Stat 541 Experimental Design Project 2

Ben DeVries, Eric Folsom, Ryan Kardoes, Alex Trauner

LEVEL 1 Header

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1. List Item 1
    1. Sublist item 1
        1. sub-sublist item 1
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- Unordered list item
    - unordered sub-list item

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: Multiple response factors measured, including serum cortisol, PGE2, SOD, and fecal IgA. 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:
        1. Serum cortisol, PGE2, SOD (measured by commercially available ELISA kit).
        2. Fecal IgA (measured by commercially available ELISA kit).
    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. Will need to run power analysis here….. could use one of the markers I have results on and just run with that for "Pilot Study". Actual power analysis was based off of joint markers for a different manuscript from this study.

Power test for approximate delta of serum cortisol found to be significantly different in a similar study, SD from current cortisol samples. Sample Size: `round(power.t.test(delta = 20000, sd = 5419.13, sig.level = 0.05, type = "two.sample", power = 0.90, alternative = "two.sided")` $= 3$

6. Specify the model: $Y_{ijht} = \mu + \alpha_i + \beta_j + T_{h(i)} + \epsilon_{ijht}$

    `Where:`

    `$Y_{ijt}$ is the response (cortisol, PGE2, SOD, or fecal IgA)`

    `$\mu$ is a constant`

    `$\alpha_i$ is main effect of treatment (CON, LOW, HIGH postbiotic supplementation)`

    `$\beta_j$ is main effect of block (sex)`

    `$T_{h(i)}$ is random effect of horse within treatment`

    `$\epsilon_{ijht}$ 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.

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 $\pm$ 25 kg BW; 562 $\pm$ 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 SCFP). 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

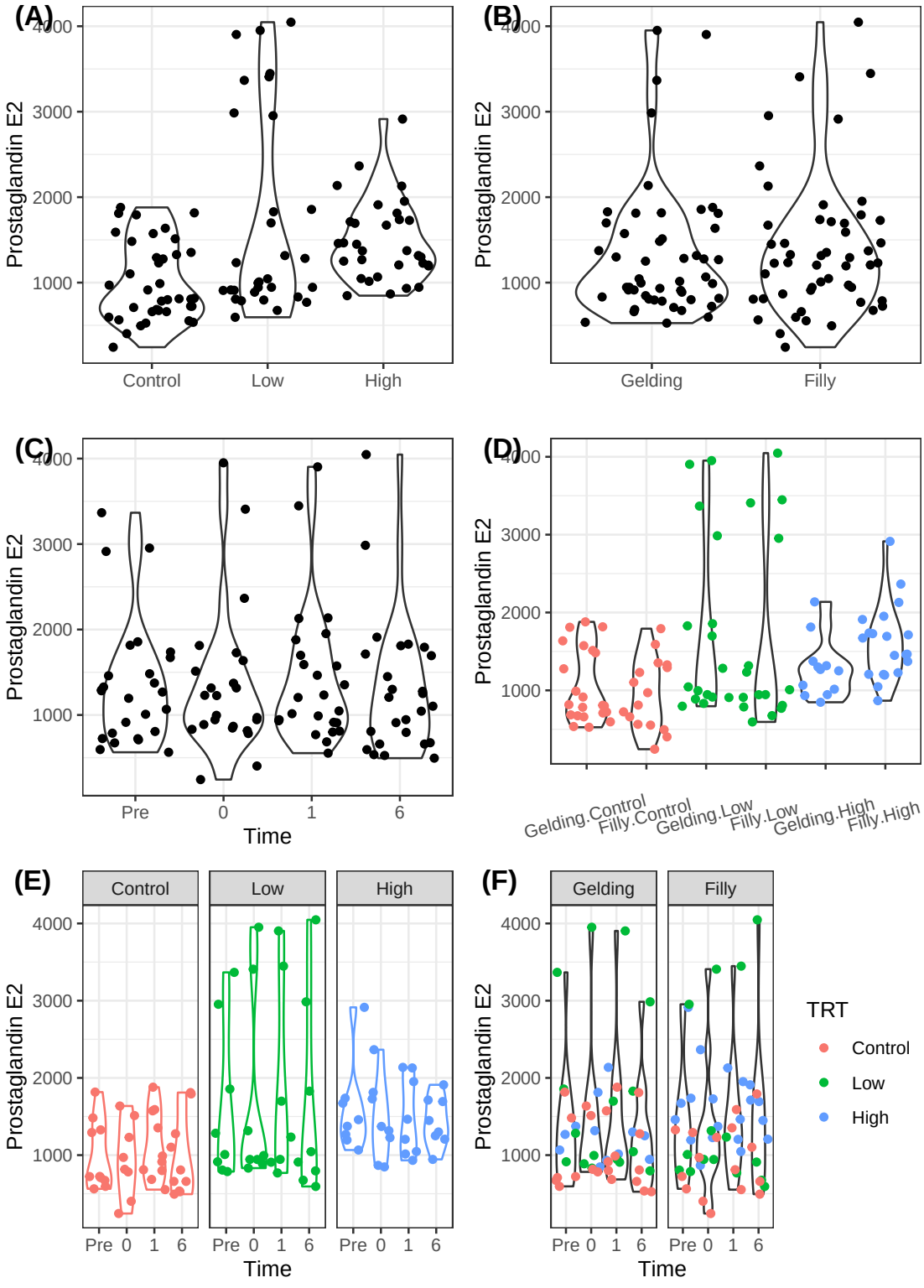
Summarize what you learned from your pilot study. Include sample size calculations - specify and defend your assumptions and choices. It is okay if you cannot achieve the desired sample as long as you justify why.

Data overview

Describe your data and present figure(s) to illustrate the response.

For our analysis of the effects of postbiotic treatments on prostaglandin E2 levels in quarterly horse yearlings, we will control for time and sex while accounting for repeated measures on

the horses. **?@fig-eda** 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 PEG2 tends to be higher for horse 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 PEG2 over time. There is no clear discernible trend, but there appears to be slightly less variation in PEG2 prior to applying treatments. This may suggest a potential treatment effect, as PEG2 measurements were more similar, prior to applying treatments. In plot (D), we see PEG2 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 PEG2 measurements over time by treatment and sex respectively. These variations are slight and provide little to no evidence of interactions.


(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

Data analysis and results

Describe your statistical methods, check assumptions and interpret your results.

After fitting our model for prostaglandin E2, we checked modelling assumptions via visual diagnostics. We see a clear violation of the assumption of homoscedasticity in the plot (A) of **?@fig-resids** where the spread of residuals tends to increase with predicted values. The Q-Q plot in (B) indicates a moderately strong violation of our assumption of normally distributed residuals, showing heavy tails. This indicates intervals from the model will be overly wide and power will be below the nominal level. Next we checked our assumptions of Linearity with the effects plot in **?@fig-fx**. The distributions of partial residuals are all roughly centered at the marginal means, suggesting Linearity is met. The partial residuals for Low in (A) show a bimodal distribution. We did not see any interaction that could explain this in our exploratory data analysis, suggesting a potentially unobserved omitted variable. Finally, we checked the assumption of normally distributed random effects with a Q-Q plot displayed in **?@fig-qq**. The plot displays a slightly light left tail, and heavy right, indicating a mild violation of normality. Intervals are likely to be slightly wider and power slightly lower than desired.

Even though our model violates multiple assumptions, we proceeded to assess the effect of probiotic treatments on 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 ($\chi^2_2 = 3.855$, p-value=0.146). Using the Bonferroni correction, we then constructed contrasts for the treatment levels. The Low probiotic treatment had a PEG2 667 points lower than the control (95% confidence interval for control-low: [-1563, 229]). The high probiotic treatment group had a PEG2 that was 497 higher than the control on average (95% confidence interval for control-high [-1405, 411]). Finally, the contrast for low minus high was estimated to be 170 (95% confidence interval: [-775, 1115]). These results do not indicate a clear difference in PEG2 among horses based on probiotic treatment, but a relationship is cerrtainly possible. Addressing modelling assumptions and increased sample size may provide stronger evidence for a probiotic effect. A log transform may address these violations.

```
Linear mixed model fit by REML ['lmerMod']
Formula: PGE2 ~ TRT + SEX + TIME + TRT:(1 | ID)
   Data: HorseDat

REML criterion at convergence: 1427

Scaled residuals:
     Min       1Q   Median       3Q      Max
-1.89508 -0.49953 -0.00625  0.32188  2.73689
```

```
Random effects:
 Groups   Name        Variance Std.Dev.
 ID       (Intercept) 539325   734.4
 Residual              83379   288.8
Number of obs: 101, groups:  ID, 26

Fixed effects:
            Estimate Std. Error t value
(Intercept)   985.20     265.60   3.709
TRTLow        667.18     356.60   1.871
TRTHigh       497.07     361.48   1.375
SEXFilly        7.00     298.96   0.023
TIME.L        -11.91      56.86  -0.209
TIME.Q        -17.49      57.75  -0.303
TIME.C        -97.67      58.70  -1.664

Correlation of Fixed Effects:
         (Intr) TRTLow TRTHgh SEXFll TIME.L TIME.Q
TRTLow   -0.556
TRTHigh  -0.501  0.451
SEXFilly -0.451 -0.082 -0.185
TIME.L   -0.005  0.004  0.002  0.003
TIME.Q   -0.008  0.002  0.005  0.002  0.006
TIME.C    0.014 -0.011 -0.006 -0.009 -0.024 -0.018


Analysis of Deviance Table (Type II Wald chisquare tests)

Response: PGE2
      Chisq Df Pr(>Chisq)
TRT  3.8548  2     0.1455
SEX  0.0005  1     0.9813
TIME 2.9404  3     0.4009


 contrast       estimate  SE df t.ratio p.value
 Control - Low      -667 357 22  -1.871  0.1708
 Control - High     -497 361 22  -1.375  0.3707
 Low - High          170 376 22   0.452  0.8940

Results are averaged over the levels of: SEX, TIME
Degrees-of-freedom method: kenward-roger
P value adjustment: tukey method for comparing a family of 3 estimates
```

```
 contrast       estimate  SE df lower.CL upper.CL
 Control - Low      -667 357 22    -1563      229
 Control - High     -497 361 22    -1405      411
 Low - High          170 376 22     -775     1115

Results are averaged over the levels of: SEX, TIME
Degrees-of-freedom method: kenward-roger
Confidence level used: 0.95
Conf-level adjustment: tukey method for comparing a family of 3 estimates
```

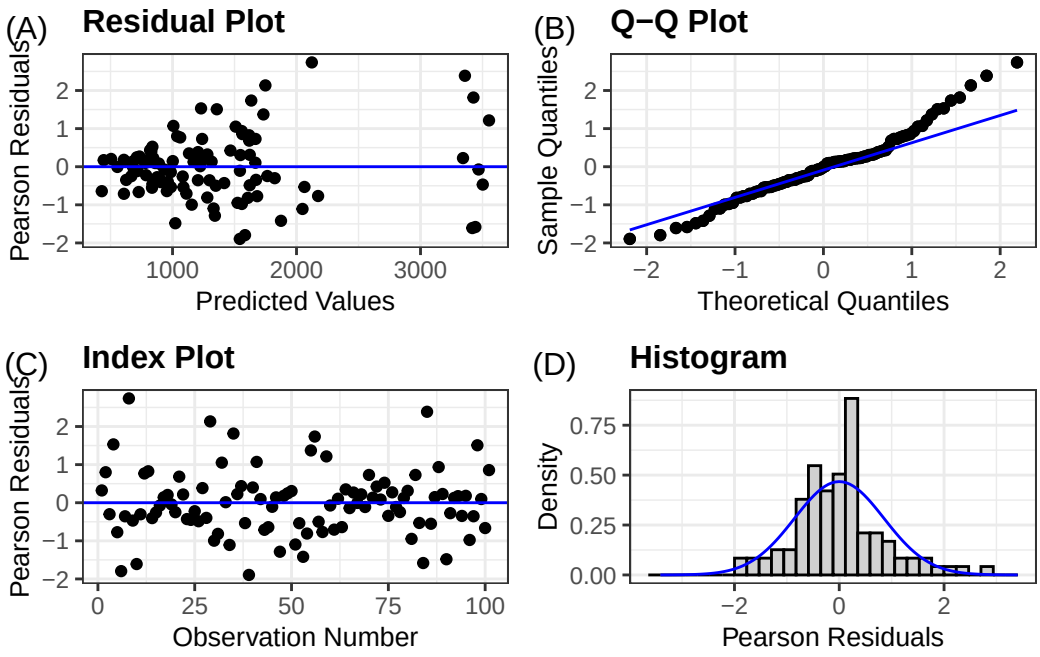


Figure 1: Panel of residual diagnostics for prostaglandin E2 model

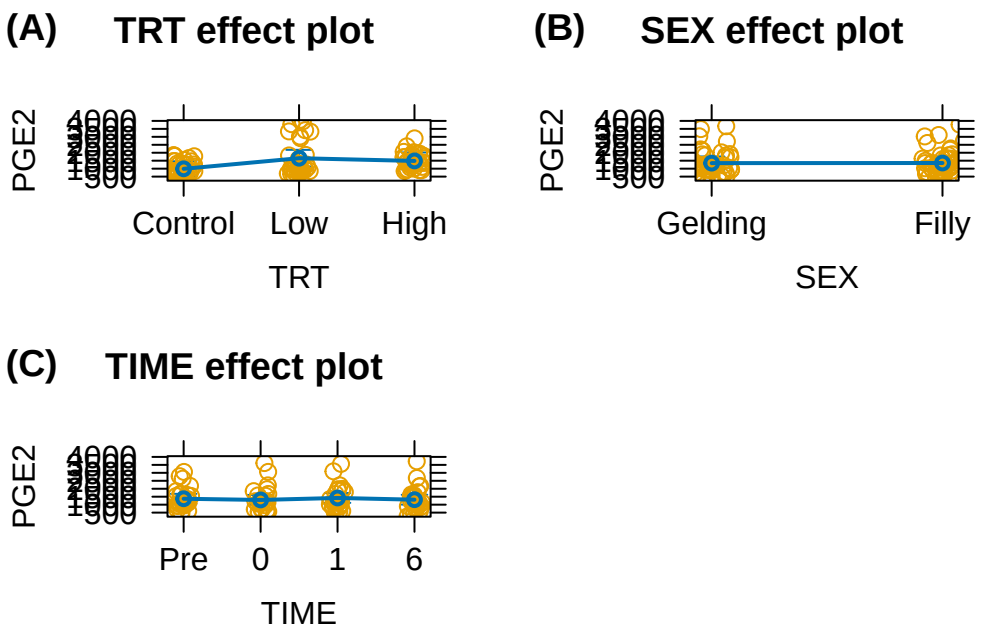


Figure 2: Panel of effects plot for prostaglandin E2 model

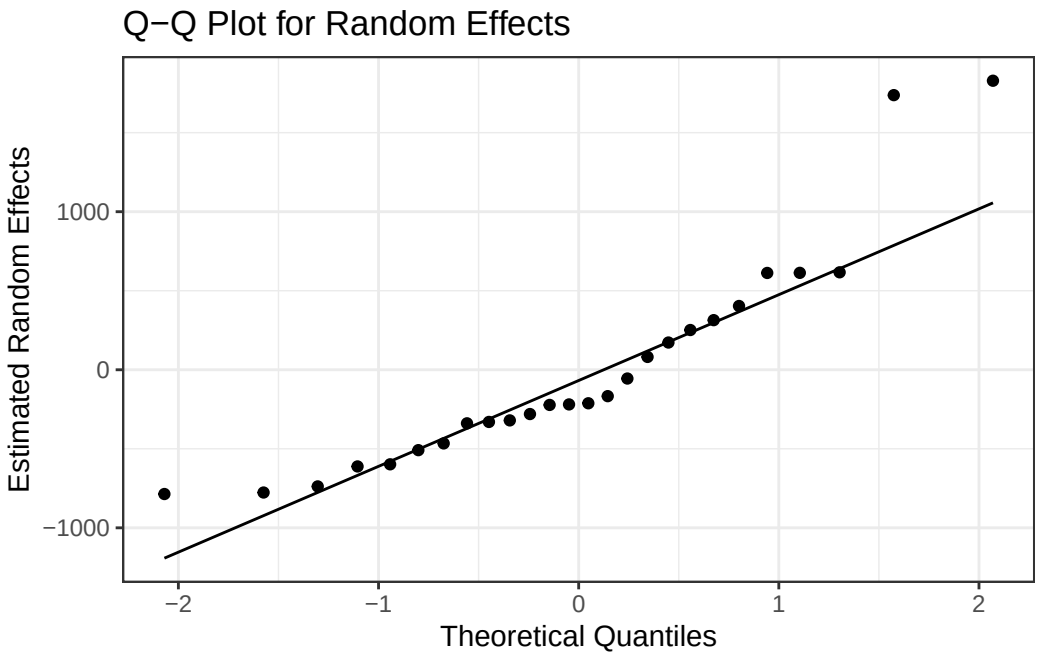


Figure 3: Quantile-Quantile plot of random intercepts for horse nested within treatment

Discussion

Provide a written discussion about the take-away points from your study. Feel free to include commentary about any lessons you learned through this process.