The Effect of Varying Salinity on Larval Survival and Pupation Rates in the Yellow Fever Mosquito (*Aedes aegypti*)

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Abstract

Aedes aegypti mosquitoes are deadly vectors of arboviral pathogens and breed in containers of freshwater associated with human habitation. We hypothesized that the salinity of the rearing environment would impact the pupation rate of *Ae. aegypti* larvae. High salinity concentrations are known to be lethal to developing *Ae. aegypti* larvae and lower salinity concentrations are known to delay larval time to pupation. We predicted that when *Ae. aegypti* Orlando larvae were placed in three salinity treatments (low, moderate, and high salinity), the higher salinities would increase time to pupation and decrease larval survival. We found that all larvae in the highest salinity treatment died before pupation and in the other two treatments there was no significant difference in survival or time to pupation compared to the control. This study should be replicated with larger sample sizes and more replicates as it has potential to yield information relevant to disease control.

Introduction

The Yellow Fever mosquito *Aedes aegypti* originated in sub-Saharan Africa and now exists in most tropical and subtropical regions of the world (Powell & Tabachnick, 2013). *Aedes aegypti* is an important vector of severe human viral diseases, such as Dengue Fever, Chikungunya, Zika, West Nile, and Yellow Fever (Lee et al., 2019) and is found associated with human settlements across a large swath of the globe (Matthews, 2019). Arboviruses spread by

mosquitoes, ticks, flies, and other arthropods have been afflicting humans for millennia and continue to cause immeasurable suffering (Powell, 2018). Mosquitoes are considered by many to be the deadliest animals in the world (Prudêncio, 2020).

Ae. aegypti have a complex life cycle involving dramatic changes in shape, function, and habitat (Levi et al., 2014). *Ae. aegypti*, as all mosquitoes, progress through four life stages: egg, larvae, pupae, and adult (Jayawardene et al., 2011). Both the larval and pupal stages are aquatic and prefer to develop in the clean water found in many types of domestic containers inside or near human dwellings (Chareonviriyaphap T et al., 2003). Larvae of the majority of mosquito species are filter feeders, consuming organic matter such as microorganisms and detritus. The length of the larval stage can vary greatly and is dependent on the availability and amount of food resources (Anoopkumar et al., 2017; Souza et al., 2019). Pupae don't require food and inhabit the pupal stage for 1-3 days before molting into their adult form (Anoopkumar et al., 2017). The larvae progress through four instars, increasing in size at each stage. In the first instar *Ae. aegypti* larva is only about 1 mm in length, whereas in the fourth instar stage it reaches a length of approximately 8 mm (Bar & Andrew, 2013). The basic morphology of *Ae. aegypti* remains similar throughout larval instars. This morphology includes a head, thorax just posterior to the head, abdomen made up of eight segments, siphon used for respiration, and anal papillae used for ion exchange (Bar & Andrew, 2013). Larvae cannot be differentiated between male and female, but female pupae are generally larger than male pupae and so can be differentiated in the pupal stage relatively easily (Benedict et al., 2009).

Ae. aegypti is a very salt-intolerant species and is considered an obligate freshwater mosquito (Ramasamy et al., 2021). Female mosquitoes 'taste' the water they may potentially lay eggs in to determine if it is an appropriate salinity. They do this by dipping their legs and

mouthparts in the water which activates the insect's sensory neurons and sends signals to its brain (Matthews et al., 2019). Gene *ppk301* allows for the mosquito to 'taste' the salinity and successfully lay their eggs in the right type of water (Matthews et al., 2019). Observations related to *Ae. aegypti* have indicated that the species occurs in salt concentrations of up to 16% (Arduino et al., 2015). At higher salinities the larval stage lasts much longer than is typical and larvae are generally much smaller despite the longer period for growth (Clark et al., 2004).

In this study, we measure time to pupation and survival rate of *Ae. aegypti* in various salinity concentrations. We hypothesized that changing salinity of the rearing environment will have an impact on time to pupation and chance of survival. We predict that the higher the salinity concentration, the longer larvae will take to pupate and the lower the larval survival rate. Rates of female and male pupation were hypothesized to be the same in all treatments. This experiment could yield information pertinent to disease control. Previous vector control has failed to prevent recent epidemics and arrest expanding geographic distribution of widespread arboviruses, such as dengue (Achee et al., 2019). As a consequence, there has been increasing necessity for new, alternative strategies for mosquito-borne arbovirus control (Achee et al., 2019). Understanding survival and time to pupation at different salinities presents a viable target for the design of novel control strategies. These strategies could include simply adding salt to containers associated with human life typically known to contain eggs, larvae, or pupae such that they are unable to develop into adult disease vectors.

Methods

Rearing Procedure

Eggs of the wild type Orlando (ORL) *Aedes aegypti* strain were transferred into a 500mL container holding approximately 200mL of dechlorinated water and placed in the bell jar of a large vacuum pump and run under vacuum for 30 minutes (Figure 1). The larvae were left to incubate for three days at 28ºC, until they were placed into treatments, feeding the larvae 0.1g of fish food every day. The day eggs were hatched was considered day zero of the experiment.

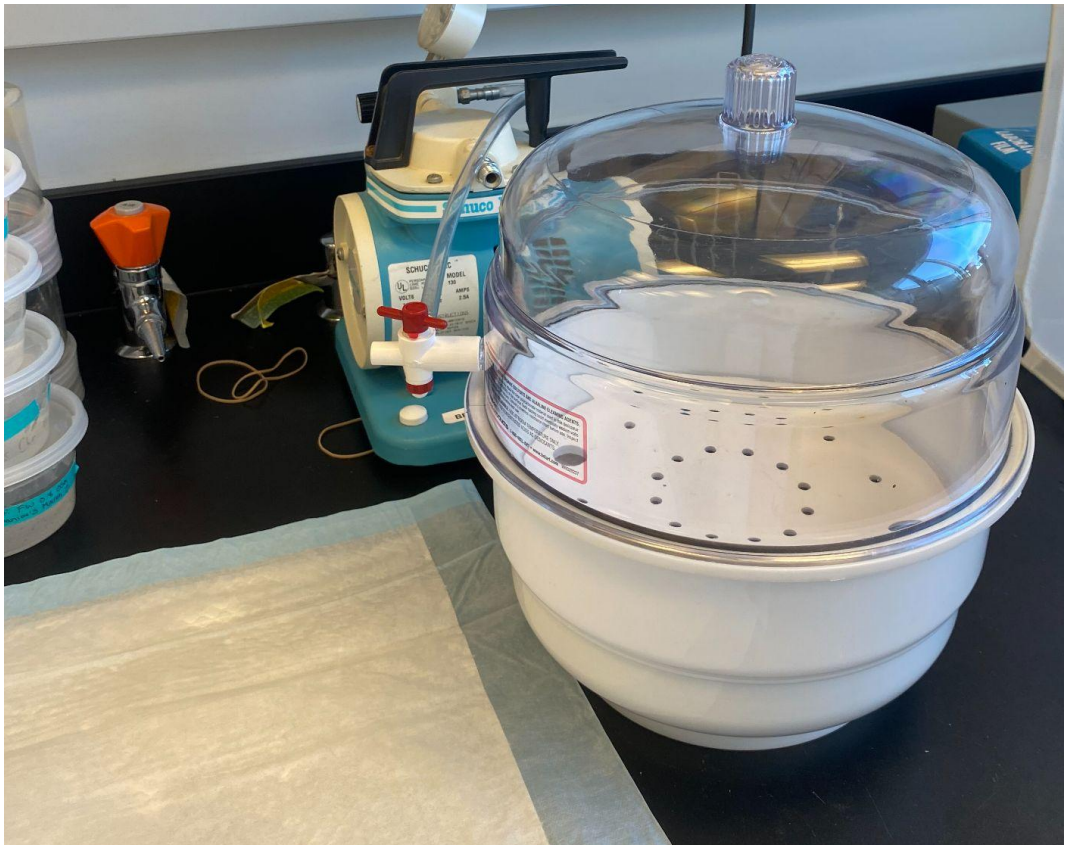

Fig. 1: The bell jar and vacuum pump setup used to hatch the eggs.

Salinity Treatments

On day three of the experiment, larvae were placed into one of four treatments: high salinity, moderate salinity, low salinity, and the control. 40 larvae were placed into each treatment. 0.2M is known to be the upper limit of salinity for survival for *Aedes aegypti* larvae (Phelan, 2022). Each treatment contained 1L of dechlorinated water. 11g of NaCl in 1L of water is ~ 0.2M NaCl and so was chosen as the high salinity treatment. 6.6g of NaCl (0.12 M NaCl) was placed into the moderate salinity treatment, and 4.4g of NaCl (0.08M NaCl) was placed into the low salinity treatment. Treatments were incubated at 28 degrees Celsius and fed 0.1g of fish food every day.

Counting and Sexing of Pupae

Each treatment was checked every day except weekends to see if any larvae had pupated. If pupae were present, they were counted, removed from the treatment, sex was determined visually, and then discarded by freezing. Counting began on day five of the experiment and ended on day 14.

Statistical Analysis

All statistical analysis was carried out using R and MATLAB coding languages. Analysis began with checking the assumptions necessitated by an Analysis of Variance (ANOVA) test: 1) the measurements in every group represent a random sample from the corresponding population, 2) the variable is normally distributed in each of the *k* populations, and 3) the variance is the same in all *k* populations. If any of the ANOVA assumptions were violated, a Kruskal-Wallis test

would be carried out instead. This test is a non-parametric alternative to ANOVA, specifically when the assumption of normality is violated. The null hypothesis for both of these tests is that there is no difference in means across all groups. Analysis was also carried out to investigate a potential relationship between pupae sex and treatment with a chi-squared contingency test.

Results

By the final day of measurement, there were N = 60 total larvae that survived to pupation, with n = 23 in the control, n = 24 in the low salinity treatment, n = 13 in the moderate salinity treatment, and n = 0 in the high salinity treatment (Figure 2). In addition, there were 37 total male pupae and 23 total female pupae across treatment groups (Figure 3).

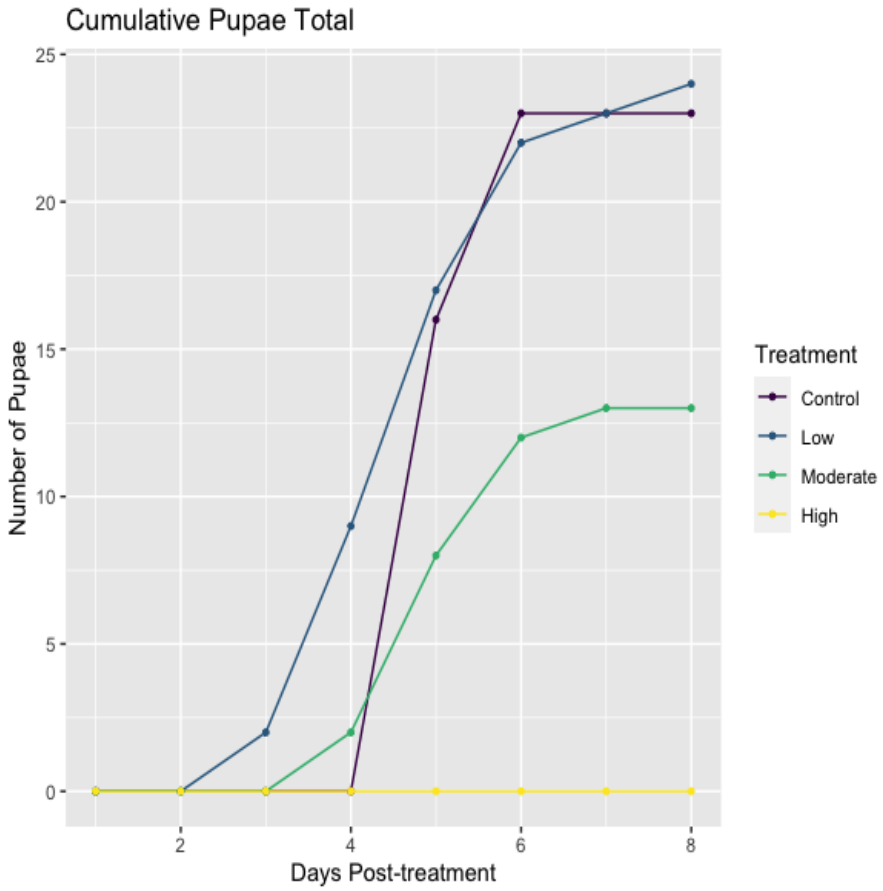

Fig. 2 Cumulative sum of pupae over time, with 60 total larvae surviving to pupation on the final day of measurement. Statistics performed by Kruskal-Wallis, testing whether group means are significantly different, returned a result of $p = 0.3611$, $n = 60$.

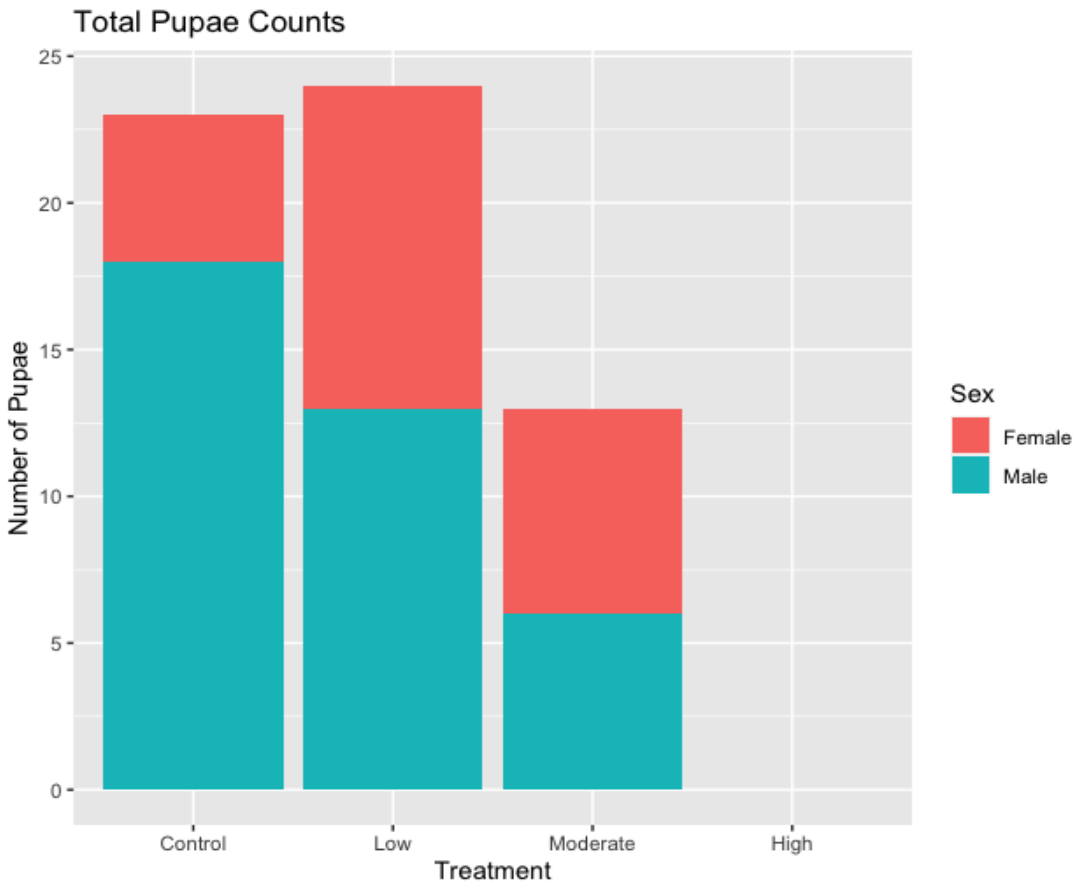

Fig. 3 Final total pupae counts per treatment and distinguished by sex. Statistics performed by chi-squared contingency test, judging the goodness of fit between expected and observed results between pupae sex and treatment group, returned a result of $p = 0.101583$, $n = 60$.

All statistical tests were performed with a significance level of $\alpha = 0.05$. The three assumptions of ANOVA, outlined in the Methods, were tested first. The first assumption, random sample, was affirmed by the nature of our experimental set-up. The latter two assumptions needed to be confirmed with further statistical tests. To test the assumption of normality, QQ plots were constructed on the variables of interest in each of the treatment groups. All three groups, as well as the total sample (N = 60), failed the assumption of normality. The data were

not able to be transformed due to small sample size; most statistics texts recommend data transformations to a Normal distribution only if each group sample size exceeds n = 20. The final assumption, that each group has, statistically, the same variance, was verified using the Levene's test, which is more robust to violations in the assumption of normality. Levene's test returned an insignificant result; with a p-value of 0.111, at the 95% confidence interval, the null hypothesis that the three groups have a different variance could not be rejected (Figure 4). With two of three ANOVA assumptions being violated, the test could not be performed under good statistical practice. Instead, a Kruskal-Wallis test was performed. The Kruskal-Wallis test, testing whether there were differences in pupation rate across treatment groups, returned an insignificant result; with a p-value of 0.3611, at the 95% confidence interval. Thus we failed to reject the null hypothesis and found that the pupation rate across the two treatment groups (moderate and low salinity) and control were not significantly different from each other (Figure 2).

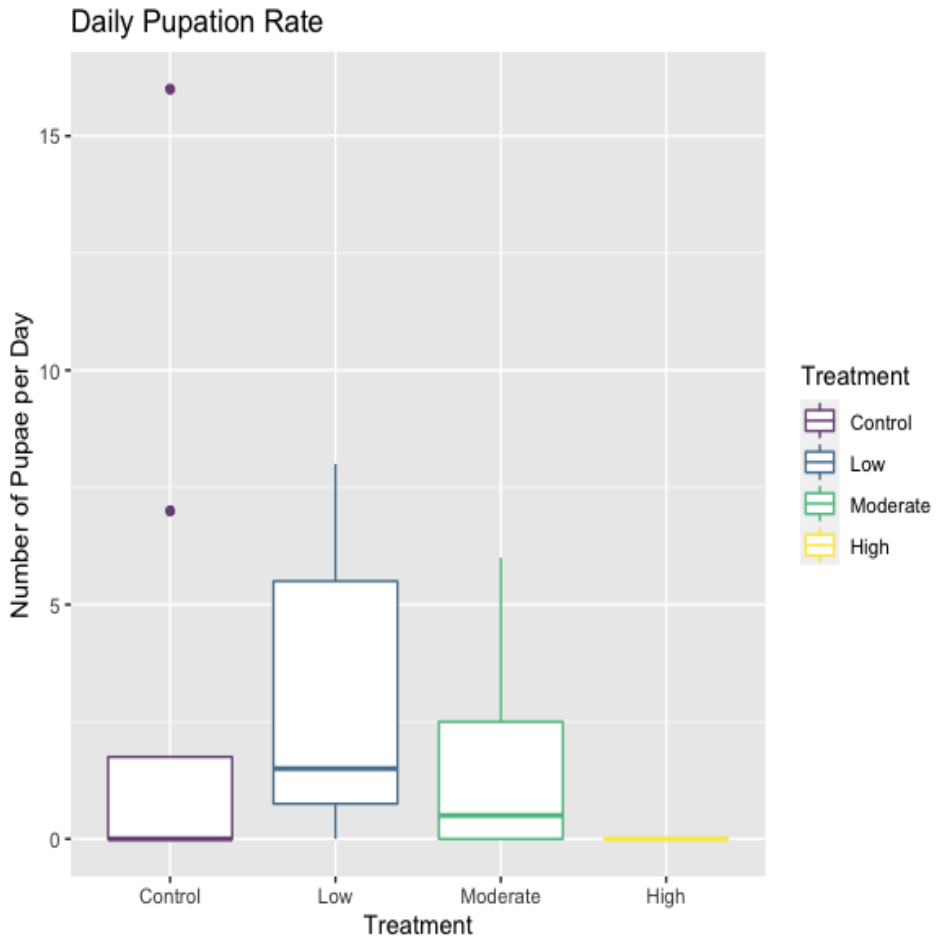

Fig. 4 Daily pupation rate per treatment group, with median bars representing median pupae per day, boxes representing the interquartile range for each group, and whiskers representing relative extrema for each group. Statistics performed by Levene's test, testing whether the four samples have equal variances, returned a result of $p = 0.111$, $N = 160$.

A chi-squared contingency test was performed to investigate the relationship between pupae sex and treatment group. The chi-squared contingency test returned an insignificant result; with a p-value of 0.102, at the 95% confidence interval, the null hypothesis cannot be rejected (Figure 3). Thus, we conclude that there was no significant relationship between pupae sex and treatment group, when analyzing the the total number of pupae in each treatment.

Discussion

Ae. aegypti have a complex life cycle involving four life stages: egg, larvae, pupae and adult (Jayawardene et al., 2011). Our study focuses on better understanding the larval stage of the *Ae. aegypti* life cycle and the external factors that may affect it. Mosquito larvae gain mass through the absorption of nutrients and will not pupate until the larvae become a certain size or gain a significant amount of nutrients (Clements, 2000). Environmental factors which inflict stress on larvae, such as salinity and temperature, have been known to negatively affect larval growth rate and therefore their time to pupate (Clark et al., 2004, Nayar, 1969). Our investigation focuses on how increased salinity affects time to pupation for *Ae. aegypti.*

Our study found no significant difference in time to pupation between treatment groups of different salinities. This disagrees with our hypotheses that higher salinity treatment groups would have increased time to pupation. These findings disagree with current literature which find that at higher salinities the larval stage lasts much longer than is typical for *Ae. aegypti* (Clark et al., 2004). However, rates of male and female pupation were found to be statistically the same, supporting that aspect of our hypothesis.

The discrepancies between the findings by Clark et al. (2004) and ours potentially stems from limitations in our study design. Our first limitation is that we only had access to the larvae during business days. Larvae were reared on a Wednesday (day 0), treated with salinity on Friday (day 2) and then data collection occurred on days 5-9 and days 12-14. On days 10 and 11, which fell on the weekend, the larvae were not fed and no pupation data could not be collected. This introduces uncertainty for data collected on days 12-14. Due to this uncertainty, we excluded data after day 10 from analysis.

Another limitation was our small sample size and lack of replicates. Although we began treatments with 40 larvae each, less than 25 larvae survived to pupation in the control group (0 g/l NaCl) and low salinity treatment (4.4 g/l NaCl). Only 13 larvae pupated in the moderate salinity treatment (6.6 g/l NaCl) and none of the larvae survived to pupation in the high salinity treatment group (11 g/l NaCl). Due to limited time for data collection, only one replicate was completed. Clark et al. (2004) found the most pronounced effects of time to pupation between salinity treatments of 7 g/l and 14 g/l, however since our high treatment group failed, we were not able to observe similar trends in our data. It is likely that our moderate treatment group (6.6 g/l NaCl) was not sufficiently high enough salinity to reproduce the findings from Clark et al. (2004). This likely contributed to our failure to reject the null hypothesis for our data.

Although our current data did not show any significant difference between treatment groups, more replicates at various salinity concentrations are needed to further investigate associations between time to pupation and increased salinity for *Ae. aegypti* larvae.

Conclusion

Mosquitoes not only disturb people's daily life and work, but also spread a variety of serious diseases, such as malaria, filariasis, chikungunya and dengue fever (Ramasamy et al., 2021). Thus, researchers need to understand the influence of environmental factors such as salinity on the mosquito life cycle and growth to provide knowledge for future disease prevention and control. This study was unable to conclude significant correlations between the various salinity concentrations and the pupation time and survival of *Ae. aegypti* larvae. The

findings from the viable treatments suggest that higher salinity does not significantly extend the time for pupation nor survival to pupae for *Ae. aegypti;* Due to the failure of the high salinity treatment we were unable to include it in our analysis. Future research should increase the number of treatment groups with different salinity concentrations other than the 4.4 g/L, 6.6 g/L and 11 g/L NaCl. In addition, future experiments should also increase the sample size and replicates for each treatment, thus reducing the likelihood of type II errors.

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Appendix

Table 1: Raw data from the experimental trials. N = 40 per treatment group. M = male, F = female. (Control = 0g NaCl, Low = 4.4g NaCl, Moderate = 6.6g M NaCl. High = 11g NaCl).

		Treatment Group														
		Control			Low			Moderate			High					
Day (Post-seeding)	Day (post Treatment)	M	F	**Total**	M	F	**Total**	M	F	**Total**	M	F	**Total**	Daily M Total	Daily F Total	**Daily Total**
2 (treatment added)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	3	0	0	0	0	0	0	0	0	0	N/A	N/A	0	0	0	0
6	4	0	0	0	0	0	0	0	0	0	N/A	N/A	0	0	0	0
7	5	0	0	0	1	1	2	0	0	0	N/A	N/A	0	1	1	2
8	6	0	0	0	4	3	7	0	2	2	N/A	N/A	0	4	5	9
9	7	12	4	16	5	3	8	4	2	6	N/A	N/A	0	21	9	30
12	10	6	1	7	2	3	5	2	2	4	N/A	N/A	0	10	6	16
13	11	0	0	0	0	1	1	0	1	1	N/A	N/A	0	0	2	2
14	12	0	0	0	1	0	1	0	0	0	N/A	N/A	0	1	0	1
Experimental Totals				23			24			13			0	37	23	60

Statement of contribution:

Abstract: Written by Hannah and Maryann

Intro: Written by Maryann

Methods: Written by Maryann and Marisa

Results: Written by Marisa and Hannah

Discussion: Written by Hailey

Conclusion: Written by Faith

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