0.53, 0.95)

eff_list_log <- allEffects(fit.lm.PGE2.log, residuals = T)
pfx.log <- lapply(eff_list_log, plot)
ggarrange(plotlist = pfx.log, labels = c("(A)", "(B)", "(C)"), ncol = 2, nrow = 2)

ggplot() +
  geom_qq(aes(sample = ranef(fit.lm.PGE2.log)$ID$`(Intercept)`)) +
  geom_qq_line(aes(sample = ranef(fit.lm.PGE2.log)$ID$`(Intercept)`)) +
  labs(title = "Q-Q Plot for Random Effects", x = "Theoretical Quantiles",
       y = "Estimated Random Effects")
```

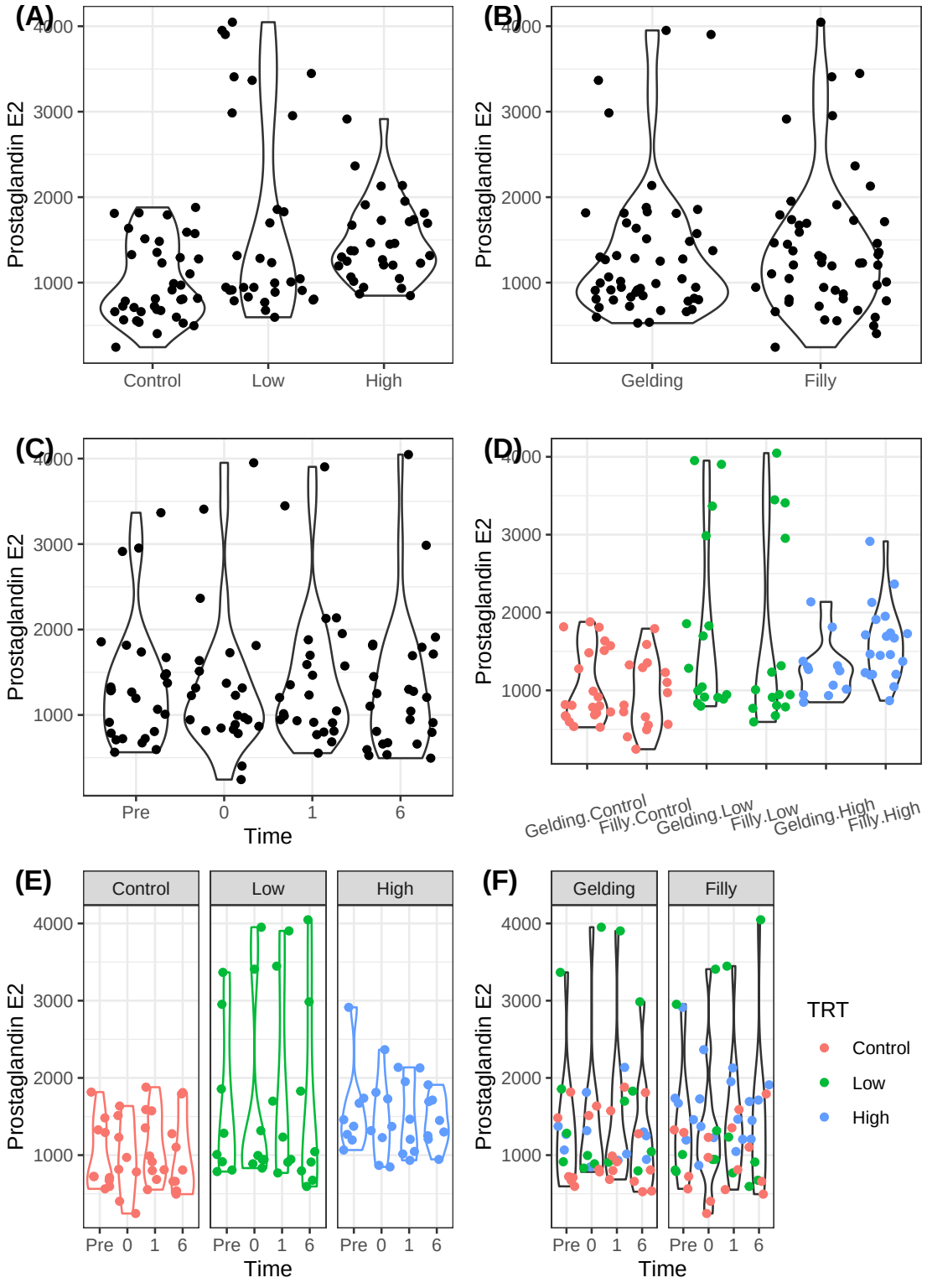
(A)
(B)
(C)
(D)
(E)
(F)
Prostaglandin E2
4000
3000
2000
1000
Control
Low
High
Gelding
Filly
Pre
0
1
6
Time
Gelding.Control
Filly.Control
Gelding.Low
Filly.Low
Gelding.High
Filly.High
TRT
Control
Low
High

Figure 1

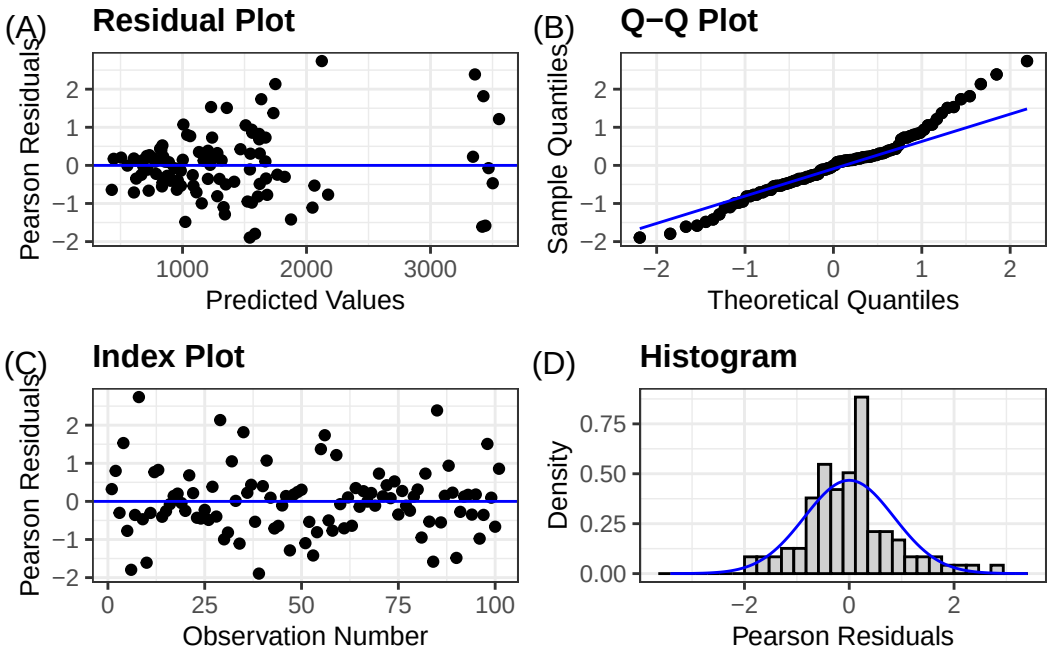

Figure 2: Panel of residual diagnostics for prostaglandin E2 model

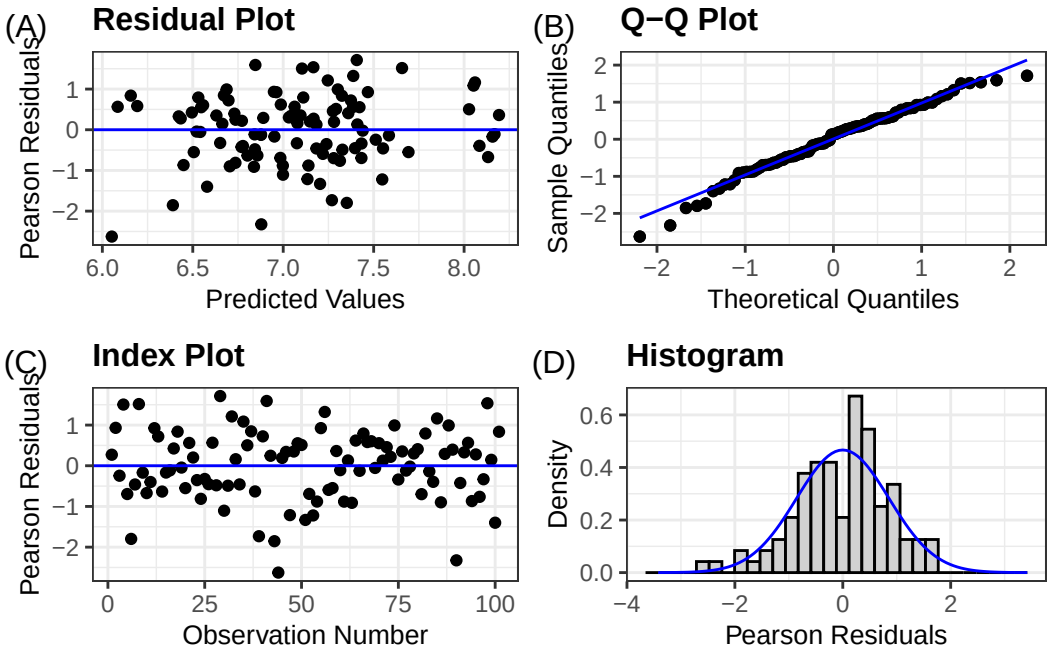

Figure 3: Panel of residual diagnostics for prostaglandin E2 model (Log Transformed)

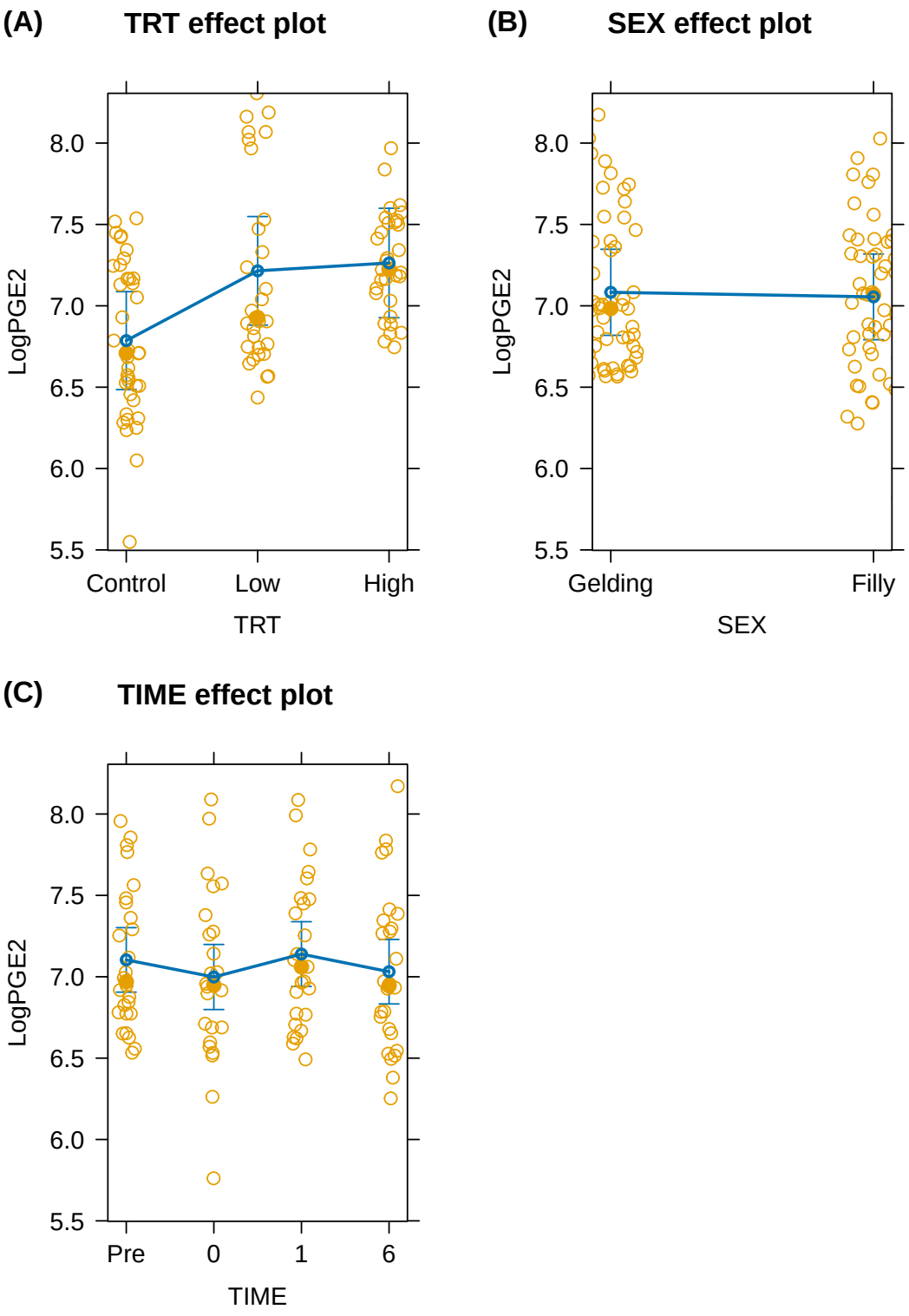

Figure 4: Panel of effects plot for prostaglandin E2 model (Log Transformed)

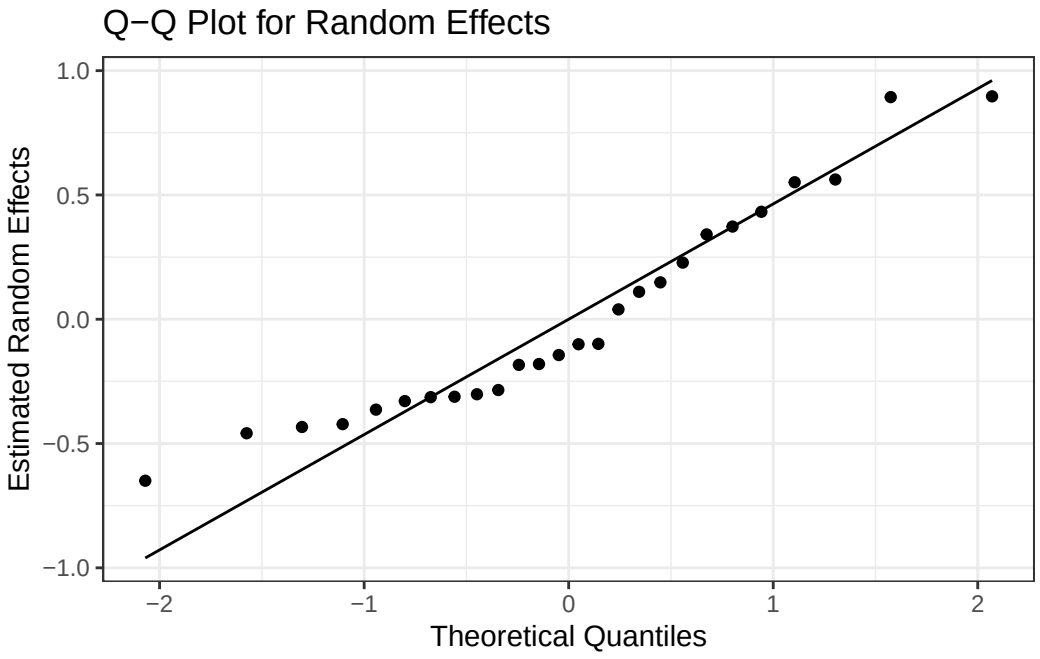

Figure 5: Quantile-Quantile plot of random intercepts for horse nested within treatment (Log Transformed)