the Berkeley Carroll School

Proceedings of the Berkeley Carroll

7TH INDEPENDENT RESEARCH CONFERENCE

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In addition, we would like to acknowledge the support and assistance of the following members of the Berkeley Carroll Administration, without whom this program would not have been possible:

Jane Moore
Director of Upper School

Robert Vitalo
Head of School

Welcome from the Editor

The classic "nine dot" puzzle asks you to connect three rows of equally spaced dots by three line segments that can be drawn without lifting your pencil.

The solution requires you to extend two of the line segments outside the artificial square formed by dots.

Consequently, problem solving that requires you to consider options outside conventional boundaries has come to be known as "thinking outside the box".

The funny thing about this year's journal (our seventh if you can imagine that!) is that many of our young scientists have practiced outside the box thinking by utilizing new kinds of – well – boxes.

Ian Ellis, for example, investigated the feeding habits of Russian tortoises by building an enclosure in which the tortoises could be raised and cared for. Ellie Pike looked for patterns in group behavior of ants as they hunted for food in much smaller enclosures – a half dozen tiny plastic ant colonies.

Katrina Fuller and Julia Harrison each explored variations in gene expression using a real time polymerase chain reaction (PCR) machine.

And some Berkeley Carroll heavy hitters couldn't get close to making contact with knuckleballs thrown inside the batter's cage as they participated in Sara Tobias' experiment looking at how vertical illusions vary from pitch to pitch.

All these literal boxes – the enclosures for ants and tortoises, the batting cage, and the PCR machine – illustrate some of the ways our students have utilized the new classroom and lab space and the new resources here at Berkeley Carroll. With the advent of the Beta Lab, our Science Research and Design students now have plenty of new options for investigating the experimental questions on their minds.

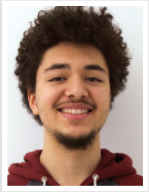
That's not to say they can't still reach out to external mentors or conduct long term studies in psychology or athletics. Carolyn Khoury, for example, investigated the neurogenetics of language at the Rockefeller University Summer Science Research Program last summer and Kian Sadeghi carried out several CRISPR (Clustered Regularly Interspaced Palindromic Repeats) experiments last year at Genspace, while Alyssa Pierre, Nysa Stiell, Imogen Micklewhite, James Pierce, and Lukas Yurasits used longitudinal surveys to look at emotion and memory, academic honesty, mood and diet, mood and exercise, and the effectiveness of energy supplements, respectively.

As our school and program continue to expand, the scientific avenues our young scientists can explore dramatically increase. With each year, we learn how to support them more and more.

How far they choose to explore outside the box is limited only by their imagination.

Until next year,
Scott Rubin
Upper School Science Chair

Science Research and Design
SENIOR RESEARCHERS



Ian Ellis '18

Ian has been researching animal behavior since entering the program, and he gave his 10th grade presentation on the behavior of African wild dogs when water is scarce. Always drawn to herpetology (the study of reptiles and amphibians), Ian has been conducting research looking at Steppe Tortoise dietary behavior, and more specifically dietary specialization, for the past two years. Ian is presenting his findings from exposing Steppe tortoises to daylight cycles of differing lengths. He hopes to continue studying these topics next year in college.

Katrina Fuller '18

Beginning in her sophomore year, Katrina was mentored by Dr. Ivan Hernández of the neuropathology department at SUNY Downstate Medical Center. During the second half of sophomore year, Katrina developed an understanding of staining and imaging techniques used frequently in biochemistry. She assisted Dr. Hernández on behavioral data collection and imaging for a study on the role of the enzyme PARP in synaptic plasticity. At Downstate, Katrina developed a curiosity for a specific compound recently found to enhance memory and relieve the cognitive deficits characteristic of Alzheimer's Disease. Last year, she researched the effects of this compound on cell structure. This year she used the school's real-time polymerase chain reaction (RT-PCR) machine to simulate the quantification of rRNA expression.

Julia Harrison '18

Julia's initial interest in psychology and the effect of childhood influences on adult behavior led her to epigenetics. This rising scientific field focuses on the mechanisms by which genes are "turned on" or "turned off". Julia's interest in microbiology and genetics grew through junior year as she read twin studies and studied genetically identical plants which display different phenotypes, such as the aspen. Julia has spent this year researching how methylation, an epigenetic process, affects phenotype in plants, and how specific drugs can induce demethylation and subsequently alter phenotype.

Carolyn Khoury '18

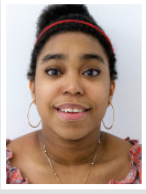
Carolyn's primary scientific interest is computational biology – the intersection of biology and computer science. She was exposed to this field after seven rigorous weeks conducting research at Rockefeller University Summer Science Research Program. There, she worked at the Laboratory of the Neurogenetics of Language under the mentorship of Dr. James Cahill and Dr. Erich Jarvis (the laboratory's principle investigator). One of the lab's main goals is to identify genes responsible for vocal-learning in humans by first locating them in certain avian species that have also acquired this trait. They hypothesize that these genes, when mutated in humans, cause speech delay and autism spectrum disorders. Ultimately, identification of these genes in humans could lead to gene therapies for such disorders. Carolyn's current SRD project is based upon her work at Rockefeller University; she has built a computer program using the Python programming language that identifies false positive signals of natural selection (paralogs) in an alignment of 33 avian species. This tool weeds out genes that have been wrongly identified as candidate vocal learning genes, refining the lab's search. She has found 952 of such paralogs.

Imogen Micklewhite '18

In 10th grade Imogen was researching the use of dance as a treatment method for Parkinson patients. During her junior year she spent her first semester at the Maine Coast Semester School where she worked on the school's farm and experienced a direct farm to table relationship with whole foods. This sparked Imogen's interest in nutrition and the effects that natural foods and processed foods have on mood. For her final project, Imogen looked specifically at naturally found sugars and processed sugars. Her experiment tested whether there was a positive or negative change in mood 20 to 30 minutes or 3 to 5 hours after consuming either a smoothie or soda.

James Pierce '18

During his first year in the Science Research and Design program, James studied the fields of Marine Biology and Psychology, specifically the effects of violent videogames on young children. Over the last 2 years he has researched the effects of exercise and mood. In this study he tested how calisthenic, aerobic, and resistance training affected the mood of Berkeley Carroll seniors over the span of 24 hours. He shares both his findings and what he learned from the experiment.

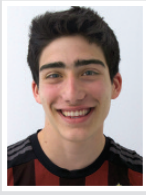


Alyssa Pierre '18

Since joining the Science Research and Design program as a sophomore, Alyssa has been studying the topic of memory, sparked by her grand-uncle's Alzheimer's diagnosis. During her sophomore year, she focused on gaining background information on memory and trying to deepen her understanding and knowledge of the topic. As a junior, Alyssa investigated if memory retention has a correlation to race. She conducted a study with Berkeley Carroll sophomores and freshmen that tested which races retained specific emotions more than others. Now as a senior, Alyssa has refined her study to test which emotions are retained by certain races. She hopes to continue her research in college, and further explore memory in accordance to race and other factors.

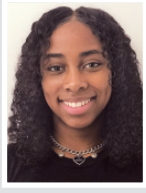
Ellie Pike '18

In Ellie's sophomore year, she became interested in social organisms after reading an article about colony collapse disorder among honey bee populations. Throughout junior year she zeroed in on the behaviors of social insects, specifically focusing on ants. As well as general information about ants, Ellie was especially interested in colony organization and the various forms of communication, specifically pheromone communication, utilized by ants. For her final project she knew she wanted to observe ants in action! Initially, she planned to center her final study around ant foraging and pheromone communication, but after talking with Dr. Gentile, she decided to focus instead on agar ant farms, where ants could tunnel and be observed. Today, Ellie is presenting her findings from several different studies and trials with the agar ant farms.



Kian Sadeghi '18

Kian is a lifer at Berkeley Carroll and began researching about CRISPR in the 10th grade. After reading various articles about CRISPR and presenting a research paper about scientists using the complex to correct the point mutation that causes Retinitis Pigmentosa, he was captivated by the power of the gene-editing device. He continued to learn about CRISPR through the 11th grade and eventually worked in a community lab called Genspace where he and his advisor Ms. Sefchovich disrupted the ADE2 gene in brewer's yeast making the organism red. Kian and Ms. Sefchovich were featured in the *Wall Street Journal* for their work at Genspace and, this year, conducted another experiment using CRISPR which involved correcting a point mutation to make bacteria resistant to an antibiotic called streptomycin. Kian hopes to continue studying genetic engineering in college.

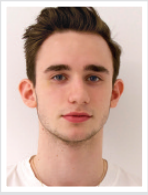


Nysa Stiell '18

Over the past three years Nysa's field of research in the Science Research and Design program first focused on general dishonesty and now specifically examines academic honesty. Her interest stemmed from her realization that understanding the process behind lying could benefit her school community. This year, her research took a double-pronged approach in a two part experimental procedure. One part allowed her to identify differences in what 10th, 11th and 12th graders are willing to admit regarding academic dishonesty. The second part required her to monitor students' heart rate in order to determine whether their heart rate decreases the more they lie.

Sara Tobias '18

Sara's experience playing baseball led to her interest in examining the differences between specific types of pitches. Since her first year in the Science Research and Design program, Sara has been working on different ways of answering the question "is there a difference in vertical illusions between knuckleball and traditional pitch sequences?" She spent her junior year working with Major League Baseball statistics to try and find discrepancies between knuckleball and traditional pitchers. During her senior year, she worked with high school baseball players and a former college pitcher to gather live data though video and a swing sensor. Though Sara's data did not yield significant results, it leaves a clear path for continued research in the future.



Lukas Yurasits '18

Lukas started looking into the field of nutrition in his sophomore year and then gradually moved to nutrition in sports in his junior and senior year. His interest originated when his father, who is a chef, had to figure out the best ways to make the most nutritious, healthiest, and easiest foods for some of his clients like Novak Djokovic, a professional tennis player. Being a hardcore athlete himself, he wanted to figure out the best products to help athletes perform at their peak. He first investigated the sports drink question to see if Gatorade and Powerade really affected the running performance of athletes after a routine workout. After encountering challenges gathering high school students after school, he moved on to experimenting on his basketball teammates. In his final experiment he wanted to examine the importance of protein and sugar, by themselves and together, in the energy and recovery levels of these high school basketball players.

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Steppe Tortoise *(Testudo horsfieldii)* Dietary Specialization While Receiving Differing Daylight Cycles

by Ian Ellis

Abstract

Dietary specialization is one of the most prominent dietary behaviours in species, and has been seen to be variable across certain conditions. A prime example of species whose dietary specialization variates across certain conditions are tortoises. The Steppe tortoise, *Testudo horsfieldii,* is a small species of tortoise that, while abundant and well known through the pet trade, has been poorly documented thus far. One condition that hasn't been researched in depth is the effect of circadian rhythms, and specifically daylight cycles, on dietary behaviour in the species. In this study we asked if there is a difference in captive Steppe Tortoise dietary specialization when receiving a longer daylight cycle versus a shorter daylight cycle. We looked into this through a three month study where one captive *Testudo horsfieldii* was kept under a 12 hour daylight cycle for the first month, a 14 hour daylight cycle for the second month, and a 7 hour daylight cycle for the third month. The tortoise was given four different accessible green food resources each day, and was given a value representative of its dietary specialization by taking the number of resources consumed divided by the total resources available. We found that across the board, the tortoise was a specialist, regardless of daylight cycle length. Furthermore, statistical analysis led to the acceptance of the null hypothesis showing no significant difference in the tortoise's dietary specialization throughout the differing daylight cycles. Sample size, time of food presentation and food familiarity seemed to be the largest sources of error. It was concluded that the change in daylight cycles either did not affect the tortoise's circadian rhythms, therefore not affecting its behaviour, or it affected the tortoise's circadian rhythms, but did not affect the tortoise's dietary behaviour.

Background

Dietary specialization is how much a species picks and chooses its food resources for consumption [Hutchinson 1957]. A species can either be a dietary specialist, one that eats a specific range of resources, or a dietary generalist, one that eats a broad range of resources [Roughgarden 1972]. Dietary specialization is seen to be essential to species health [Tracy et al, 2006], but has been seen to be variable across abiotic conditions. A prime example of a species whose dietary specialization variates across certain conditions are tortoises.

One example of a variation is that tortoise species have been found to be increasingly specialized as the precipitation in their surrounding area increases (in other words, across an increasing precipitation gradient), which then allows the more specialized tortoises to eat in a more nutritionally con-

scious manner [Murray IW, Wolf BO, 2013]. Researchers were able to quantify specialization and nutrition in desert tortoises by looking at their shells. By analyzing the keratin in desert tortoise shells via a continuous flow isotope ratio mass spectrometer, the researchers were able to find carbon isotope values. These carbon isotope values let the researchers know what photosynthetic groups the tortoises had consumed in their lifetime, and therefore, the plants they ate and how nutritious these plants were. In other words, the isotope values ingrained in the tortoise shells served as lifelong dietary logs for the tortoises. As mentioned earlier, the study [Murray IW, Wolf BO, 2013] found that the tortoises become more specialized as the precipitation gradient increased. The researchers came to this conclusion as analysis of the tortoise shells revealed the tortoises were more nutritionally oriented and more specialized when the tortoise was located in an area with higher precipitation.

Another study has found that tortoise species specialize differently throughout different phenological periods in the spring for optimal nutritional consumption [Jennings WB, Berry KH, 2015]. Phenological periods are the parts of life cycles in living organisms, and in this case, different flowering and sprouting times for certain plants in the spring. By observing 18 tortoises take over 35,000 bites, the number of times the tortoise bit at and consumed a food resource, in the spring foraging season, and then estimating the relative abundance of potential food plants, the researchers found that the tortoises chose food plants specifically throughout the spring foraging season, thus showing specialization. Focusing on the plants selected by the tortoises, researchers discovered that these plants had high nitrogen levels, signifying a higher nutritional value for the tortoises [Jennings WB, Berry KH, 2015].

One condition that thus far hasn't been researched in depth is the effect of circadian rhythms, and specifically daylight cycles, on dietary specialization. Circadian rhythms are rhythms that persist in constant conditions in 24 hour cycles [Tosini et al., 2000]. The most powerful zeitgeber, or environmental cue, for circadian rhythms are those that trigger daylight cycles (when an animal is awake) [Aschoff, 1981]. Established daylight cycles are important in reptiles [Pough, 1991] and have been shown to be especially important in other Testudines, the order of tortoises and turtles [Sakamoto et al., 1990].

The Steppe tortoise, *Testudo horsfieldii,* is a small species of tortoise naturally found across Afghanistan, Northern Pakistan, Northern and Eastern Iran, and North Western China. Their small size, large temperature tolerance, active nature, and eager appetite make *Testudo horsfieldii* a hardy, convenient and therefore ideal tortoise species to work with in captivity [Heinen, 2000]. Although it is one of the most abundant tortoise species in the world, the feeding behaviour of *Testudo horsfieldii* has been poorly documented [Ernst and Barbour 1989, Iverson 1992, Lagarde et al 2003]. However, the small amount of research that has been done on specialization has put *Testudo horsfieldii* midway between a specialist and a generalist [Lagarde et al 2003].

Tortoise specialization is a documented, but generally new topic of research [Murray IW, Wolf BO, 2013]. Because of this, there is a lot to be learned about captive *Testudo horsfieldii* specialization. In addition, while it has been assumed that most species will modify their circadian rhythms based on when food is available [Aschoff, 1981], it hasn't been specifically studied in reptiles (and therefore tortoises), nor have I found any published research specifically relating daylight cycles to dietary behavior in tortoises. This all leads to my experimental question: Is there a difference in captive Steppe Tortoise *(Testudo horsfieldii)* dietary specialization when receiving a longer daylight cycle versus a shorter daylight cycle?

Figure 1

A Captive Steppe Tortoise *(Testudo horsfieldii)*

Question:

Is there a difference in captive Steppe Tortoise *(Testudo horsfieldii)* dietary specialization when receiving a longer daylight cycle versus a shorter daylight cycle?

Methods

To operationalize the independent variable, the length of daylight cycle the tortoise received, a three month study was designed where the tortoise was kept under a 12 hour daylight cycle for the month, a 14 hour daylight cycle for the second month, and a 7 hour daylight cycle for the third month.

The tortoise was given four different accessible green food resources each day: dandelions, kale, collard greens and arugula. To operationalize the dependent variable, the specialization of the Steppe Tortoise, we adapted a dietary specialization scoring system based off previous tortoise dietary research models [Murray IW, Wolf BO, 2013]. If the tortoise ate only one of the four of the resources one day, the assumption could be made that the tortoise was acting as a heavy specialist. Conversely, if the tortoise ate all four of the resources in one day, the assumption could be made that the tortoise was acting as a heavy generalist. To reflect this in the data, dietary specialization was scored by taking the amount of resources eaten and put it over the total number of resources. Values closer to 0.25 signified a heavily specialist, as a score of 0.25 means the tortoise only ate 1 out of 4 resources, while val-

ues closer to 1 signified a heavy generalist, as that would mean the tortoise ate all four of the resources. For a food resource to be considered 'consumed', all the tortoise had to do was take at least one bite. In addition to this score, which particular food resources the tortoise consumed was recorded to get a better sense of any dietary trends.

To review and process this data, a camera recorded the tortoise during the day to to capture the tortoise eating. Having a camera removed ambiguity in the data as it allowed confirmation via film on how many and which resources the tortoise ate. Only one tortoise was used in this study primarily due to personal and facility based conflicts.

Figure 2

***Testudo horsfieldii* Enclosure Used in the Study**

Procedure

1. Set up the tortoise enclosure:
   i. Assemble the Zoo Med Tortoise House.
   ii. Fill the enclosure with a 3 inch layer of Zoo Med Forest Floor Bedding.
   iii. Set up tortoise's heat and UVB by setting up Zoo Med 100 watt Powersun UVB bulb into Zoo Med Mini Deep Dome Heat Fixture. Attach the fixture onto the Zoo Med Small Lamp Stand and plug the light into Zoo Med Aqua-sun Aquarium Controller (this is a timer; use will be detailed later). Position light so that it covers open part of the enclosure.
   iv. Place the water bowl in the enclosure. Fill with water and condition with Zoo Med ReptiSafe® Water Conditioner every day.
   v. Place the four food bowls against the back wall across from the hide.

2. Place the tortoise in the enclosure.
3. Plug in the Amcrest ProHD, connect it to wifi, turn it on and place it so it gets a bird's eye view of the tortoise's four food resources (refer to the Amcrest ProHD manual for specifics on camera setup). Keep the camera on for the duration of the study.
4. Set the tortoise up under a 12 hour daylight cycle. Do this by setting the timer for the enclosure lights running from 7:00 AM to 7:00 PM.
5. When the light turns on, give the tortoise access to the 4 different green food resources: dandelions, kale, collard greens and arugula. Place each different resource in its own food bowl.
6. When the light turns off, remove all food from the enclosure.
7. Go to the Amcrest Cloud website (again refer to the Amcrest ProHD manual for specifics on camera) and review the Amcrest ProHD recording for the duration of the day's daylight cycle. Record the tortoise's dietary specialization for the day using the Specialization Scoring System (see below).

   In addition, record the particular resources that were consumed.
   i. If the tortoise ate 1 out of the 4 resources, it would get a non-weighted dietary specialization score of 1/4 (signifying of a specialist) for the day, and so on.
8. Record data (steps 4-7) for 30 days.
9. At the end of the 30 days, set up the tortoise under a 14 hour daylight cycle. Do this by setting the timer for the enclosure lights running from 6:00 AM to 8:00 PM.
10. Repeat steps 5-8 for a 14 hour daylight cycle.
11. At the end of the 30 days, set up the tortoise under a 7 hour daylight cycle. Do this by setting the timer for the enclosure lights running from 9:30 AM to 4:30 PM.
12. Repeat steps 5-8 for a 7 hour daylight cycle.

Equations

Equation for Dietary Specialization

$$\textit{Dietary Specialization} = \frac{\textit{Number of Food Resources Consumed}}{\textit{Number of Food Resources Available}}$$

$x > 0.625 \rightarrow$ ***Generalist***
$x < 0.625 \rightarrow$ ***Specialist***
$x = 0.625 \rightarrow$ ***Further Analysis Needed***

Data Tables

Figure 3

Captive *Testudo horsfieldii* Food Source Consumption Under 7 Hour Light Cycle

This table shows the dietary specialization of the tortoise over 30 days under a 7 hour daylight cycle. Each day, the tortoise was given a dietary specialization score. In addition, whether or not a food resource was consumed each day was recorded, where 1/1 signified consumption of the food resource and 0/1 signified no consumption. An average specialization score is presented at the bottom as well as the percentage of days each resource was consumed. With an average score value of 0.57, the tortoise was more of a specialist under this daylight cycle.

7 HOUR CYCLE						
Day	**Score** (Fraction)	**Score** (Decimal)	**Kale Eaten?**	**Collards Eaten?**	**Dandelions Eaten?**	**Arugula Eaten?**
1	3/4	0.75	1/1	0/1	1/1	1/1
2	2/4	0.5	1/1	0/1	1/1	0/1
3	2/4	0.5	1/1	0/1	1/1	0/1
4	1/4	0.25	0/1	0/1	1/1	0/1
5	2/4	0.5	1/1	0/1	1/1	0/1
6	2/4	0.5	1/1	0/1	1/1	0/1
7	2/4	0.5	1/1	0/1	1/1	0/1
8	3/4	0.75	1/1	1/1	1/1	0/1
9	3/4	0.75	1/1	1/1	1/1	0/1
10	2/4	0.5	1/1	0/1	1/1	0/1
11	3/4	0.75	1/1	1/1	1/1	0/1
12	2/4	0.5	1/1	0/1	1/1	0/1
13	3/4	0.75	1/1	1/1	1/1	0/1
14	4/4	1	1/1	1/1	1/1	1/1
15	2/4	0.5	1/1	0/1	1/1	0/1
16	2/4	0.5	1/1	0/1	1/1	0/1
17	2/4	0.5	1/1	0/1	1/1	0/1
18	2/4	0.5	1/1	0/1	1/1	0/1
19	1/4	0.25	0/1	0/1	1/1	0/1
20	2/4	0.5	0/1	1/1	1/1	0/1
21	2/4	0.5	1/1	0/1	1/1	0/1
22	3/4	0.75	1/1	0/1	1/1	1/1
23	2/4	0.5	1/1	0/1	1/1	0/1
24	2/4	0.5	1/1	0/1	1/1	0/1
25	2/4	0.5	1/1	0/1	1/1	0/1
26	3/4	0.75	1/1	1/1	1/1	0/1
27	3/4	0.75	1/1	1/1	1/1	0/1
28	2/4	0.5	0/1	0/1	1/1	1/1
29	3/4	0.75	1/1	0/1	1/1	1/1
30	1/4	0.25	0/1	0/1	1/1	0/1
Average		0.566	25/30 = 83.3%	8/30 = 26.7%	30/30 = 100%	5/30 = 16.7%

Figure 4

Captive *Testudo horsfieldii* Food Source Consumption Under 12 Hour Light Cycle

This table shows the dietary specialization of the tortoise over 30 days under a 12 hour daylight cycle. Each day, the tortoise was given a dietary specialization score. In addition, whether or not a food resource was consumed each day was recorded, where 1/1 signified consumption of the food resource and 0/1 signified no consumption. An average specialization score is presented at the bottom as well as the percentage of days each resource was consumed. With an average score value of 0.58, the tortoise was more of a specialist under this daylight cycle.

12 HOUR CYCLE						
Day	**Score** (Fraction)	**Score** (Decimal)	**Kale Eaten?**	**Collards Eaten?**	**Dandelions Eaten?**	**Arugula Eaten?**
1	3/4	0.75	1/1	1/1	1/1	0/1
2	2/4	0.5	1/1	0/1	1/1	0/1
3	2/4	0.5	1/1	0/1	1/1	0/1
4	2/4	0.5	1/1	0/1	1/1	0/1
5	3/4	0.75	1/1	1/1	1/1	0/1
6	2/4	0.5	1/1	0/1	1/1	0/1
7	2/4	0.5	1/1	0/1	1/1	0/1
8	3/4	0.75	1/1	0/1	1/1	1/1
9	2/4	0.5	1/1	0/1	1/1	0/1
10	3/4	0.75	1/1	0/1	1/1	1/1
11	3/4	0.75	1/1	0/1	1/1	1/1
12	3/4	0.75	1/1	0/1	1/1	1/1
13	2/4	0.5	1/1	0/1	1/1	0/1
14	2/4	0.5	1/1	0/1	1/1	0/1
15	3/4	0.75	1/1	0/1	1/1	1/1
16	3/4	0.75	1/1	0/1	1/1	1/1
17	2/4	0.5	1/1	0/1	1/1	1/1
18	2/4	0.5	1/1	0/1	1/1	1/1
19	2/4	0.5	1/1	0/1	1/1	1/1
20	2/4	0.5	1/1	0/1	1/1	1/1
21	2/4	0.5	1/1	0/1	1/1	1/1
22	1/4	0.25	1/1	0/1	1/1	1/1
23	2/4	0.5	1/1	0/1	1/1	1/1
24	3/4	0.75	1/1	1/1	1/1	0/1
25	2/4	0.5	1/1	0/1	0/1	1/1
26	2/4	0.5	1/1	0/1	1/1	1/1
27	2/4	0.5	1/1	0/1	1/1	1/1
28	2/4	0.5	1/1	0/1	1/1	1/1
29	3/4	0.75	1/1	0/1	1/1	1/1
30	2/4	0.5	1/1	0/1	1/1	1/1
Average		0.575	30/30 = 100%	3/30 = 10%	28/30 = 93.3%	8/30 = 26.7%

Figure 5

Captive *Testudo horsfieldii* Food Source Consumption Under 14 Hour Light Cycle

This table shows the dietary specialization of the tortoise over 30 days under a 14 hour daylight cycle. Each day, the tortoise was given a dietary specialization score. In addition, whether or not a food resource was consumed each day was recorded, where 1/1 signified consumption of the food resource and 0/1 signified no consumption. An average specialization score is presented at the bottom as well as the percentage of days each resource was consumed. With an average score value of 0.54, the tortoise was more of a specialist under this daylight cycle.

14 HOUR CYCLE						
Day	**Score** (Fraction)	**Score** (Decimal)	**Kale Eaten?**	**Collards Eaten?**	**Dandelions Eaten?**	**Arugula Eaten?**
1	3/4	0.75	1/1	0/1	1/1	1/1
2	3/4	0.75	1/1	0/1	1/1	1/1
3	2/4	0.5	1/1	0/1	1/1	0/1
4	2/4	0.5	1/1	0/1	1/1	0/1
5	2/4	0.5	1/1	0/1	1/1	0/1
6	2/4	0.5	1/1	0/1	1/1	0/1
7	3/4	0.75	1/1	0/1	1/1	1/1
8	2/4	0.5	1/1	0/1	1/1	0/1
9	3/4	0.75	1/1	0/1	1/1	1/1
10	2/4	0.5	1/1	0/1	1/1	0/1
11	2/4	0.5	1/1	0/1	1/1	0/1
12	2/4	0.5	1/1	0/1	1/1	0/1
13	2/4	0.5	1/1	0/1	1/1	0/1
14	2/4	0.5	1/1	0/1	1/1	0/1
15	2/4	0.5	1/1	0/1	1/1	0/1
16	2/4	0.5	1/1	0/1	1/1	0/1
17	2/4	0.5	1/1	0/1	1/1	0/1
18	2/4	0.5	1/1	0/1	1/1	0/1
19	2/4	0.5	1/1	0/1	1/1	0/1
20	2/4	0.5	1/1	0/1	1/1	0/1
21	2/4	0.5	1/1	0/1	1/1	0/1
22	3/4	0.75	1/1	0/1	1/1	1/1
23	2/4	0.5	1/1	0/1	1/1	0/1
24	2/4	0.5	1/1	0/1	1/1	0/1
25	2/4	0.5	1/1	0/1	1/1	0/1
26	2/4	0.5	1/1	0/1	1/1	0/1
27	1/4	0.25	1/1	0/1	0/1	0/1
28	2/4	0.5	1/1	0/1	1/1	0/1
29	2/4	0.5	1/1	1/1	0/1	0/1
30	3/4	0.75	1/1	0/1	1/1	1/1
Average		0.541	30/30 = 100%	1/30 = 3.3%	28/30 = 93.3%	6/30 = 20%

Graphs

Figure 6

Dietary Specialization Under a 7 Hour, 12 Hour and 14 Hour Daylight Cycle

This graph shows the tortoise's average dietary specialization scores across the three daylight cycles. Under all three light cycles, the tortoise received a value under 0.625, which is representative of a specialist. Error bars of the SEM show that there was no significant difference in the tortoise's dietary specialization under the three day light cycles.

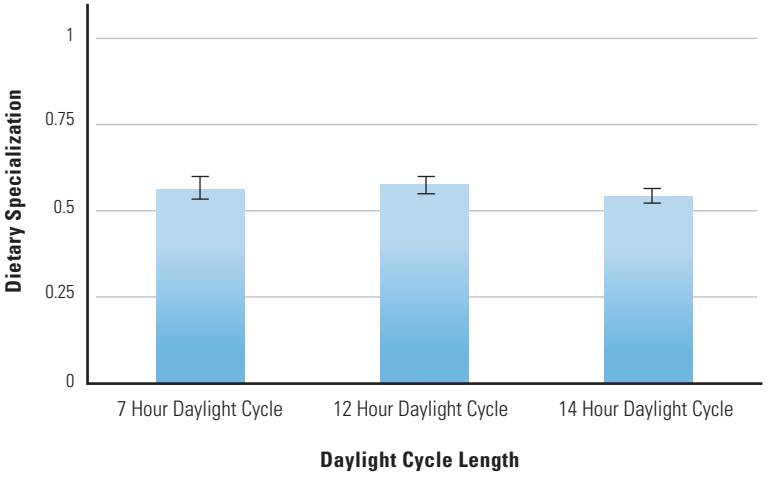
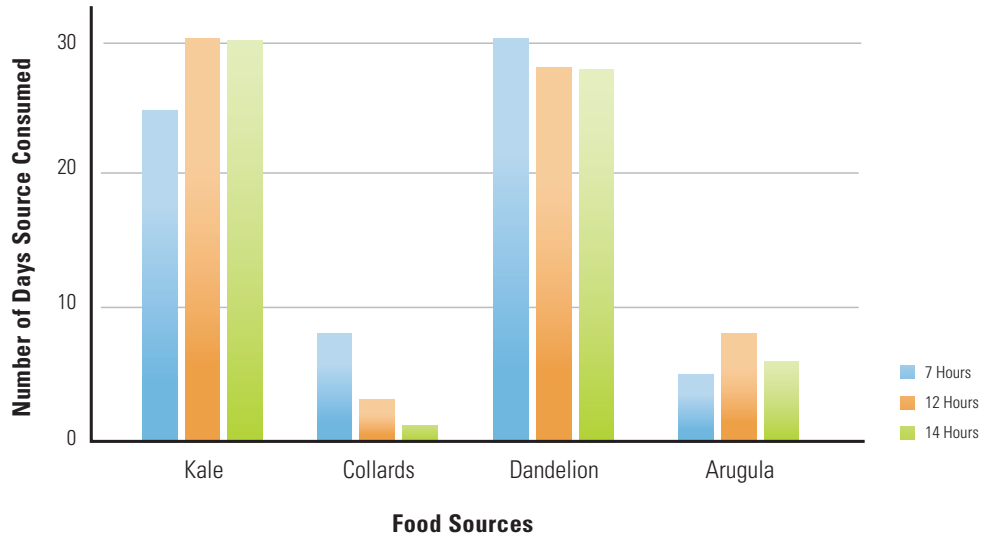


Figure 7

Captive *Testudo horsfieldii* Food Source Consumption Under Light Cycles of Varying Length

This graph shows the tortoise's consumption of each individual food resource across the three daylight cycles. The tortoise in general stayed to a similar dietary pattern regardless of daylight cycle, where consuming kale and dandelion often while consuming collards and arugula rarely



Results of t-test

Value after a two tailed independent t-test comparing 12 to 14: 0.305510
0.305510 > 0.05 → accept H0

Value after a two tailed independent t-test comparing 7 to 12: 0.834404
0.834404 > 0.05 → accept H0

Value after a two tailed independent t-test comparing 7 to 14: 0.511866
0.511866 > 0.05 → accept H0

Data Analysis

Looking at Data Tables #1, #2 and #3, we can see how *Testudo horsfieldii,* across the board, was a specialist. The tortoise received an average specialization score of 0.567 under a 7 hour daylight cycle, an average specialization score of 0.575 under a 12 hour daylight cycle, and an average specialization score of 0.542 under a 14 hour daylight cycle. In other words, under all three light cycles, the tortoise received a specialization score of less than 0.625, therefore signaling a dietary specialist.

While data initially suggests that the tortoise was overall more of a specialist, if we look deeper into the data we can see how there were some signs of generalist behaviour under the 7 hour daylight cycle. If we look at Graph #2, we can see how under the 12 hour and 14 hour daylight cycles, the tortoise ate its food at nearly the same ratio and at a ratio as an obvious specialist; it ate the kale and dandelions most often while it ate the arugula and collards least often. But as we look closer into the 7 hour daylight cycle group, we can see that despite having a more specialized dietary score than the 12 hour group, the tortoise seemed to eat the most balanced diet under the 7 hour daylight cycle. The tortoise's balanced diet here can be seen in how the tortoise ate each resource at least 15% of the days under the 7 hour cycle. This more balanced approach suggests that the shorter daylight cycle did lead to the tortoise being more of a generalist, although the data didn't reflect such because it measured on a day by day basis (if the tortoise ate 2/4 resources one day and the other 2/4 resources the next day, it would still get an average score of 2/4 despite consuming all 4 food resources).

I ran three two-tailed independent t-tests with my data in order to get threes p-value to determine if there was a significant difference in my data. The p-values I received after running a t-test between the three groups were 0.834404 (7 hours & 12 hours), 0.511866 (7 hours & 14 hours) and 0.305510 (12 hours & 14 hours). All three values are greater than 0.05, showing no significant difference in my data. This leads to me accept to my null hypothesis: there is no difference in captive Russian Tortoise *(Testudo horsfieldii)* dietary specialization when receiving longer daylight cycles versus shorter daylight cycles. The null hypothesis can also be further supported after looking at Figure #6, as the error bars, a representation of +/- Standard Error Mean (SEM), are all overlapping. This data does not support my initial hypothesis that there would be some type of difference due to the differing daylight cycles altering the circadian rhythms of the tortoise.

While the study did a good job controlling external variables, some uncertainty may have entered. The three main places where experimental uncertainty may have occurred were food resources not being presented immediately when daylight cycles began, my small sample size, and the tortoise's previous familiarity with some of the food resources.

Ideally, (so that the tortoise would receive access to its food resources for the entirety of the daylight cycle) I would give the tortoise its food resources the moment its lights turned on. This did not end up happening as food was usually presented within the first 15 minutes of the daylight turning on,

as presenting food immediately when the lights turned on proved difficult due to other morning conflicts. This could have caused some uncertainty in the results as the tortoise might have behaved differently when consistently provided food immediately when the daylight cycle, and circadian rhythms, kicked in.

Another potential error was my sample size. Different tortoises in a population can have different dietary preferences (Van Valen, 1965), so the dietary preferences of my tortoise might not be representative of a tortoise population. This is something that I could have controlled better had I increased my sample size ($n = 1$ for my study), but this was unreasonable due to constraints such as the fact that I did not have the facilities to accommodate more than one tortoise during the time of the study. While increasing sample size is usually just a good next step in scientific studies to further validify significance, the lack of a large sample size in my study could have resulted in a high level of uncertainty as there might be discrepancies between my test tortoise's specific dietary preferences and captive *Testudo horsfieldii* dietary preferences as a whole.

Finally, uncertainty may have been introduced because of previous familiarity between the tortoise and some of its food resources. In the pilot study I ran last year with the same tortoise, I used two of the same resources that I used in this study: kale and dandelions. Previous exposure to these two resources could have introduced a potential bias by the tortoise to prefer or not prefer (prefer in this case as the lowest percentage of either of those resources eaten in any of the groups was 83.3%) those two resources more so than the other less familiar food resources (arugula and collards). This could have caused uncertainty in the data because this bias would have affected the tortoise's dietary specialization more than the daylight cycles.

Conclusion/Discussion

As mentioned previously, we can conclude from the data that there was no difference in captive Steppe Tortoise *(Testudo horsfieldii)* dietary specialization when receiving a longer daylight cycle versus a shorter daylight cycle. Therefore, one of two possible conclusions can be made, both of which would require more research to be done. The first potential conclusion is that despite the differing daylight cycles, the tortoise's circadian rhythms remained unchanged, leading to no significant change in dietary behavior. This could mean that the study didn't display a large enough difference in daylight cycles to change the tortoise's circadian rhythms; increasing the difference in daylight cycles would be next step with this research.

On the other hand, the other possible conclusion is that the tortoise's circadian rhythms did change when in the different groups, but didn't significantly affect the tortoise's dietary behavior. This could mean that tortoise circadian rhythms and tortoise dietary specialization aren't linked. However, if this conclusion were the case, we would first have to look into the food presentation conditioning effect. Food presentation conditioning is the idea that since food resources were presented at around the same time as the lights turned on, presentation of the food resources could have conditioned and altered the tortoises circadian rhythms to eat when food was first presented. To check for food presentation conditioning, another future step would be to alter the time food sources were presented to see if the tortoise's circadian rhythms change because of the lights turning on and off or because of the presentation of food.

A way to check which conclusion is the most appropriate to accept would be to take note of when the tortoise first consumed food resources relative to the lights turning on. If the tortoise consumed food resources at around the first time every day, we could say that the tortoise's circadian

rhythms were not changing, which would lead us back to the first potential conclusion. If the tortoise first consumed food resources at different times every day, we could say that the tortoise's circadian rhythms were changing, however it had no effect on the tortoise's dietary specialization, which would lead us back to the second potential conclusion. It's also important to remember that both of these conclusions (except the part on food presentation conditioning) are pertinent on future data also supporting the null hypothesis.

Another important factor in this study was the role of captivity. All of the literature I read previously had conducted tortoise dietary research with wild tortoises [Jennings WB, Berry KH, 2015] [Murray IW, Wolf BO, 2013]. In my study, I used captive *Testudo horsfieldii.* The role of specialization, and behaviour as a whole, could differ between wild and captive specimens due to differing ecological stresses, such as predation, intra-species competition, precipitation and seasons [Ndaimani et al, 2016] [Murray IW, Wolf BO, 2013] [Lagarde et al 2003] [Jennings WB, Berry KH, 2015]. In addition, wild *Testudo horsfieldii* forage and eat for around three months a year, where they consume high amounts of food to meet the energy requirements to reproduce, grow and maintain homeostasis, before hibernating for 9 months [Ataev 1997, Lagarde et al. 1999], where captives do not (unless stimulated similarly to their natural environment to do such). This lack of behaviour could totally change how captives approach their dietary specialization. Future work with both captive and wild *Testudo horsfieldii* would provide better insight into the role of captivity on specialization.

In addition, it's important to consider species in this study. Most of the literature I have read thus far has concerned *Gopherus agassizii,* another species of tortoise distributed across desert regions in North America [Murray IW, Wolf BO, 2013]. The role of specialization could differ between species due to different species-specific physiological needs. However it was found that *Gopherus agassizii* has similar foraging and dietary behaviours to *Testudo horsfieldii*, despite different ecological stresses (Such as *Gopherus agassizii* not undergoing the hibernation that *Testudo horsfieldii* does) [Lagarde et al 2003]. In addition to *Gopherus agassizii*, multiple other tortoise species have been to be found to have similar dietary behaviors to *Testudo horsfieldii* (McDonald, Mushinsky 1988, Mason et al. 1999, Bjorndal 1989, Moskovits, Bjorndal 1990). Future research with differing Chelonian species would bring better insight into trends in tortoise dietary specialization, however continued research with *Testudo horsfieldii* may in itself help provide more insight into the dietary behavior of other similar Chelonian species.

One variable I would like to manipulate if I were to replicate this study again would be food sources. I chose to use all green resources as in a preliminary study I carried out last year, and the tortoise vastly prefered the two green resources available (dandelions and kale). In addition, I chose these resources off of suggested and common green resources given to captive *Testudo horsfieldii* [Heinen 2000]. Presenting the tortoise with food resources from its natural environment might trigger different dietary behaviors and specializations. In addition to type of food resource, amount of food resource would be something to look at Dietary preference in in *Testudo horsfieldii* has been found be be closely linked to food availability [McDonald, Mushinsky 1988] [Moskovits, Bjorndal 1990] [Mason et al. 1999]. Increasing the amount of resources wouldn't just increase the potential range of results, but also might show a difference in *Testudo horsfieldii* specialization.

Another variable I would like to manipulate if I were to replicate this study again would be the variable of the spatial range. The tortoise was kept in a 37" x 25" x 13" enclosure, which is close to the minimum for that species of tortoise. In a future study, increasing the enclosure size would be a priority. More space would lead to the tortoise moving more and therefore using up more energy. This could change the tortoise's physiological needs, leading to a change in dietary behaviour. In addition, by increasing the tortoise's spatial range, I could change the the placement of the food resources presented. In this study, all four resources were presented together. Optimal foraging theory says that animals

should minimize costs and maximize intake when foraging [Pyke, 1984]. Conversely, wild *Testudo horsfieldii* have been found to spend surprisingly very little time foraging [Lagarde et al 2003]. Changing the placement of different resources could potentially deter or attract the tortoise to eat food resources and therefore affect how it specializes.

Wild *Testudo horsfieldii* dietary behaviour has been closely associated with seasonal changes [McDonald, Mushinsky 1988] [Moskovits, Bjorndal 1990] [Mason et al. 1999]. One of the largest parts of seasonal changes is the shortening or elongation of daylight cycles. A study using daylight cycles to mimic seasons and the change of seasons might show a change of dietary behaviour in *Testudo horsfieldii*.

Overall, this study should continue to build the behavioural profile for *Testudo horsfieldii*. With this species being so common as household captives and abundant as wild specimens, future research should be done to continue documentation and improve knowledge of this small desert tortoise.

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How Does rRNA Expression Differ in Tissue Sample Treated with an mTOR Activator, CX-5461, a mTOR Activator and CX-5461, or a Control Solution?

by Katrina Fuller

Abstract

In the present study, I aimed to distinguish whether rRNA expression differed in tissue samples treated with an mTOR activator, CX-5461, an mTOR activator and CX-5461, or a control solution. To do so, I would isolate rRNA, generate complementary DNA via reverse transcription, and conduct SYBR® Green RT-PCR. However, due to time-constraints, this question was left unanswered. Instead, I sought to simulate varying levels of rRNA through creating a dilution series of known DNA [1/100, 1/10,000, and no template control (NTC)] and quantifying them using the *Bio-Rad* RT-PCR CSI Kit. To measure this DNA, dilutions were created and added to two PCR tube strips. Template DNA was added to the pair of corresponding PCR tubes including the NTC. IQ SYBR® Green supermix and the CSI kit primers were mixed and added to each of the 8 PCR reactions. The thermal cycler was programmed to amplify the detected PCR until the reaction ran out of reagents. Results showed that the 1/100 dilution displayed a curve that amplified after 10 cycles, that the 1/10,000 dilution displayed a curve that amplified after 18 cycles, and that the NTC dilution displayed a curve that amplified after 40 cycles, giving me a sense of what I would have seen with rRNA expression.

Background

Alzheimer's disease (AD) is a neurodegenerative disorder defined by neuropathological lesions of plaques and tangles and associated with progressive memory loss and cognitive impairment. A number of studies suggest an altered protein synthesis machinery in AD: total protein synthesis (P. Bustany, 1982), (N. S. Langstrom, 1989), number of ribosomes, and ribosomal translation efficiency are reduced (Q. Ding, 2005). Furthermore, the site of ribosomal biogenesis (the nucleolus) is compromised in AD: The nucleolus is smaller (D. Iacono, 2008, 2009), nucleolar protein content is altered (J. Zeng, 2016), (K. Hernández-Ortega, 2015), and the ribosomal RNA (rRNA) genes are epigenetically silenced (M. Pietrzak, 2011). While these studies provide evidence that the protein synthesis machinery progressively declines in AD, the mechanism by which these alterations impact cognitive decline is still being worked out.

Protein synthesis has been well studied in synaptic plasticity and learning and memory, but most research focuses on gene expression of protein-encoding genes. Many studies focus on the transcription, processing, and translation of messenger RNA (mRNA), excluding other factors of gene expression

that are necessary for protein synthesis, such as ribosomal RNA (rRNA). Ribosomal RNA is the RNA component of the ribosome, which synthesizes proteins by translating a (mRNA) copy of the DNA template in the cell nucleus. Because rRNA is essential for protein synthesis, its decreased biogenesis and the shrinking of the nucleolus (the rRNA site of production) in AD may indicate that an impairment in rRNA production machinery is a factor in memory loss. Recently, it was discovered that new ribosome biosynthesis is a key process for synaptic mechanisms correlated with forming new memories (K. D. Allen, 2014). Therefore, it is likely that when memory is impaired, some mechanism of new rRNA production is impaired as well. It is possible that chemically activating protein synthesis rRNA production in AD brains may alleviate some of the memory loss, and allow the brain to produce more new ribosomes.

To begin to explore these hypotheses, researchers at SUNY Downstate tested whether an increase in the ribosome biogenesis pathway is important for memory formation by using a mTOR activator to induce rRNA and protein synthesis *in vivo* (preliminary data) (Dr. Kim Allen and Dr. Ivan Hernández, personal communication, 2018). mTOR is a kinase that regulates metabolic processes – one being autophagy, which is critical for the maintenance of cellular homeostasis. Autophagy allows a cell to send degraded proteins to lysosomes, which recycle them into new cellular components and provide the cells with nutrients. During cellular stress, the process of autophagy is upscaled, which is why dysfunction of mTOR is a factor of AD. When autophagy becomes dysregulated, it breaks down the beta-amyloid polypeptide precursor protein too much, causing it to aggregate and form the plaques and neurofibrillary tangles characteristic of AD. These neurons then waste away and cell communication is inhibited. However, this uncontrolled autophagy can be suppressed by the activation of mTOR (D, G., 2014). This mTOR activator also lowers levels of overproduced beta-amyloid and aliens the cognitive deficits common as a result of AD (L, Wei., 2012).

Researchers at SUNY Downstate are measuring whether the effect of this mTOR activator requires polymerase 1 to enhance memory and increase ribosome production (Dr. Kim Allen and Dr. Ivan Hernández, personal communication, 2018). Polymerase 1 is an enzyme that catalyzes the transcription of DNA to synthesize precursors of rRNA. Polymerase 2 transcribes mRNA. If this mTOR activator is able to enhance memory when polymerase 1 is blocked, it will be clear that it does not require rRNA synthesis to increase memory and mRNA may play a larger role in memory enhancement than previously thought.

In order to test this, researchers used CX-5461, a drug that inhibits polymerase 1, which is responsible for the transcription of new rRNA. Interference with ribosomal synthesis would show up in animal behavioral studies.

Four groups of mice were trained in the Active Place Avoidance Task, a fear-inducing test used to evaluate memory retention. In this test, subjects learned to avoid an environment in which a foot-shock was previously delivered. To serve as a baseline, group 1 was treated with two saline control solutions that have no therapeutic action. Group 2 was treated with the same saline control solution and the mTOR activator, group 3 was treated with the saline solution and CX-5461, and group 4 was treated with the mTOR activator and CX-5461.

Before this *in vivo* data could be interpreted, it would need to be confirmed at the molecular level whether CX-5461 had in fact blocked polymerase 1 and whether the mTOR activator had enhanced rRNA transcription. RNA would be isolated from the samples, primers would be designed and reverse transcription would be conducted via polymerase chain reaction (PCR) to make complementary DNA (cDNA) that only reflected the genes expressed. cDNA is more stable and easier to work with than RNA, which is susceptible to degradation by RNAses (enzymes that break down RNA). By designing

primers specific to different types of RNA, the specific RNA genes activated by learning as well as the efficiency of the mTOR activator as an rRNA transcription-enhancer would be measured.

Due to time constraints, I was unable to measure rRNA expression with RT-PCR. Instead, I tested whether using the *Bio-Rad* RT-PCR CSI Kit could generate results that would be similar to the expected results of the quantified rRNA expression.

Conventional PCR is able to measure exactly what DNA is present. It involves both forward and reverse primers surrounding the target region, nucleotides, and a DNA polymerase. This approach is often used for genomic and gene expression analysis, diagnosing genetic diseases and detecting contaminants in environmental samples (Bustin, 2004). Through the use of a reverse transcription reaction, conventional PCR has also become useful for the detection of RNA targets, useful for gene expression analysis and diagnosis.

PCR that is used to measure what DNA is present as well as how much is known as real-time PCR (RT-PCR). It is based on the same principles as conventional PCR but it allows for the amplified product to be measured as the reaction progresses. RT-PCR also uses fluorescence chemistry, which allows product amplification to be detected for the entire time, using Bio-Rad thermal cyclers with fluorescence detection instruments. Using the primers, the SYBR® Green dye would bind to the double-stranded DNA produced by the PCR. The fluorescence measured equals the amount of product. Because the amount of DNA or RNA after each cycle multiplies exponentially in proportion to the starting amount of template in the sample, RT-PCR allows you to determine how much material is initially present. In a potential experiment, this technique could be applied to measure how memory-enhancing drugs affect rRNA expression – where changes in rRNA expression would be measured against a gene that does not undergo changes in transcription, also known as "housekeeping genes".

Materials

- CSI Kit: Suspect DNA sample, Crime Scene DNA sample
- iQ SYBR Green® Supermix
- PCR-grade water
- PCR tube strips
- Optical flat caps
- RT-PCR Instrument
- Vortex device

Methods

A series of dilutions were created to represent different levels of initial rRNA. First, 990 µl of water was added to each of three 1.5 ml microfuge tubes. For the 1/100 dilution, I added 10 µl of concentrated Suspect DNA to the first tube. For the 1/10,000 dilution, I vortexed the tube, then transferred 10 µl of diluted Crime Scene DNA from the first tube to the second tube. The third tube had no template DNA added and served as the No Template Control (NTC). An eight-well PCR tube strip was used to prepare the duplicate PCR reactions. Next, 12.5 µl of template DNA from each tube of the dilution series was added to the pair of corresponding PCR tubes including a sample with undiluted template DNA. To prepare the PCR Master Mix, 110 µl of iQ SYBR® Green supermix was pipetted to a new 1.5 ml microtube. Then, 2.2 µl of the Crime Scene Investigator kit primers was added to the tube and vortexed. Finally, 12.5 µl of the mastermix was added to each of the 8 PCR reactions.

Figure 1

Plate Setup

A (crime scene DNA sample); B (suspect DNA sample); NTC (no template control)

	A	B	C	D	E	F
1	A	A	B	B	NTC	NTC
2	A	A	B	B	NTC	NTC

Before it was run, the RT-PCR thermal cycler was programmed with the following protocol:

Cycle 1: 94ºC for 2 min: initial denaturation of DNA

Cycle 2: 94ºC for 30 seconds: denaturation
52ºC for 30 seconds: annealing
72ºC for 1 minute: extension – collect data after this step

Repeat Cycle 2 for 40 cycles

Instrument programmed to heat samples from 65ºC (for 10 seconds) to 95ºC (for 5 seconds) and have the instrument collect data after each incremental step for melt-curve analysis.

Figure 2

Thermal Cycler Program

With every cycle of the reaction, the amount of DNA increased exponentially along with the fluorescence. DNA was detected by the PCR machine until the reaction ran out of reagents.

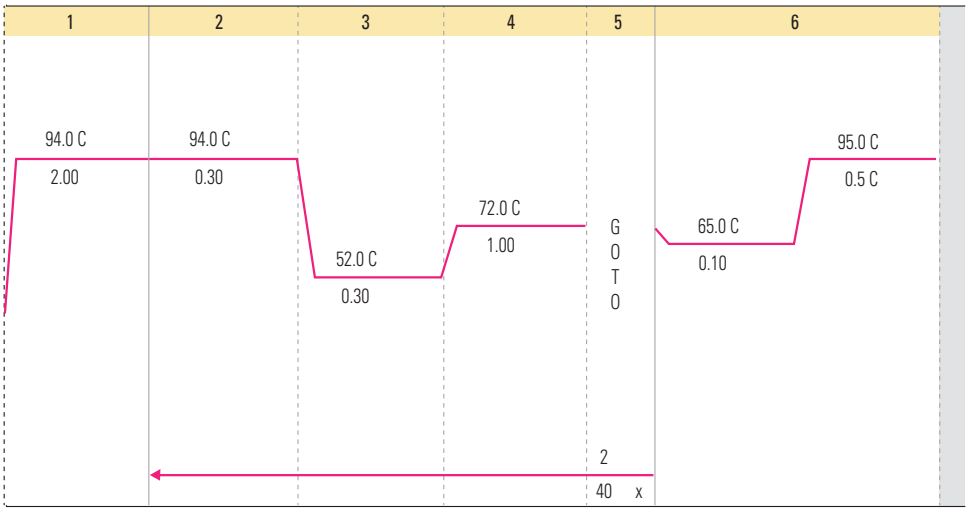


Figure 3a

Amplification Plot

This graph shows relative fluorescence units (RFU) in relation to number of cycles. Curve 1 = 1/100 dilution, Curve 2 = 1/10,000 dilution, Curve 3 = NTC.

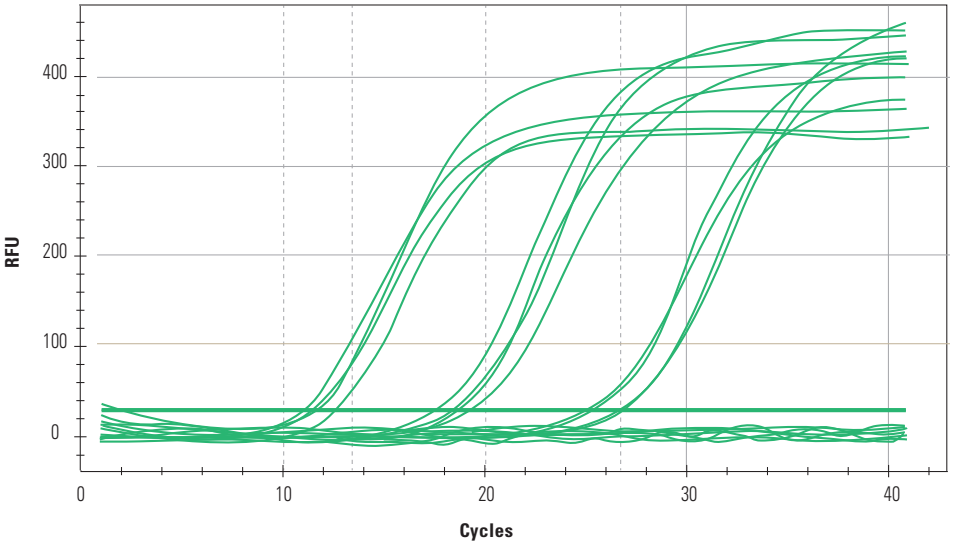


Figure 3b

Melt Curve of Reaction Product

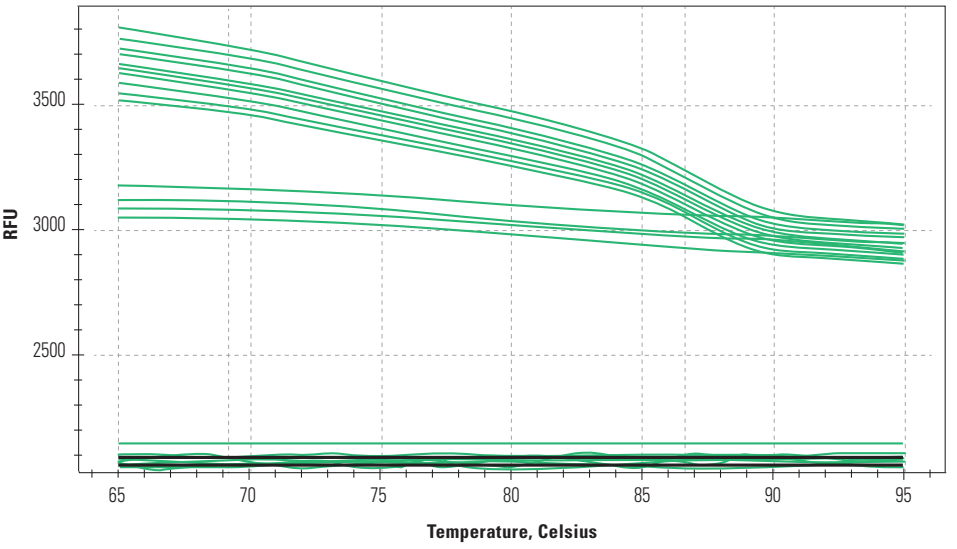
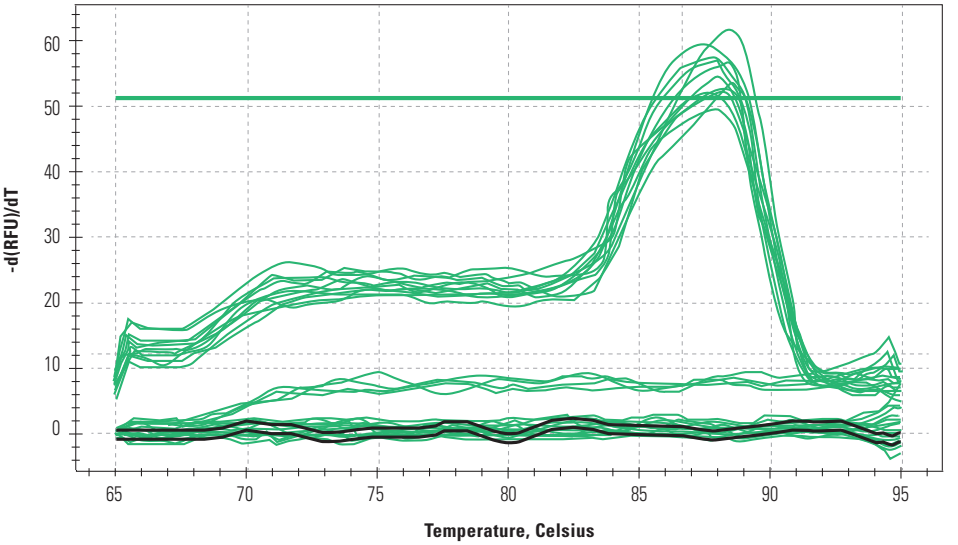


Figure 3c

Melt Peak Data



Data Analysis

Figure 3a shows what the fluorescence of the PCR reaction looks like, measured in real time as the CSI PCR reactions are amplified. The total fluorescence is on the y axis and the number of cycles is on the x axis. Figure 3a shows three curves: the curve that begins to amplify at significant levels after cycle 10 has the 1/100 dilution, the curve that began to rise at 18 has the 1/10,000 dilution, and the final curve is the NTC. The curve representing the 1/100 dilution entered the plateau phase by cycle 24 and the curve representing the 1/10,000 dilution plateaued at cycle 31. The NTC samples showed amplification as late as cycle 40 because these products were PCR primer-dimers, which result from the primers themselves serving as DNA templates. The plateau phase indicates that the PCR process had finished because there was no more possible amplification. This graph shows how samples with higher concentrations of initial DNA have curves that rise earlier. These curves cross the threshold cycle (CT value), which tells us the starting quantity of template DNA. Figure 3b shows the temperature at which the double-stranded DNA dissociates into the single-stranded DNA during the denaturation phase. This process releases SYBR Green, resulting in a decrease in fluorescence. The total fluorescence is on the y axis and the temperature in Celsius is on the x axis. The 1/100 dilution is represented by the curve that begins at values greater than 3,500 RFU that decrease to about 3,000. The 10,000 dilution is represented by the curve that begins at values greater than 3,200 RFU that decreases to about 3,000, and the NTC sample is represented by the line along the x axis. Figure 3c shows that the curve representing the 1/100 dilution reaches a primer dimer peak at 60 RFU, the 1/10,000 dilution is represented by the line that reaches 10 RFU, and the NTC sample is represented by the line along the x axis.

Conclusion

From observing that the 1/100 dilution displayed a curve that amplified after 10 cycles, that the 1/10,000 dilution displayed a curve that amplified after 18 cycles, and that the NTC dilution displayed a curve that amplified after 40 cycles, I was able to get a sense of the kind of curves I would have seen with varying rRNA levels. I would have expected that the control group treated with two saline solutions would express mediocre memory retention and low rRNA production because three rounds of

training are typically required for memory consolidation. I would have expected that the group treated with the mTOR activator and a vehicle would show enhanced memory and increased rRNA production. And I would have expected that the group treated with CX-5461 and a vehicle would show a decrease in memory and rRNA production. If indeed the mTOR activator required polymerase 1 to function, the group treated with the mTOR activator and CX-5461 would have shown suppressed or no memory and relatively low rRNA production. These values would appear as different levels of rRNA, which – as Figure 3a and 3b demonstrate – would appear as curves that correspond to the different amounts present. If the expected results are statistically significant, it will be clear that the effect of this mTOR activator requires polymerase 1 to enhance memory and increase ribosome production. This would mean that this mTOR activator does not require rRNA synthesis to increase memory and mRNA may play a larger role in memory enhancement than previously thought, and may one day be used to activate mTOR and rRNA production, and alleviate the devastating cognitive deficits present in Alzheimer's Disease.

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The Effect of Methylation on Growth Rates in Plants

by Julia Harrison

Abstract

Phenotypic difference in plants is determined not only by genetic means but also by the epigenome. DNA methylation, an epigenetic process, is one of the ways in which the phenotype may be altered. There are some drugs which target the epigenome, specifically methylation. One of these drugs is 5-Aza-2'-deoxycytidine (decitabine). Decitabine has been linked to hypomethylation (Bender, 1998). The aim of this study is to find the phenotypic response in *Brassica rapa* to exposure to decitabine at early developmental stages. Along with phenotypic response, we examined survival rates and different responses between *Brassica rapa* varieties. We exposed three different types of *Brassica rapa* plants to a decitabine solution while they germinated, and measured heights and observed appearances of the plants. We found significant differences between growth rates for all three types of the plant at some point in their growth cycle; the untreated plants were always taller. In addition, we found a higher survival rate among the untreated plants, and different physical traits between the three species. We can conclude that the decitabine had an effect on the phenotype of these plants. In order to determine that the phenotypic response was in fact due to a change in methylation, rather than unrelated toxicity of decitabine, we did a complementary study on *Arabidopsis thaliana* plants with naturally occurring difference in phenotype. We extracted DNA from each variety of these plants to determine if the natural variation was due to methylation. Unfortunately, our results in this study were inconclusive.

Keywords

• Epigenetics • DNA methylation • 5-Aza-2'-deoxycytidine • *Brassica rapa*
• Phenotypic plasticity • *Arabidopsis thaliana*

Introduction

One of our fundamental understandings of biology is that heritable phenotypic traits allow for variation among species, and that the only way for traits to be transferred through generations is genetic. This process is crucial to the ability of species to adapt and change. We have come to understand that there is another way for phenotypic traits to be coded within our DNA – epigenetics.

Epigenetics is a relatively new field under the umbrella of biology. The term "epigenetic" was only coined in 1942; it refers to mechanisms within cells to silence or promote genes. These mechanisms include DNA methylation, RNA interference, and histone modification (Bossdorf, 2010). One of the key and most-studied processes of epigenetic modification is the addition of a methyl group to the proteins, histones, that DNA is wound around (Bird and Wolffe, 1999). This is called DNA methylation. Methylation tightens the DNA around the histones, so they can't be accessed by RNA polymerase –

genes which are methylated can no longer be transcribed, effectively silencing them. Methylation in eukaryotes happens almost exclusively on cytosine 5' to guanine, known as CpG dinucleotides. Areas on the genome rich in CpG dinucleotides are called CpG islands and they are prone to epigenetic modification (Herman, 1996). Collectively, the set of epigenetic modifications in an organism is referred to as the epigenome, in much the same was as the set of genetic material in an organism is called the genome.

It is evident that the epigenome plays a significant role in the natural lives of organisms. Epigenetic variation, unlike genetic variation, is changeable within a single individual through any of these processes. This allows for rapid adaptation to environments, a characteristic which is particularly important to the survival of plants. As they are sedentary, it is crucial that they are able to tolerate and adapt to stressors (Boyko and Kovulchuk, 2008). Plants which have been exposed to a stressor may be more resistant in the future due to methylation as well as other epigenetic functions (Bruce *et al.,* 2007). Plants with identical genomes have been shown to exhibit different phenotypes based on the conditions they were grown in (Raj *et al.,* 2011). Two populations of the same species of plant, grown in different conditions, will exhibit vastly different phenotypes across all individuals in the population (Lira-Medeiros, *et al.,* 2010). Epigenetic variation is more heritable in plants than animals, via both sexual and asexual reproduction, as in plants, epigenetic markers are not systematically erased in embryogenesis (Smulders *et al.,* 2011).

Epigenetic modifications play a role in plants' life cycle. There is evidence that mutations in the epigenome can delay growth or progression onto the next stage of life. It has been shown that in *Arabidopsis,* histone methylation plays a key role in flowering time. (Bastow *et al.,* 2004). Significantly more histone methylation was found on the plants which flowered on time as compared to the late flowering plants. This is an example of naturally occurring hypermethylation.

As part of the continuing research in the developing field of epigenetics, it is important to distinguish between the genome and the epigenome and identify ways of modifying the epigenome. One such way is to induce de-methylation on the genome. Use of certain drugs to alter the methylation has been found to create phenotypic changes. Recent research has shown that with the use of the drug 5-azacytidine, which has been found to alter gene expression (Jones, 1985), it is possible to hypomethylate *Arabidopsis* plants, change their phenotype, and delay growth (Bossdorf, 2010).

The purpose of this study is to test the efficacy of a different drug in hypomethylating plants. 5-Aza-2'-deoxycytidine (decitabine) has also been found to be a demethylating agent (Christman, 2002). Decitabine has been used in low doses as a cancer drug, reversing *de novo* methylation and controlling unchecked growth (Bender, 1998).

Here, we manipulated the epigenome of a set of three different species of *Brassica rapa* plants and observed changes in physical appearance as well as growth cycle. We exposed the plants to a decitabine solution in the germination stage of seed development. Our inquiry is based on these tquestions: Does exposure to decitabine alter phenotype? Does exposure to decitabine alter mortality rate in *Brassica rapa?* Do different species of *Brassica rapa* respond differently to exposure to decitabine?

The subsequent study on *Arabidopsis thaliana* was intended to verify that the phenotypic response was due to methylation on the plant. One of the primary findings of our first study was that growth was delayed by the demethylating agent. We chose to test flowering time, a pivotal point in the growth cycle of most plants, as it relates to methylation in our second study. *Arabidopsis,* known commonly as thale cress, is a small plant which normally flowers a few weeks after it is planted. *Arabidopsis* and *Brassica rapa* are part of the same family, *Brassicaceae,* which is why we found it appropriate to complete the study on *Arabidopsis.* The *FWA* gene in the plant encodes for a transcrip-

tion factor, which controls flowering. The repression of this gene allows for development in the plant. Without methylation, the plant would flower two to three weeks late. We acquired two different varieties, one known for its late flowering, and tested methylation on both. Our study follows this question: Does hypomethylation induce late flowering in *Arabidopsis?*

Plant Material

We obtained three variations of *Brassica rapa* fastplants. The first, Standard (S) is bred to have a short lifecycle, but no other variations. All plants are less than a foot tall, with yellow flowers. The second, Purple Stem, Hairy (PSH) expresses the purple pigment, *anthocyanin,* strongly, and has additional *trichome,* or hairs. The third, AstroPlant (AP), is more compact and shorter. The supplier does not provide information on how the plants differ genetically or epigenetically.

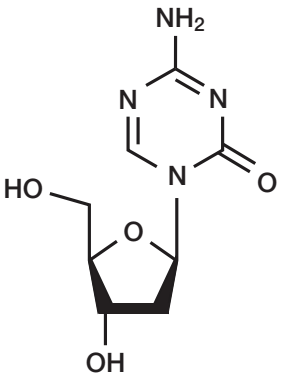
We used two varieties of the model plant *Arabidopsis thaliana*. One is the wild type, Landsberg *erecta* (Ler-0) and the other is a mutant that exhibits delayed growth and flowering. The mutation is thought to be on the *flowering wageningen* (FWA) gene, and therefore in this paper the mutant variation will be referred to as *fwa-1*.

Drug

The 5-Aza-2'-deoxycytidine is a modifier which inhibits methyltransferase activity. DNA methyltransferases are a group of enzymes which bind methyl, donated from S-adenosylmethionine, to the 5'-carbon base of cytosine, consequently preventing transcription and expression of the gene. The addition of decitabine reduces methylation on the genome and allows RNA transcription to take place, activating the genes that it targets. It has been used as a cancer drug in an effort to hypomethylate genes which restrict growth. The sample we used was ≥97% pure, and water soluble.

Figure 1

Chemical Structure of 5-Aza 2'-deoxycytidine ("5-Aza-2 -Deoxycytidine", n.d.)



Methods

We germinated each variety soaked either in water or a demethylating agent. To do this, we created a 50 μM solution of decitabine. We split twenty seeds from each variation equally into two petri dishes between two pieces of filter paper. One petri dish we soaked with 1.4 mL of water, and the other we soaked with 1.4 mL deoxycytidine solution. Thus, there were six groups (S-, S+, PSH-, PSH+, AP-, AP+; + denotes the addition of the decitabine solution).

The seeds were germinated in these conditions in complete darkness for 74 hours. After this period of time, measurements of growth were taken and the plants were potted and placed under 24-hour fluorescent light, less than a foot away from the light source.

The plants were watered every other day, and height measurements were taken and photographic data collected. Survival rates were determined based on the number of plants exhibiting life. In the study, days are defined as "days since seeds were first exposed to the drug or control", and additional measurements were taken on the day the seeds were transferred from petri dishes to containers with soil.

We used Analysis of Variance statistical tests to find the p-value.

We planted *Arabidopsis* flowers, both wild-type (ler-0) and mutant (fwa-1). The wild type flowered 23 days after planting, and the mutant *Arabidopsis* flowered 15 days after the wild-type, 38 days after planting.

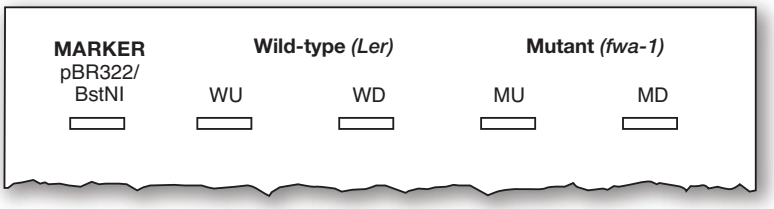
Once the *fwa-1* plants flowered, leaf samples were collected from both plants. We extracted DNA from both, using a nuclei lysis solution to dissolve organelles within the cell and access the DNA, and RNAse enzymes to break down the RNA, so that it would not be amplified in Polymerase Chain Reaction (PCR). PCR is used to reproduce sections of DNA. The DNA was microcentrifuged to concentrate it, and washed using an ethanol solution.

Then, we added a McrBC restriction enzyme, as well as its buffer, to half of the DNA, so that we had samples of both the wild-type and mutant DNA with and without the enzyme. This restriction buffer is modification-dependent. Thus, the McrBC enzyme will cut DNA which is methylated, and will not cut DNA which is unmethylated: the mutant type should remain uncut, while the wild type will be cut. The next step was to amplify the DNA using PCR. In this step, the cut DNA should not be amplified, leaving the mutant type with the enzyme (MD), the mutant type without (MU), and the wild-type without the enzyme (WU) to be amplified.

Once the PCR was completed, we analyzed the DNA using gel electrophoresis. The samples were loaded into the cells like so:

Figure 2

Organization of DNA Samples in Gel



Procedures

Procedure for Brassica rapa *primary experiment*

This procedure is divided into three parts.

I – Create 50 µM 5-Aza-2'-deoxycytidine solution

1. Acquire 5 g 5-Aza-2'-deoxycytidine.
2. Add 440 µL distilled water, to create a 50 M solution.
3. Using a micropipette, add 1.4 µL of this solution to a clean microtube.
4. Add 1.4 mL distilled water.
5. Repeat steps 3-4 three times.
6. Label these three tubes S+, PSH+, AP+. *+ denotes the addition of the decitabine.*
7. Add 1.4 µL of distilled water to a clean microtube
8. Add 1.4 mL distilled water.
9. Repeat steps 3-4 three times.
10. Label these three tubes S-, PSH-, AP-. *- denotes the control.*

II – Germinate seeds

1. Split 20 Standard (S) seeds equally between two petri dishes; each dish should have 10 seeds.
2. Place seeds between two pieces of filter paper.
3. Repeat steps 1-2 using the Purple Stem/Hairy (PSH) variety as well as the AstroPlant (AP) variety.
4. Label one of the dishes with the S seeds as S- and the other as S+. Label one PSH dish with PSH- and the other with PSH+. Label on AP dish with AP- and the other with AP+.
5. Add the contents of each microtube (labeled S-, S+, PSH-, PSH+, AP-, AP+) to the appropriate petri dish, marked with the same label.
6. Store the petri dishes in the dark for 3 days to simulate growth under soil.

III – Grow Plants

1. Add soil to eighteen separate containers in 6 groups. Label each group: S-, S+, PSH-, PSH+, AP-, AP+
2. Take germinated seeds. Measure the length of them, in centimeters.
3. Separate individuals from one another.
4. Place seeds in their designated group, 3-4 to a container.
5. Add equal amounts of water to each container.
6. Water every other day, or as needed.
7. Take measurements and photos every six days*.

*Days are measured as "days after seeds were first exposed to the drug or control", and additional measurements were taken on the day the seeds were transferred from petri dishes to containers with soil.

Procedure for* Arabidopsis *secondary experiment

(This procedure is divided into five parts.)

I - Plant Arabidopsis seeds

1. Moisten the potting soil. Divide the flats into their three-celled sections. Label half of the sections "ler-o" and the rest, "fwa-1." Fill planting cells evenly with soil, but do not pack tightly.
2. Fit the planting cells into the tray, but leave empty space to facilitate watering.
3. Carefully scatter seeds evenly on top of the soil. Plant each seed type in the appropriately labeled sections.
   a. Fold a 4- x 4-inch sheet of paper in half.
   b. Place the seeds into the fold of the paper, and gently tap them onto the soil.
   c. Provide space between seeds (3/4-inch radius around the seeds), so they will grow better and the plant phenotypes can be readily observed. Plant approximately 2–3 seeds per planting cell.
   d. Record the date of planting.
4. At an empty space, add 1/2 inch of water to the tray. Water regularly to keep soil damp, but do not allow soil to remain soggy.
5. Cover planting cells with the plastic dome lid to assist germination. (Remove the cover 1–2 days after germination.)
6. Provide a constant (24 hours/day) fluorescent light source about 1 foot directly above the plants.
7. Measure flowering time for wild-type Ler and mutant fwa-1 plants. Record flowering time as the number of days from planting to the emergence of anthers, the male structures producing yellow pollen. (Examine plants regularly, looking for signs that the plant is developing a stem. Once the stem begins to emerge, or bolt, check daily for flowers.)
8. Continue on to Part II as soon as the late-flowering phenotype is detected in mutant fwa-1 plants.

II - Isolate Genomic DNA from Arabidopsis

1. Obtain wild-type Ler and a mutant *fwa-1* samples.
2. Take a tissue sample from each wild-type *Ler* and mutant *fwa-1* sample, approximately 0.25 inches in diameter. Do not cross-contaminate samples.
3. Put each sample in a 1.5-mL tube, labeled with genotype.
4. Add 100 µL nuclei lysis solution to each tube.
5. Use a clean plastic pestle to grind each solution inside the 1.5 mL tubes for 2 minutes.
6. Add 500 µL more nuclei lysis solution to each tube.
7. Incubate tubes in a water bath or heat block at 65 degrees Celsius for 15 minutes.
8. Add 200 µL RNAse to each tube. Mix by rapidly inverting the tubes.
9. Incubate tubes in a water bath or heat block at 37 degrees Celsius for 15 minutes.
10. Add 200 µL of the protein precipitation solution.
11. Vortex each tube for 5 seconds by holding the top and gently flicking the bottom.
12. Chill each tube on ice for 5 minutes.
13. Put the tubes in a microcentrifuge at maximum speed for 4 minutes.
14. Transfer 600 µL of the supernatant from each tube into a clean 1.5 mL tube. Be sure not to disturb the precipitate. Label the genotype

15. Add 600 µL of isopropanol to each tube. Mix, with the caps closed, by rapidly inverting several times.
16. Put the tubes in a microcentrifuge at maximum speed for 1 minute to pellet the DNA.
17. Using a micropipette, remove the supernatant.
18. Add 600 µL of 70% ethanol. Close the caps, and flick the bottom of the tubes several times to wash the pellet.
19. Centrifuge the tubes for 1 minute at maximum speed.
20. Using the micropipette, remove the ethanol.
21. Let the solid at the bottom dry for 10-15 minutes.
22. Add a rehydration solution to the tubes and allow the samples to rehydrate at 65 degrees C for 60 minutes OR 4 degrees C overnight.
23. Store samples at -20 degrees C.

III - Digest PCR Products with McrBC

1. Transfer DNA to a clean 1.5 mL tube. Label DNA type. Either:
   a. WU (wild-type undigested)
   b. WD (wild-type digested)
   c. MU (mutant-type undigested)
   d. MD (mutant-type digested)
2. Use the table below as a checklist to add reagents to each tube. Be sure to change micropipette tips and use a sterile one for each addition:

Tube	Sterile dH2O	Restriction Buffer	McrBC	Wild-type DNA	Mutant DNA
WU	10 µL	3 µL	–	12 µL	–
WD	8 µL	3 µL	2 µL	12 µL	–
MU	10 µL	3 µL	–	–	12 µL
MD	8 µL	3 µL	2 µL	–	12 µL

3. Mix and then pool reagents by pulsing tubes in a microcentrifuge.
4. Incubate overnight in a water bath or heat block at 37 degrees C.
5. Inactivate the sample by incubating it at 65 degrees C; samples can be stored at -20 degrees C.

IV - Amplify DNA by PCR

1. Label four PCR tubes:
   a. WU (wild-type undigested)
   b. WD (wild-type digested)
   c. MU (mutant-type undigested)
   d. MD (mutant-type digested)
2. Add 22.5 µL of the FWA Primer Mix to each of the four tubes.
3. Add 2.5 µL of the WU DNA type to the tube marked WU.
4. Add 2.5 µL of the WD DNA type to the tube marked WD.
5. Add 2.5 µL of the MU DNA type to the tube marked MU.
6. Add 2.5 µL of the MD DNA type to the tube marked MD.
7. Store reagents on ice until they are to be used.

8. Program the following profile into the PCR machine for 35 cycles:
   Denaturing: 94 degrees Celsius, 30 seconds
   Annealing: 60 degrees Celsius, 30 seconds
   Extending: 72 degrees Celsius, 30 seconds
   After 35 cycles have been completed, the contents can be held at 4 degrees Celsius.
9. Amplify in thermal cycler (PCR).
10. Store on ice, or at -20 degrees Celsius.

V - Analyze PCR Products by Gel Electrophoresis.

1. Seal the ends of the gel casting tray, and insert a well-forming comb.
2. Pour 2% agarose solution into the tray, so that it covers approximately one-third to one-half of the height of the comb teeth.
3. Allow it to set completely (20 minutes).
4. Pour enough 1x TBE buffer to cover the surface.
5. Carefully remove the comb and add additional 1x TBE buffer to fill the wells and cover the gel, creating a smooth buffer surface.
6. Use a micropipette to fill the the left-most well with 20 µL of the pBR322/BstNI size marker.
7. Fill the two middle wells with the WU and WD samples, and the two right-most wells with the MU and MD samples. Be sure to clearly mark which well each sample is located in.
8. Electrophorese samples at 130 V for 30 minutes. There will be sufficient separation when the cresol red dye front has moved 50 mm.

Data

Data for* Brassica rapa *primary experiment

Table 1

Survival rates of each group (%)

	S -	S +	PSH -	PSH +	AP -	AP +
Day 3	100	90	90	100	100	90
Day 6	90	80	60	50	80	50
Day 12	90	60	50	60	80	50
Day 18	90	60	50	50	80	50

Table 2

Average heights of plants (cm)

	S -	S +	PSH -	PSH +	AP -	AP +
Day 3	4.55	2.56	2.00	2.25	3.45	2.28
Day 6	3.39	1.25	3.17	1.20	2.06	1.20
Day 12	5.39	2.00	5.20	1.58	2.50	2.10
Day 18	9.17	5.67	8.80	4.20	3.81	3.10

Figure 3

Survival Rates of Each Group (%)

The highest survival rates are in S- and AP- populations, neither of which received the drug. These data may indicate that the addition of decitabine increases mortality rates in plants.

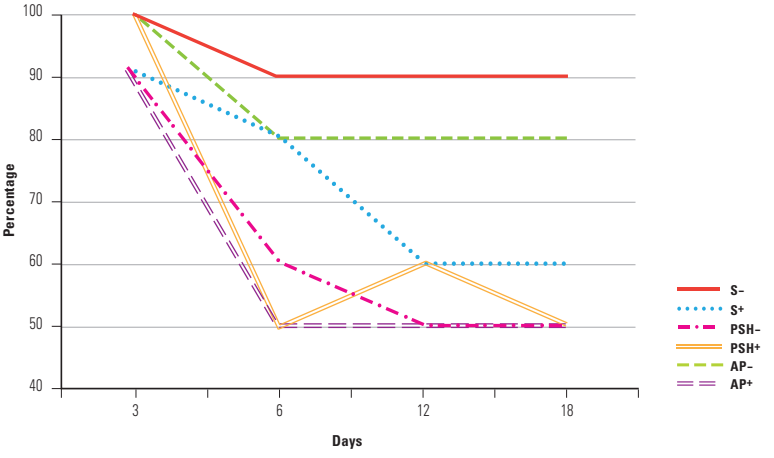


Figure 4

Average Height or Length of Plants at Different Points in their Growth (cm)

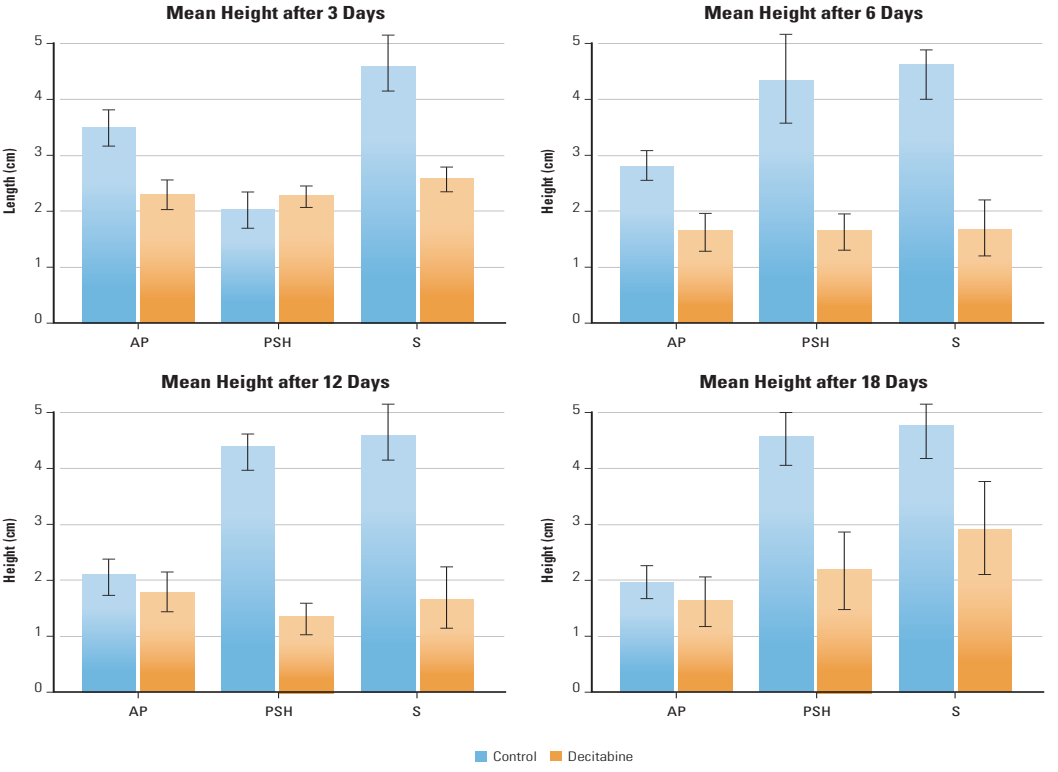


Figure 5

Germinated S- vs. S+. There is more growth in S- than S+ ($p < 0.01$).

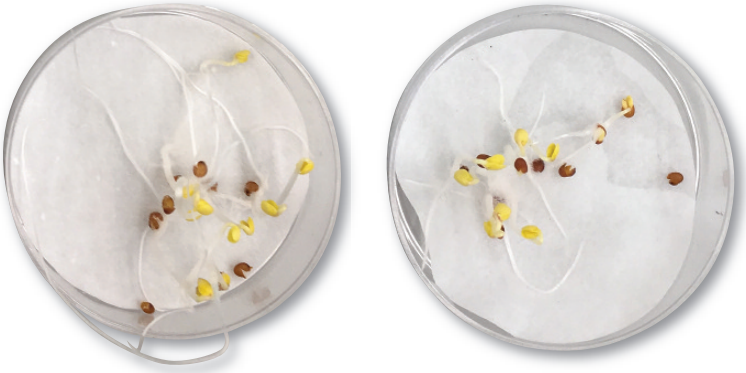
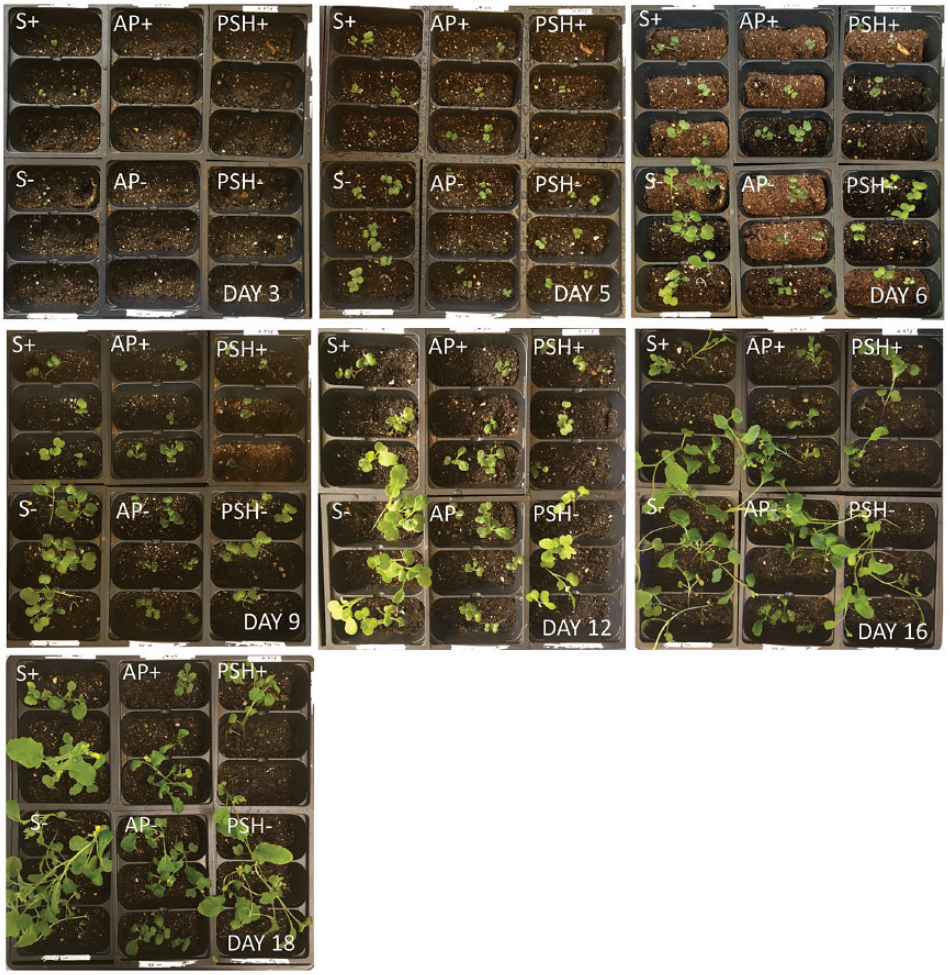


Figure 6

Growth of Plants over 15 Days. The Controls Exhibited Greater Growth than the Plants with Decitabine

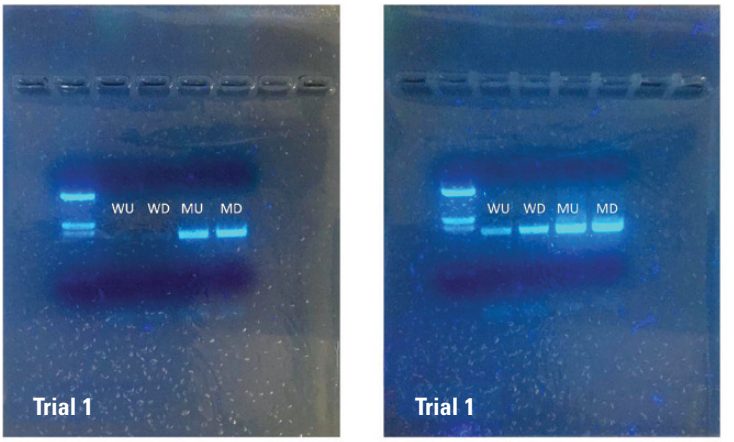


Data for* Arabidopsis *Secondary Experiment

Figure 7

Gel Electrophoresis PCR Results for Both Trials

(Neither trial was conclusive.)



Analysis

Of the 60 seeds germinated, 57 survived germination (95%), and 38 survived the 18 days past initial exposure (63.3%). The most viable population was S-, from which 9 individuals survived (90%). Also viable was AP- with 8 individuals surviving (80%). Of the remaining group, S+ had a 60% survival rate, and all others had a 50% survival rate. There was an anomaly in that PSH+ appears to have a survival rate of 50% on day 6, but seemingly 10% of the plants come back to life by day 12. It is likely that some of the plants had not yet reached the surface of the soil and therefore were erroneously counted as not having survived.

There was a significant difference between the growth of the plants germinated without decitabine and plants germinated with decitabine. On average, the germinated seed length for S- was 1.99 cm greater than the germinated seed length for S+ ($p<0.01$). The average germinated seed length for PSH- was 0.25 cm less than PSH+ and was not statistically significant. The average germinated seed length for AP- was 1.17 cm greater than the germinated seed length for AP+ ($p<0.02$).

The average height of S- plants 15 days after planting was 3.5 cm greater than that of S+ plants ($p<0.05$). The average height of PSH- plants 15 days after planting was 4.6 cm greater than that of PSH+ plants ($p<0.02$), and the average height of AP- plants 15 days after planting was 0.75 cm greater than that AP+, but the difference was not significant.

S- exhibited a significantly greater height than S+ at every point that measurements were taken ($p<0.05$). AP- exhibited significantly greater heights at germination up to three days after planting ($p<0.03$), but after Day 3, the difference between the plants' heights became insignificant. However, AP- had a survival rate 40% higher than that of AP+. Conversely, the height difference between PSH- and PSH+ became significantly different only on Day 3 of the trial, and remained that way until Day 15 ($p<0.03$).

We noticed that while generally the plants exhibited the same physical characteristics, the leaves of AP+ became wrinkled towards the end of the experiment. None of the other plants exhibited this characteristic.

Interestingly, while all of the AstroPlants (AP) exhibited reduced growth and a more compact size as their variety is intended to, the Purple Stem/Hairy (PSH) plants only had a slightly purple stem and were not hairy.

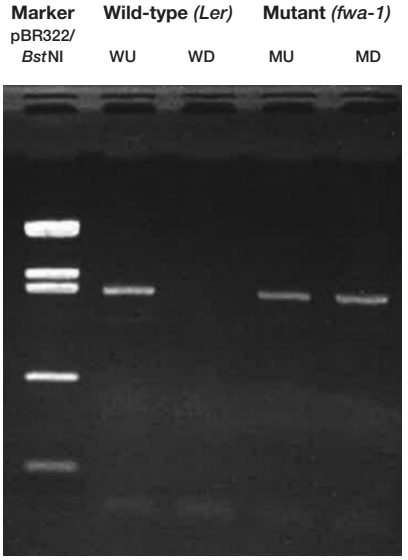
Our gel electrophoresis results were inconclusive for the *Arabidopsis* study. In the first trial, neither of the wild-type DNA samples were amplified in PCR, but both of the mutant type were. In the second trial, all DNA samples were amplified in the PCR.

Ideally, our gels would have looked like this:

Figure 8

Conclusive DNA Amplification Results for the *Arabidopsis* Study

("Detecting Epigenetics", 2013)



The gel in Fig. 8 demonstrates both that the enzyme works in general, due to the fact that only the WU sample was amplified, and that the enzyme does not work on the fwa-1 sample because it is methylated.

While the results we gathered are good in that both the cut and uncut mutant DNA appear on the gel in both trials, it is impossible to tell whether the enzyme is working, because there isn't a distinction between the wild type with and without the enzyme on both trials.

Discussion

To better understand the ways in which phenotypic plasticity is linked to epigenetic modification, we raised different *Brassica rapa* varieties with the demethylating agent decitabine, in order to manipulate the plants epigenome and therefore phenotype.

In this study, we did not verify whether the epigenome was was modified using DNA analysis techniques, so there is a chance that the phenotypic changes observed were due to the observed toxicity of decitabine in mammalian cells rather than a change to the methylation on the plant DNA (Jüttermann, 1994). However, given the wealth of information connecting decitabine to hypomethyla-

tion, and the continued differences in growth after the decitabine was not in contact with the plants, we abide by our assumption that the phenotypic changes were a result of epigenetic changes.

We observed that germinating the *Brassica rapa* plants using decitabine significantly affected the plants' growth and survival rate. We also observed some changes in physical appearance of the plants. The differences in height between the control groups and decitabine groups indicates that the use of decitabine in germinating the plants has a lasting effect on the plants' phenotype, in all likelihood altering their epigenome. The results of this study suggest that natural methylation in *Brassica rapa* affects speed of maturation as well as growth, a result found in other studies (Bossdorf, 2010; Raj *et al.*, 2011).

The greatest sustained difference in height was between that of the Standard varieties, and the least difference in heights was that of the AstroPlants, which are bred to be compact and exhibit slower growth. This may point to the idea that AstroPlants are modified epigenetically, which is why the demethylating agent would do the least work on them. However, the control AP group had a much higher survival rate than the AP+ group, which may mean that it is more vulnerable to demethylation elsewhere on its genome, leading to increased mortality due to hypomethylation. Demethylation may more seriously affect biological processes within the plant. Moreover, the AP+ group exhibited the most obvious physical difference of all groups, which is that the leaves began to wrinkle. The AP- group did not exhibit these changes. Based on this, we can assume that there are parts of the AstroPlants' epigenetic code that is dissimilar to all other varieties.

Decitabine is a drug that is used to control growth for cancer treatment. While we did not target specifically any parts of the DNA for demethylation, it may be that the genes which code for growth patterns have CpG islands within them, and are therefore more likely to be methylated.

There are a few reasons that we may have gotten inconclusive results with the *Arabidopsis* study. Potentially, the wild-type samples with and without the enzyme didn't appear in Trial 1 because of the amount of DNA. It is likely that there wasn't enough wild-type DNA for the PCR to effectively amplify. Alternatively, the DNA may have been damaged at some point in the process. For example, if the samples weren't kept at -20° C after the DNA was isolated, nucleases in the solution could have fragmented the DNA. However, this situation seems unlikely.

On the other hand, it is possible that both wild-type samples appeared in Trial 2 because there was *too much* DNA. Most likely, the DNA overwhelmed the McrBC, and there wasn't enough of the enzyme to cut all of the wild-type DNA. The amount that avoided being digested went through PCR amplification. This would explain why the wild type samples are less distinct than the mutant samples in the Trial 2 gel. There would be less DNA, so it would not show up as clearly.

There is a remote possibility that samples got confused between trials, and that the wild-type without enzyme from Trial 1 was switched with the wild-type with enzyme from Trial 2.

Because in both trials the mutant sample was clearly amplified, we can understand that the enzyme did not cut the unmethylated mutant DNA. Unfortunately, it is impossible to verify that the McrBC enzyme worked properly, and the data has to be classified as inconclusive.

In the future, we intend to do follow up genetic verification on the actual plant material, rather than an analogous study, that the phenotypic changes in the *Brassica rapa* plant were due to methylation changes, rather than toxicity of decitabine unrelated to epigenetics.

In conclusion, we found that the growth of Brassica rapa plants was altered by use of the demethylating agent 5-Aza-2'-deoxycytidine, and that different varieties are susceptible in different ways. Altering the epigenetic code may open up new passages for genetic modification.

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Developing a Program to Identify False-Positive Identification of Vocal-Learning Genes

by Carolyn Khoury

Abstract

Autism spectrum disorders (ASD) and speech delay are conditions that are affected by genetic mutations. It has been hypothesized that the genes being mutated to cause these disorders are vocal learning genes (Petkov & Jarvis 2012). Vocal learning is a key component of spoken language which is critically important to human social interaction. In recent years, the field of genome sequencing has expanded, making it possible to identify key evolutionary changes underlying vocal learning at the genetic level by identifying adaptations shared between different phylogenetic clades that independently evolved vocal learning. Ultimately, identification of these genes in birds can shed light on the genetics of these disorders in humans. I have obtained a 450 million base pair whole genome alignment of the sequenced genomes of thirty-three avian species, including those of three vocal learning clades: Oscine Songbirds (Order: *Passeriformes),* Parrots (Order: *Psittaciformes),* and Hummingbirds (Family: *Trochilidae)* from the Laboratory of the Neurogenetics of Language at Rockefeller University. The overarching goal of this project is to figure out what these alignments contain, as well as what errors (in the form of paralogous gene loci) are present in the alignment. In particular, the lab aims to determine which components of each genome were included and excluded from the alignment. However, the current problem we face is paralogs (duplicated genes), since they lead to the false identification of adaptation. In this project, I have developed a computer program in the Python programming language, in tandem with Bash scripting, which can identify paralogous gene loci in the alignment. From this program, I identified 952 paralogous loci in the alignment. I then built phylogenetic trees for twenty-one of these paralogs, which allowed me to qualitatively identify paralogous loci, including candidate gene loci of key evolutionary changes for vocal learning. Developing this paralog identification program to exclude false positive signals of adaptation is an important step in the effort to identify vocal learning genes and ultimately develop a gene therapy for ASD and speech delay.

Introduction

Vocal learning is the ability of an animal to produce learned sounds that have meaning (Petkov & Jarvis 2012). However, relatively few animals have this functionality. Vocal learning must not be confused with auditory learning, a less evolutionarily advanced trait found in all vertebrates that involves the memory of heard sounds (Petkov & Jarvis 2012). Identified vocal learners include bats, cetaceans (i.e. dolphins), seals, and elephants. However, the vocal learning phenotype is most notably advanced in humans and certain avian species including **Oscine songbirds, Parrots,** and **Hummingbirds**, each of which are represented in Figure 1 on the right.

Figure 1

(Below) Images of a Gold-crested Oscine Songbird, a Macaw Parrot, and a Western Emerald Hummingbird

FRANKLIN 2015

VIATOUR 2009

THOMPSON 2010

Humans and birds such as these can produce exceptionally complex and organized vocalizations (Petkov & Jarvis 2012). Most other avian species don't have this complex trait, meaning there must be key genetic differences between vocal learning and non-vocal learning avian species. The genetic differences unique to vocal learning birds are particularly interesting to our research since their identification may bring us closer to finding the homologous genes responsible for vocal learning in humans, which has great implications in the search for the causes of genetic vocal and speech disorders in humans.

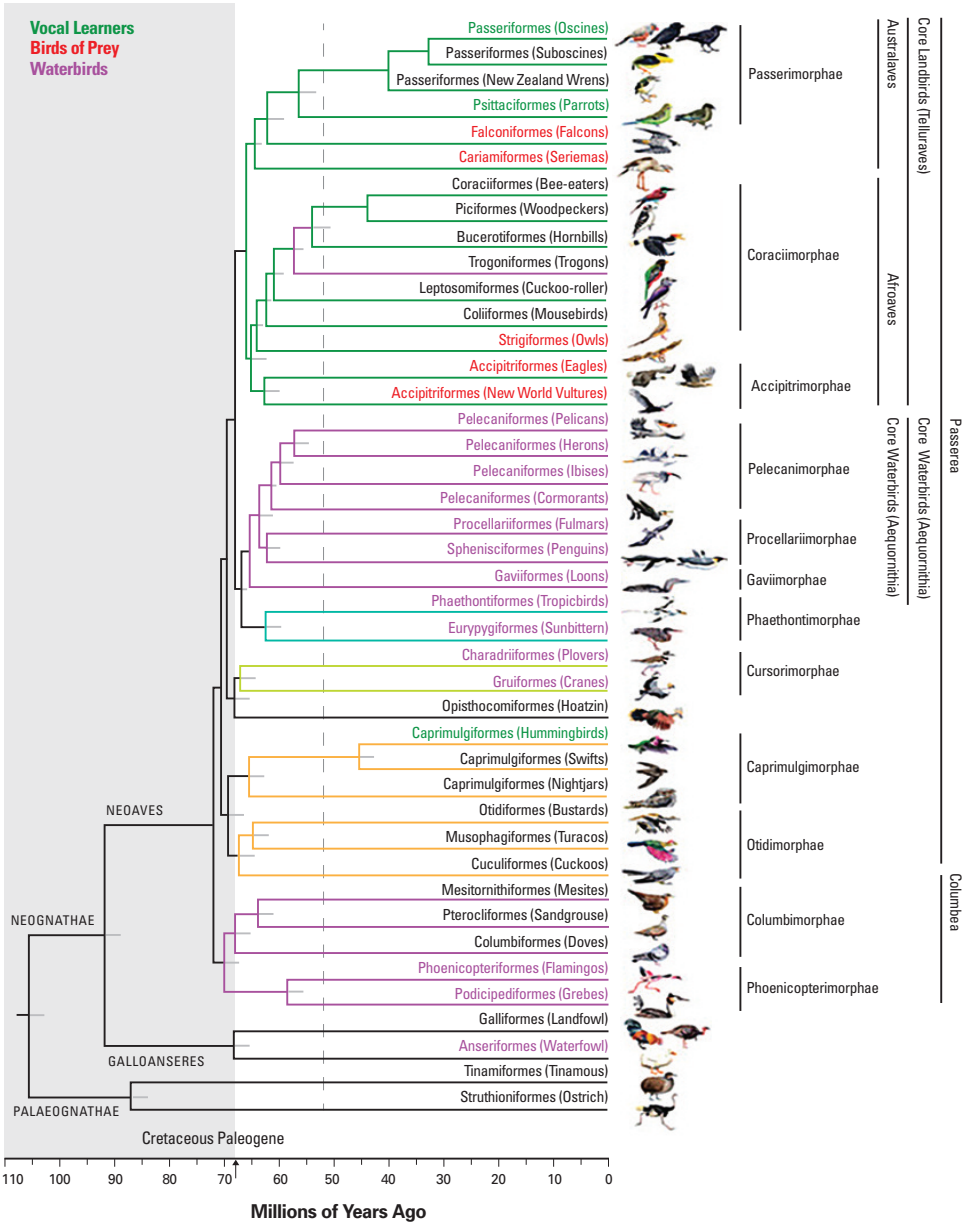
In order to understand the genomics of the vocal learning phenotype, one must start by understanding how and why this phenotype manifests itself in particular avian species and clades. To do so, we must look into the evolutionary trends of vocal learning in the context of avian evolution as a whole. Over 150 million years ago, the first birds evolved from the theropod dinosaurs, making them the only living descendants of dinosaurs. However, the dinosaurs were completely wiped out about 66 million years ago in an event known as the **Cretaceous-Paleogene (K-Pg) extinction event,** in which a massive asteroid slammed into Earth, causing worldwide environmental destruction (Zhang et. al. 2014). This created a huge vacancy in diverse ecological niches that would be quickly filled by mammalian and avian species. This, in turn, caused rapid evolution of these two groups, resulting in the modern human, as well as the over 10,500 living species of birds (Neoaves) we see today (Zhang et. al. 2014). This rapid evolution in the avian lineage led to a widely diverse set of genes and resulting phenotypes, one of which is the vocal learning phenotype found in the three avian vocal learning groups mentioned earlier.

The way in which genes evolve – for abilities like vocal learning – can be traced through **comparative genomics,** in which particular genomic features of different species can be compared side-by-side in order to identify particular trends in the evolution of a given feature (Zhang et. al. 2014). The genetic sequences of the various species being compared make up what is known as an **alignment,**

which allows for significant genetic similarities and differences between species to be identified (Lipman, Altschul, & Kececioglu 1989). For example, the alignment used in this project was a whole genome alignment of thirty-three avian species, and it compared the genomes of each species in the alignment to the rest. The result of such analysis using a given alignment allows us to identify relative

Figure 2

Genome-scale Phylogeny of Birds (Jarvis, et al. 2014)



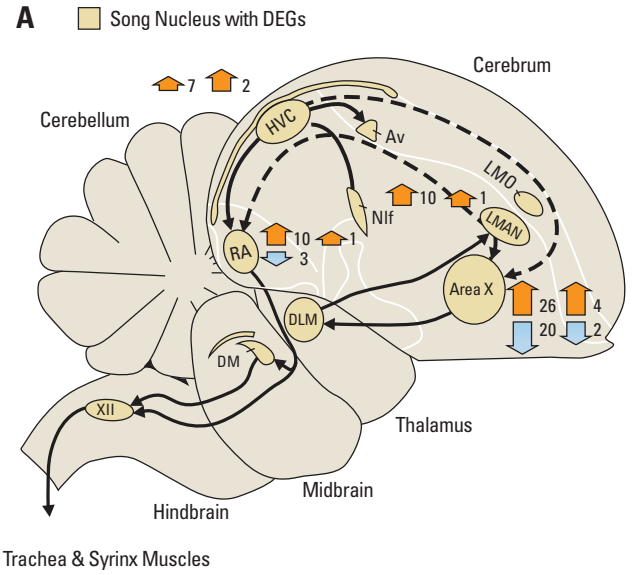
relationships between species, and create what is known as a phylogenetic tree. A **phylogenetic tree** or **phylogeny** is a diagram that depicts evolutionary descent of different species from a common ancestor in the context of a given gene or DNA sequence (Jarvis et. al. 2014). Each branch in the phylogeny represents a new avian species that evolves from another. For example, Figure 2 (left), is a genome-scale phylogenetic tree of birds, depicting how closely related each species is from the rest.

Comparative genomics also allows for the identification of conserved elements and accelerated regions. **Conserved elements** are sequences of DNA that have been well conserved throughout the evolution of a given lineage. The conservation of certain DNA sequences across distantly related species reflects that the sequence has an important function in the lineage. Therefore, such elements are said to evolve at a "slower than neutral rate" (Zhang et. al. 2014). Conversely, there are accelerated regions. **Accelerated regions** are segments of DNA that, unlike conserved elements, evolve at a faster than neutral rate in some species, but are highly conserved in others. In turn, these accelerated regions are likely to be the result of natural selection and may have a positive functional effect on the survival of a species. This is suggested to be the case with the vocal learning genes in songbirds, parrots, and hummingbirds, particularly in genes involved in regions of their brain known as **song nuclei** (Zhang et. al. 2014), depicted in Figure 3 below. These song nuclei are specialized vocal learning forebrain circuits – similar to those of humans – and are not present in non-vocal-learning birds. Because they are unique to vocal learning species and have been found to be accelerated, these song nuclei genes are strong candidates for vocal learning genes.

Identification of these accelerated regions in the genomes of vocal learning birds is crucial to the greater implications of this research: identifying **homologs** (human versions) of these vocal learning genes in humans to treat disorders caused by their mutations. If mutated, these vocal learning

Figure 3

Songbird Brain Diagram Showing the Specialized Forebrain Song-learning Nuclei (Yellow)
(Zhang. et al., 2014)



genes may cause developmental and/or speech disorders such as **autism spectrum disorders (ASD)** and **speech delays.** Some potential candidate genes have already been identified, including SOX5, NRXN1, AUTS2, and others (Jarvis et. al. 2014). However, the identification of such genes as being accelerated in vocal learning birds isn't entirely certain. This is due to the high risk of obtaining false positive results, known as **paralogs,** which are randomly duplicated sections of a genome. Paralogs do not all occur in the same locus on every genome. Therefore, when the genomes of two different species in the alignment are being compared (via previous bioinformatic computer programs) at a specific gene locus where they are typically identical, if one of the genomes contains a paralog at that locus, then the comparison program will detect a large difference between the two species. This confounds our analysis because the paralogous alignments make closely related species appear highly diverged, leading to a false positive signal of natural selection. Thus, this negative impact on our analysis is precisely the problem which this project aims to solve using bioinformatics and comparative genomics techniques.

Methods

Every aspect of the quantitative analysis of this project was conducted through code. I first looked for frequency of genetic divergence in each pairwise comparison between genomes in the alignment. To do this, I created code that reads through the genetic information of two given species in the alignment, one base pair at a time, and detects whether or not it can be considered an observable site. A base pair is considered an observable site if it contains either "A," "T," "C," or "G" which represent the DNA nucleotides adenine, thymine, cytosine, and guanine respectively. An unobservable site would contain an "N" or an asterisk ("*") which indicates that the DNA sequencer (at The Rockefeller University) was unable to determine the identity of the nucleotide in question. If it is an observable site, genetic divergence is determined based on whether or not there is a difference between the nucleotide letter present at the location on two given genome sequences being compared. If there is a difference, it would be considered a genetic divergence. The code then stores the total number of observable sites (for every 1000 base pairs) into an array list, and stores the number of divergences into another array list. Frequency is then determined by finding the ratio of genetic divergences to observable sites for each comparison. Each of these frequencies was then put in a list for statistical analysis. Summary statistics of the divergence frequency data was conducted for each pairwise comparison. To do this, I created code that finds each decile (every tenth percentile) of the list. This was done using the "numpy" function in Python. The goal of obtaining the divergence frequency data point at each decile is to locate unusually large differences between the number of detected divergences at one region of the genome and another in the data set. These large differences are indicative of paralogous regions.

I created code that analyzes this data by scanning each decile of the divergence frequency data generated by the summary statistics in search of values above a threshold of 0.3 genetic divergences per observable site (300 for every 1000 base pairs) between each of these deciles, which is a relatively large number of divergences. Any value greater than this threshold would be considered a paralogous gene loci since paralogs tend to generate exceptionally large signals of genetic divergence – hence the reason they confound the ultimate search for genetic signals of adaptation when comparing these avian genomes. A portion of this code can be seen in Figure 4.

The decile intervals within which the paralogs were found were automatically recorded and input into a separate file for organization. Because the divergence frequency tests were conducted in 1000-base pair sections of each genome, and the coordinates of each of these locations was recorded with the summary statistics, I was able to find the general genetic locus of each identified paralog using the "msa_view" package of Bash commands, which has a variety of options specially created for computational genomics using genome alignments. Using the database of sequenced avian genes, their

Figure 4

Python Code that Sets Paralog Threshold of 0.3 Divergences Per Observable Site

```
#Carolyn Khoury
#This will apply a formula that will consistently quantify the size of the "jumps" between
consecutive percentiles. The threshold for a significant paralogous "jump" is 0.3.

import sys,os
file=open(sys.argv[1])
for line in file:
        line=line.replace("  ", " ")
        line=line.replace("  ", " ")
        line=line.replace("  ", " ")
        data=line.split(" ")
        n=6
        n2=n-1
        while (n<=13):
                if(((float(data[n])-float(data[n-1]))/float(data[13]))>=0.3):
                        print(line, "There is a paralog between items %d and %d" %(n,n2))
                n+=1
                n2+=1
```

coordinates, and their function (provided by The Rockefeller University), I was able to match the given gene coordinates with an identified paralog, giving each paralog a functional and more tangible identity. This information was then transferred to a final file, with each paralogous gene and its coordinate having a separate line.

Data & Analysis

Using a simple Bash command that counts the number of lines in a file ("wc -l"), I was able to find that there were precisely **952** paralogous gene loci that were greater than or equal to the 0.3 divergence threshold. A graphical representation of this threshold at work can be seen in Figures 5 and 6 below.

Figure 5

Frequency of Genetic Divergence Between Species Relative to the NR5A2 Gene, Identified by My Code to be Paralogous

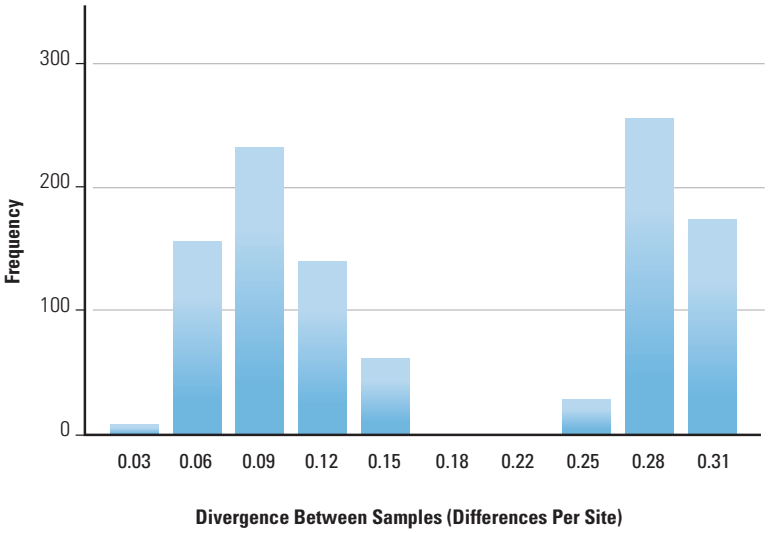
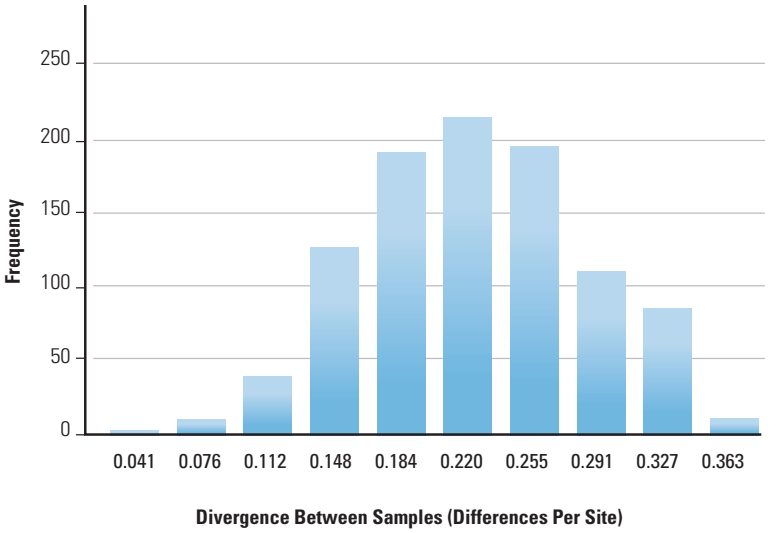


Figure 6

Frequency of Genetic Divergence Between Species Relative to a Random Non-paralogous Gene



The graphs above depict the frequency of each divergence magnitude – in other words, the frequency of various divergence frequencies. Figure 5 is in the context of a paralog while Figure 6 is in the context of a non-paralogous gene locus. While in Figure 5 there seems to be a relatively high incidence of the 0.09 genetic divergence frequency along with higher frequencies such as 0.28 and 0.31, this is simply an indication of the fact that every organism (even if they are in the same species) has a unique genetic makeup, which in turn suggests minor and negligible genetic differences for the purpose of this project. However, the way in which the frequency drastically increases at around 0.28 divergences per site indicates that there is even higher incidence of very large genetic divergence – the hallmark of paralogous gene loci. On the other hand Figure 6 depicts the exact opposite trend of the graph in Figure 5; as the magnitudes reach more extreme values of divergence frequency such as 0.291 and beyond, it is clear that there is much lower incidence of the more extreme and erroneously high divergence frequency values compared to the more moderate values such as 0.184 and 0.220, thus indicating that this is not a paralog.

I then conducted a qualitative analysis of this data by building phylogenetic trees of paralogs I had identified quantitatively to confirm that they were indeed paralogs. It was impractical for me to create trees for all 952 paralogs, so I only created 21. However, these 21 were representative of paralogs found in each decile interval. I constructed the maximum likelihood phylogenetic trees for each locus with a program called raxML which simply formats the data to be visualized by the TREX Newick Viewer. The TREX Newick Viewer is an online web server that is used to validate and visualize phylogenetic trees from code. Once created, the branches on the phylogenetic tree indicate the extent to which one species is related to the other; the longer the branch, the less related two species are to one another since there is greater genetic divergence between the two. Through this method, one of the qualitatively strongest paralogs was found to be the NR5A2 gene paralog, whose phylogenetic tree can be seen in Figure 7 below. What is shown in this tree is a visualization of a paralog's behavior and effects, evinced by the distinct grouping and an excessively long branch between two species. This long branch is indicative of an erroneously large amount of genetic differences – the hallmark of a paralog.

Figure 7

Phylogenetic Tree Depicting Relationships Between Avian Species Relative to the NR5A2 Paralog

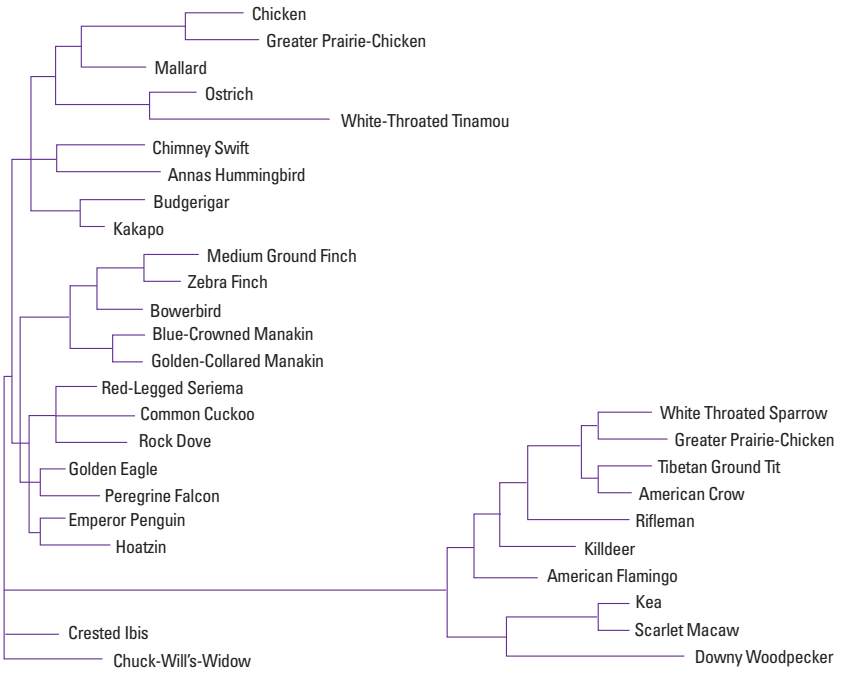
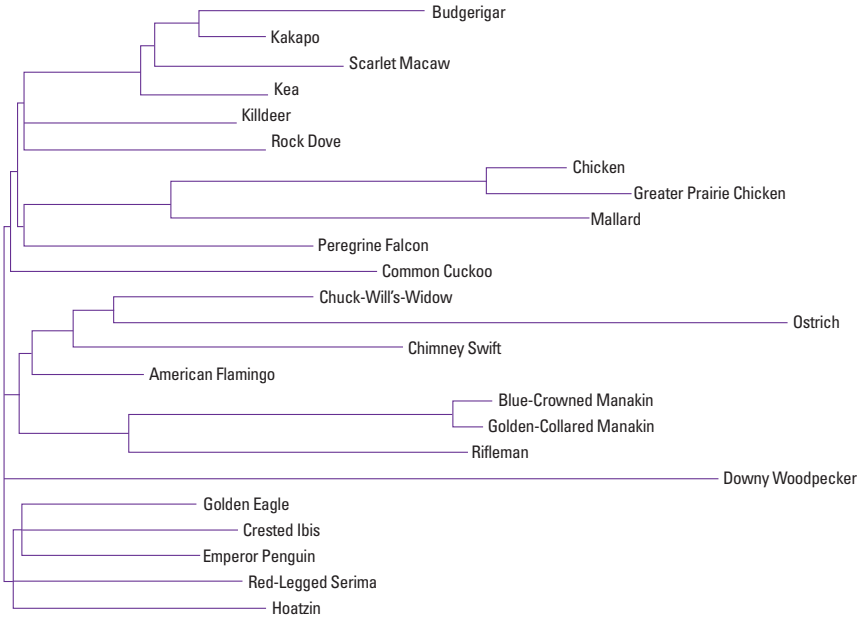


Figure 8

Phylogenetic Tree Depicting Relationships Between Avian Species Relative to a Random Non-paralogous Gene

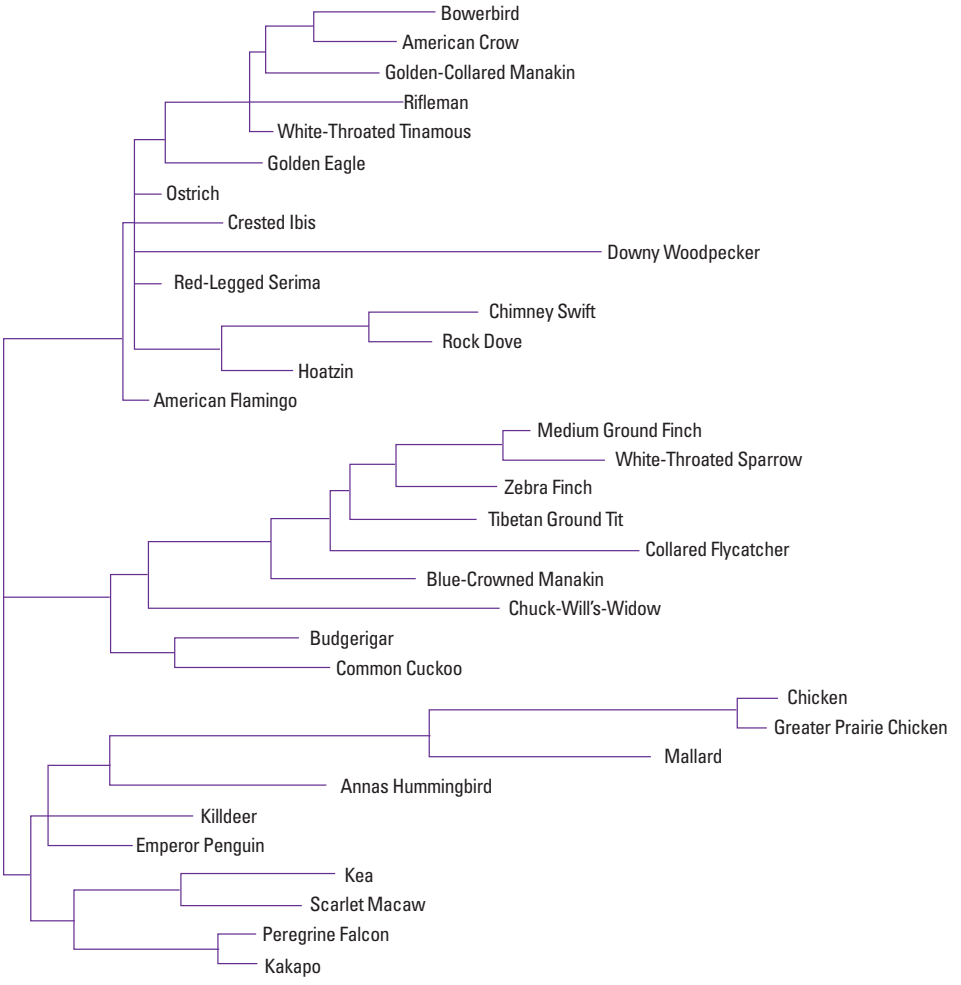


This qualitative analysis of NR5A2 confirms the quantitative analysis of the divergence frequencies taken earlier. In contrast, the phylogenetic tree of a random non-paralogous gene, such as that in Figure 8, has branches that are rather uniform in length, and no evident grouping or separation of species. Juxtaposing the phylogenetic tree of NR5A2 and that of Figure 8 will further highlight the paralogous nature of NR5A2 and thus confirm the efficacy of the paralog threshold of 0.3 genetic divergences per site.

Through this qualitative approach, I also found that the most obvious and clear paralogous loci tended to turn up in the middle of the divergence frequency data set of each comparison between approximately the 20th through 70th percentiles, such as NR5A2, which appeared between the 50th

Figure 9

Phylogenetic Tree of Paralogous Locus LOC100858107, Occurs Between 10th and 20th Percentiles



and 60th percentiles, as compared with paralog phylogenies such as those shown below, which occur between the 10th and 20th percentiles (Figure 9) and the 70th and 80th percentiles (Figure 10) of the data. While there is some minor grouping that can be detected in these two trees, it isn't stark, and the branch-lengths aren't excessively long. In fact, they arguably resemble the phylogenetic tree of the non-paralogous gene locus more than the NR5A2 paralog. This could indicate that the divergence frequency threshold of an average of 0.3 divergences per base-pair might have been too large, and may have detected paralogs where there may not necessarily be any. This overestimation can be dangerous to the overarching goals of the project: the search for signals of significant adaptation between vocal learning and non vocal learning avian species. The goal is to find a threshold that finds a balance between significant genetic divergence and erroneously large genetic discrepancies.

Figure 10

Phylogenetic Tree of Paralogous Locus POC1A, Occurs Between the 70th and 80th Percentiles

Discussion/Conclusion

Developing this paralog identification program to exclude false positive results is an important step in our effort to identify vocal learning adaptations. In the search for vocal learning genes in songbirds, parrots, and hummingbirds, this program will significantly reduce the risk that accelerated regions will be incorrectly identified due to the presence of paralogous loci. This program will bring us one step closer to identifying accelerated genes involved in vocal learning. Ultimately, this will help us learn more about the genetic tendencies of the homologs of these genes in humans in order to treat developmental disorders that occur due to mutations in these genes.

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The Effect of Naturally Found Sugars and Processed Sugars on Mood Measured by the Positive and Negative Affect Schedule (PANAS)

by Imogen Micklewhite

Abstract

This experiment tests the question: Is there a short term or long term change in positive or negative mood measured by PANAS (the Positive and Negative Affect Schedule) in test subjects who consume naturally found sugar (glucose and fructose) and subjects who consume processed sugars (sucrose, invert sugar syrup, and high fructose corn syrup)? The experiment was conducted with two groups: Experimental Group A was assigned to drink 180 mL of Fanta soda and Experimental Group B was assigned to drink 180 mL of a fruit smoothie. Before the experiment began, both groups completed the PANAS questionnaire then drank their assigned drink. All test subjects completed the PANAS questionnaire again 30 to 40 minutes later and then once again 3 to 5 hours after consuming either the soda or the smoothie. None of the data was statistically significant with p-values larger than 0.05 except the Long Term effect of the positive PANAS score for the soda group, which decreased a statistically significant amount. This suggests there is a negative impact on mood 3 to 5 hours after consuming processed sugar.

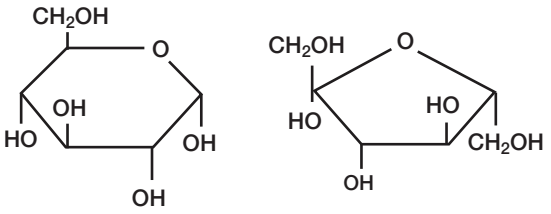
Background

The Chemical Compounds of Both Glucose and Fructose.

When talking about sugar in a nutritional context, the term 'sugar' has both broad and multiple meanings. Sugars come in the form of sucrose, invert sugars, high fructose corn syrup, crystallized glucose, crystallized fructose, and sugar alcohols (Schorin et al., 2012). Glucose and fructose are found naturally in plants and are examples of the simplest compound of sugar, monosaccharides. (Schorin et al., 2012). Figure 1 shows the chemical compounds of glucose and fructose (Berger, 2013).

Figure 1

Glucose vs. Fructose

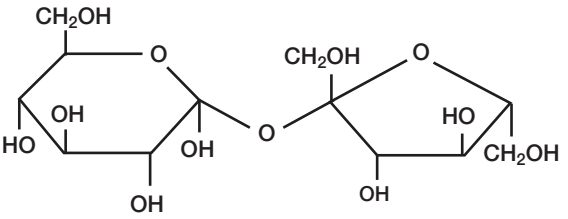


Glucose Fructose

Sucrose, invert sugar syrup, and high fructose corn syrup are all carbohydrates with some mixture of fructose and glucose, but each with different concentrations of the two ingredients (Schorin et al., 2012).

Figure 2

Sucrose



The Chemical Compound of Sucrose.

They can also exist as disaccharides, a sugar formed with two monosaccharides. Figure 2 shows the chemical bonds of sucrose (Berger, 2013). It is made up of a glucose and a fructose. Sucrose is purified from either sugar cane or sugar beets (plants that contain fructose and glucose) and is commonly known as table sugar or the sugar you use to cook (Schorin et al., 2012). While cane sugar and beet sugar are very important sources of energy for organisms, neither contains vitamins or minerals (Schorin et al., 2012). Invert sugar syrup is made by splitting sucrose into its two components and high fructose corn syrup in corn starch with an added enzyme to convert some of the glucose into fructose. Sugar alcohols or polyols are organic compounds in fruits and vegetables. Polyols are made up of multiple hydroxyl groups which are bonds between oxygen and hydrogen atoms. These sugars have about a 2 calories to 1 gram calorie count which is lower than table sugar which is 4 calories to 1 gram (Schorin et al., 2012). Therefore, since polyols have a lower calorie count they are used as sweeteners.

The term 'added sugars' is regularly used when talking about sugar in relation to nutritional facts. Added sugars are classified as any type of sugar that is added to food when processed. Understanding the terminology of different types of sugars is important in understanding how they are digested and how they affect the human body. Once in the human digestive system, sugars break down into their components and are released into the bloodstream. In most cases their components are either glucose, fructose, or some mixture of the two. Glucose is the only carbohydrate used as fuel by the brain, therefore it must constantly be kept in the bloodstream as well as stored in the body so that when glucose blood levels are low they can be replenished. The hormones that control this process are insulin and glucagon (Schorin et al., 2012). When you consume glucose it enters into your bloodstream triggering the pancreas to produce insulin which signals the cells to absorb the glucose. The glucose that is not absorbed is stored in the liver in the form of glycogen. When blood sugar levels drop, glycogen is converted to glucose and released into the bloodstream by the hormone glucagon (Hein et al., 2016). Fructose is metabolized in the liver where it is turned into glucose, but the process that starts the breakdown of fructose does not need insulin to metabolize it, like glucose (Schorin et al., 2012). Sugar alcohols are slowly absorbed into the bloodstream while in the small intestine (Schorin et al., 2012). As the researchers discuss in *The Science of Sugars, Part I: A Closer Look at Sugars,* each form of sugar is chemically identical and the body's digestive system cannot tell added sugars and natural sugars apart (Schorin et al., 2012). It is how they break down and release in the system that causes different sugars to have different effects. For example, since glucose is absorbed into the bloodstream quickly, it produces an insulin spike causing sugar highs and then shortly after, sugar crashes. Sugar alcohols, however, avoid sugar crashes because they do not require a lot of insulin. This is one of the ways eating processed food and a piece of fruit differ. Fruits contain fructose sugar alcohols and important cellular structures such as fiber that contribute to the controlled release of energy sourced from sugar (Gearing, 2015). Fiber found in whole fruits and vegetables make your body feel full because they cannot be broken down in the body, but rather help to push food through the alimentary canal. This causes you to feel

more satisfied and prevents overeating (Gearing, 2015). When the human digestive system tries to break down the fiber cells, sugars come second and are released slowly, avoiding sugar spikes (Gearing, 2015). As Gearing shows, the nutritional value of cartoned orange juice and an orange are very different. The cartoned orange juice is processed with added sugars and has no fiber. This lack of fiber causes sugar spikes and does not leave the body feeling full. The digestion, detoxification, and elimination of processed sugars also use up vitamins and minerals, leaving the body with less useful resources than before (Wilder, 1984). An orange on the other hand is full of vitamins, minerals, and fibers that fill you up and cause a controlled release of sugar.

Globally, there is an issue with overconsumption of sugar. I find it horrifying, yet not surprising, that one sixth of an American's diet is sugar; that is around 100 pounds of sugar a year (Wilder, 1984). Evolutionarily speaking, human beings who can find more sources of energy are able to thrive and reproduce. However, overconsumption of sugar is an issue. When you consume too much sugar your weight increases as well as your risk for diabetes, heart disease, and many other harmful illnesses. One alarming issue is the large presence of sugar in schools. Sometimes teachers give out candy in class either as part of an activity or just to please students. Through this, students are reinforced with the idea that sugar is a goal or a prize, making them strive to consume more sugar. In addition, giving excess sugar to students at school contributes to behavioral problems, sleepiness, difficulty focusing, hyperactivity, obesity, and even more complications (Wilder, 1984). High fructose corn syrup has a major presence in many other parts of our lives as well. You may even be surprised to know that products labeled healthy in the supermarket include this harmful ingredient and are actually not healthy.

Essentially, the added sugars in processed foods contain the same compounds as sugars in fruits, but the latter contains vitamins, minerals, and fiber while the former lacks the same nutritional value causing them to be absorbed quickly into the bloodstream. This causes sugar spikes and over computation of sugar which contribute to a multitude of mental health and physical problems. This leads me to propose the experimental question: Is there a short term (20 to 30 minutes) or long term (3 to 5 hours) change in positive or negative mood measured by PANAS in test subjects who consume naturally found sugar (glucose and fructose) and subjects who consume processed sugars (sucrose, invert sugar syrup, and high fructose corn syrup)?

Materials & Methods

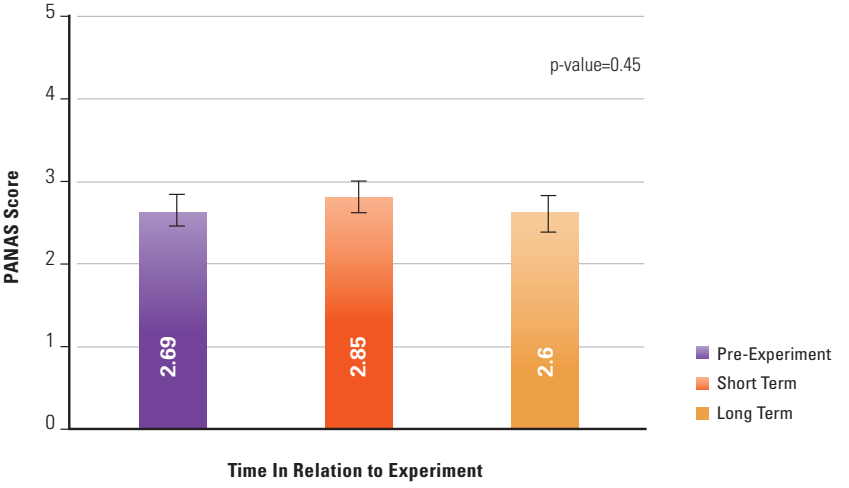
1. Purchase Fanta Soda. Fanta Soda was chosen because it had the highest sugar content out of all sodas in the general supermarket
2. Make smoothies by adding one carton of strawberries, one carton of blueberries, two peeled oranges, two apples, and about 200 mL of water to a blender.
3. Pour 180 mL of soda into cups
4. Pour 180 mL of smoothie into cups
5. Randomly split up 26 test subjects (high school students) into two experimental groups:
   a. Experimental Group A: assigned to consume 180 mL of Fanta Soda during the testing period
   b. Experimental Group B: assigned to consume 180 mL of smoothie during the testing period
   c. Subjects with dietary restrictions are placed in the appropriate group.
6. All test subjects complete the Pre-Experiment form, the Positive and Negative Affect Schedule (PANAS) questionnaire, a self-reporting questionnaire about mood (see Supplementary Materials)

7. After completing the Pre-Experiment form, test subjects in Experimental Group A drink the entire contents of 180 mL of Fanta soda and test subjects in Experimental Group B drink the entire contents of 180 mL of smoothie.
8. 30-40 minutes after consuming either the soda or the smoothie, test subjects complete also complete the PANAS. This second questionnaire is to test whether there is a short term mood change.
9. 3-5 hours after consuming either the soda or the smoothie, test subjects complete the PANAS questionnaire once again in the evening as part of their homework. This third questionnaire is to test whether there is a long term mood change.

Data

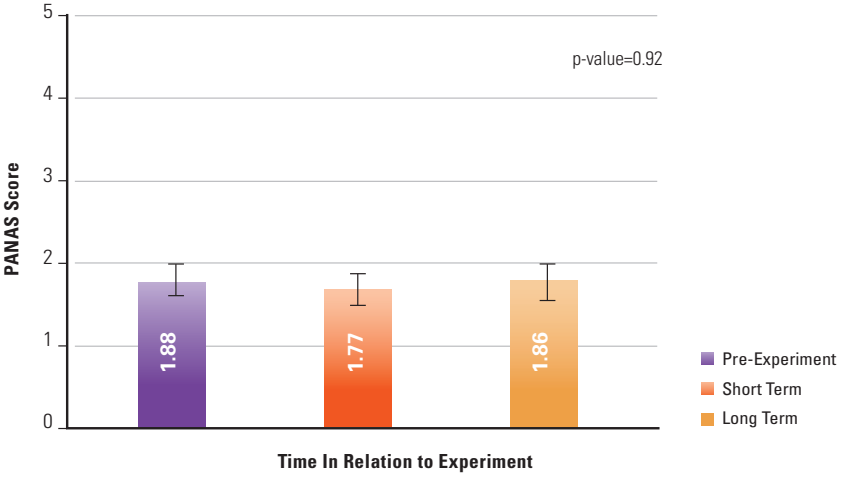
Graph 1

Positive PANAS Score for Smoothie Group



Graph 2

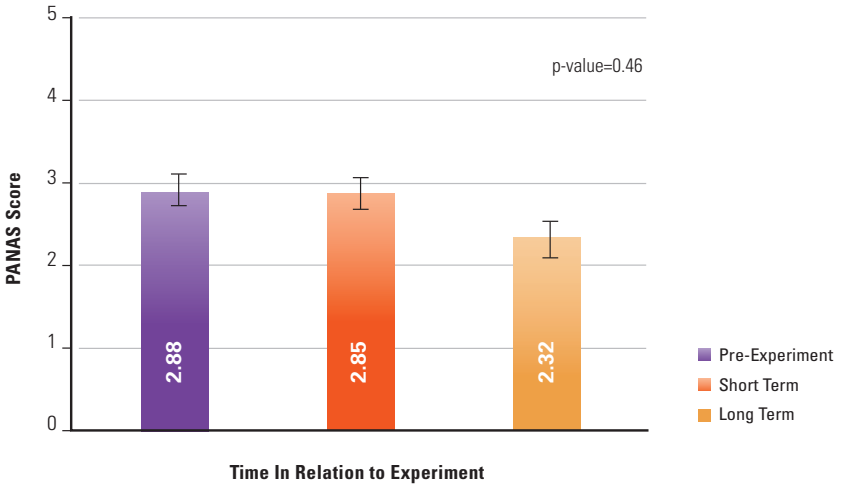
Negative PANAS Score for Smoothie Group



*Short Term: 30-40 minutes after drinking smoothie, *Long Term: 30-40 minutes after drinking smoothie

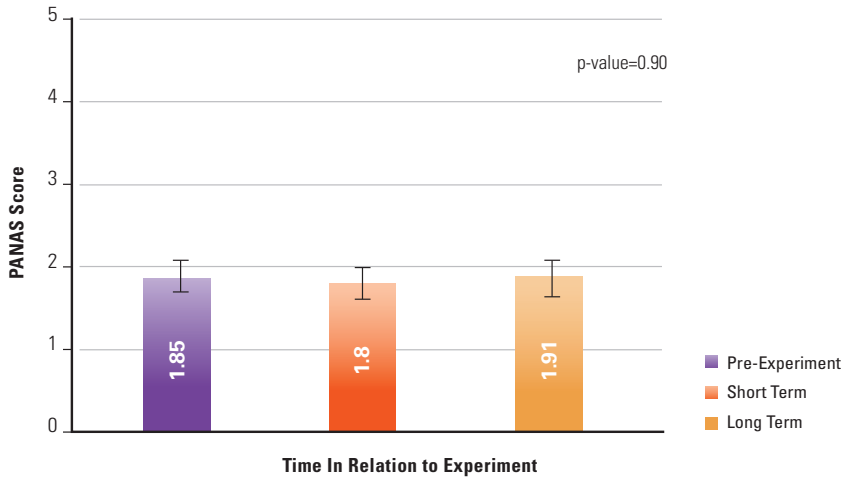
Graph 3

Positive PANAS Score for Soda Group



Graph 4

Negative PANAS Score for Soda Group



*Short Term: 30-40 minutes after drinking soda, *Long Term: 30-40 minutes after drinking soda

Data Analysis

The PANAS scores were calculated first by assigning a value to the five possible answers to the survey questions. “Not at all or very slightly” was set equal to 1, “A little” to 2, “Moderately” to 3, “Quite a bit” to 4 and “Extremely” to 5. For each round (pre-experiment, short term, and long term), both the positive and negative scores of each test subject were averaged then compared. There were 26 test subjects who are Berkeley Carroll high school students, therefore 13 subjects in the smoothie group and 13 subjects in the Soda group. I used a correlated Analysis of Variance (ANOVA) statistics test to calculate

p-value. If the p-value equals less than 0.05, the data set is significant. If it equals more than 0.05, the data is not significant and no difference in population averages can be concluded.

The positive PANAS score for the Smoothie group increased very slightly for the Short Term effect, then decreased to just below the Pre-Experiment score in the Long Term effect. The p-value for this data set is 0.45 and the error bars in the graph overlap which shows no significant difference. The negative PANAS score for the Smoothie group slightly decreased in the Short Term effect, then increased again, returning close to the original Pre-Experiment PANAS score. The data is also not significant because the p-value equals 0.92 and as the graph shows, the error bars overlap.

The positive PANAS score for the Soda group stayed the same in the Short Term effect, then decreased in the Long Term effect. The drop in the positive PANAS score was significant with a p-value of 0.046. This means a conclusion can be made that three to five hours after drinking soda, there was a lack of positive mood in the test subjects in the Soda group. Similar to the positive score in the Soda group there was no change in the Short Term effect, then the negative score slightly increased in the Long Term effect, but not significantly with a p-value of 0.90.

Conclusions/Discussions

In conclusion, most of the data I collected is not statistically significant with the exception of the positive PANAS score for the Soda group in the Long Term effect which decreased with a p-value of 0.046. The positive and negative PANAS score for the smoothie group and the negative PANAS score for the soda group showed no significant difference. For the data that did show statistical significance, the results show that there was a decrease in positive mood 3 to 5 hours after drinking Fanta Soda. This suggests that processed sugar has a negative effect of mood in the long term.

There are many possible errors that could have occured in this experiment. First of all, the effect of mood based on one factor is very difficult to measure because outside influences that could affect mood are impossible to control. For example, sleep, academic stress, family stress, medication, exercise, mental illness, and many more factors affect mood. In between the Pre-Experiment form, Short Term form, and Long Term form anything could have happened that affected a test subject's mood and skewed the data. Some examples of events that could have affected mood in between forms are being taught heavy material in class, getting a good or bad grade on a test, or winning or losing a sports game. In addition to this, it is important to note that the sample size was very small. There were only 26 test subjects in this study which decreases the chance of collecting significant data because it is much more likely that any change in mood was made by chance.

Another potential error is that during the testing period even though all test subjects were told to drink the whole 180 mL of either soda or smoothie, it is possible some test subjects did not finish the entire glass. A third potential error is that these surveys were quite tedious so test subjects could have gotten disinterested in the form and just filled it out randomly. Another factor to think about is that compared to everything else my test subjects would be eating in a whole day, 180 mL of either soda or a smoothie does not seem to be a large factor in their diets.

If I were to take future steps with this experimental question, I would focus on eliminating errors that occured in this study. I would try to control test subjects' diets by designing a meal plan that would incorporate either sugars found naturally or processed sugars more prominently. I would also create a testing environment where there would be no major events such as receiving a graded test or playing in a sports game that could affect mood.

Supplementary Material

Figure 3

PANAS Questionnaire

These are the twenty moods the questionnaire asks about. The scale for the answering the questionnaire is also at the top. (Watson et. al, 1988)

1	2	3	4	5
Very Slightly or Not at All	A Little	Moderately	Quite a Bit	Extremely

_____ 1. Interested	_____ 11. Irritable
_____ 2. Distressed	_____ 12. Alert
_____ 3. Excited	_____ 13. Ashamed
_____ 4. Upset	_____ 14. Inspired
_____ 5. Strong	_____ 15. Nervous
_____ 6. Guilty	_____ 16. Determined
_____ 7. Scared	_____ 17. Attentive
_____ 8. Hostile	_____ 18. Jittery
_____ 9. Enthusiastic	_____ 19. Active
_____ 10. Proud	_____ 20. Afraid

Scoring Instructions

Positive Affect Score:
Add the scores on items 1, 3, 5, 9, 10, 12, 14, 16, 17, and 19. Scores can range from 10 - 50, with higher scores representing higher levels of positive affect. Mean Scores: Momentary = 29.7 *(SD = 7.9);* Weekly = 33.3 *(SD = 7.2)*

Negative Affect Score:
Add the scores on items 2, 4, 6, 7, 8, 11, 13, 15, 18, and 20. Scores can range from 10 - 50, with lower scores representing lower levels of negative affect. Mean Scores: Momentary = 14.8 *(SD = 5.4);* Weekly = 17.4 *(SD = 6.2)*

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How Do Varying Types of Exercise Affect Mood Over the Span of a Day?

by James Pierce

Abstract

The positive effect exercise has on mood is undeniable (Stanton et al, 2014). Many studies have solidified a correlation between the two (Dimeo, 2001). Exercise has even become a popular alternative to medication for treating clinical depression. However, less has been studied about which types of exercise are most effective for improving mood, and when the effect of exercise kicks in. After tracking the mood of 11 subjects over the span of 3 individual workout routines consisting of resistance, calisthenic, and aerobic exercises, I was able to determine that the largest and only statistically significant change in mood came immediately after aerobic exercise (running).

Background

Over the last decade depression has been one of most studied illnesses ("Depression treatment", n.d). Depression is a mental disorder defined as "the feeling of sadness or loss of interest" ("Depression", 2017). However, there are many different types of depression, the most widely known being Major Depressive Disorder (MDD), also known as "Clinical Depression". The difference between MDD and other kinds depression is that MDD is far more persistent. Furthermore, MDD is often believed to be more severe and dangerous due to its symptoms. Symptoms of MDD include physical pains, personality changes, loss of sleep, loss of appetite, and in some instances, suicidal thoughts ("Depression", 2017). In certain cases, patients suffering from MDD will even turn to drugs, alcohol, or self-inflicted pain as a coping mechanism. While its symptoms and effects are widely known, the causes are still unclear. Some suggested causes include differences in brain chemistry, hormones, trauma (ex: loss of a loved one), or even inherited traits (Liao et al, 2013).

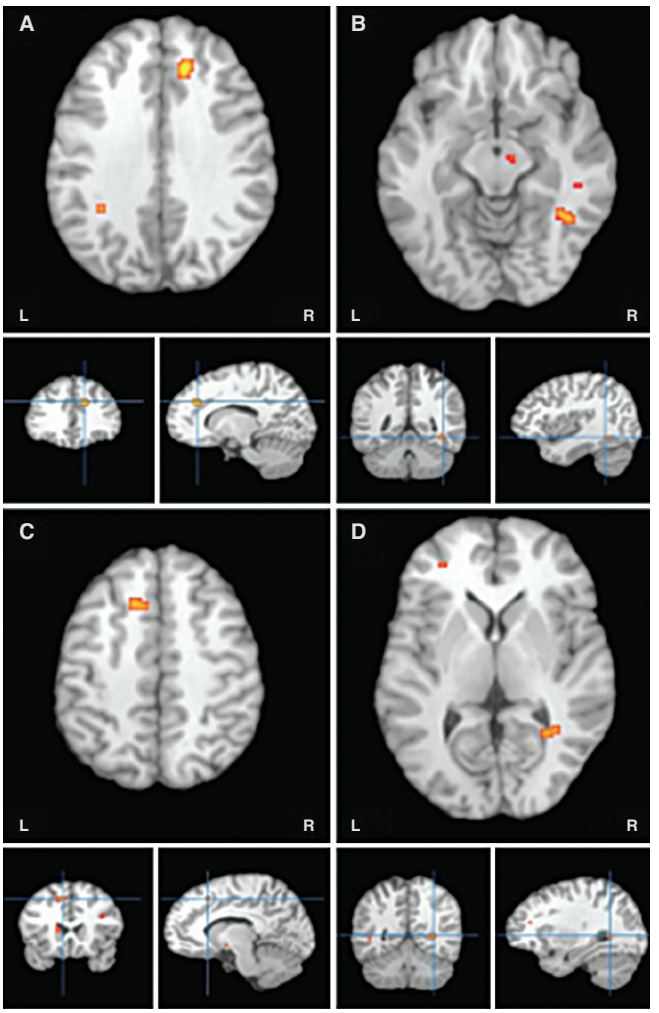
In the 2013 study, *"Is depression a disconnection syndrome? Meta-analysis of diffusion tensor imaging studies in patients with MDD",* Doctor Yi Liao of the Florida Institute of Technology led an extensive research program that observed the brain chemistry and structure of patients suffering from MDD. Dr. Liao and his team compared the brain images of 231 MDD patients and 261 "control" patients from 11 different studies to observe the composition of white matter in different areas of the brain (Liao et al, 2013). White matter is a tissue predominantly found in the "deepest and largest part of the brain" ("What Is White Matter Disease?", n.d). White matter tissue is made up nerve fibers that connect parts of the brain to the spinal cord. Essentially, white matter helps your brain communicate with the rest of the nerves in your body allowing you to "think fast or walk straight" ("What Is White Matter Disease?", n.d). In order to see the composition of white matter, doctors and researchers use diffusion tensor imaging (DTI). DTI is a method in which MRI scans and computer software are used to watch the flow of different molecules in the brain. As water molecules diffuse throughout the brain membranes and tissues,

images of the brain structure and activity become clear (Alexander et al, 2007). After receiving the images and scans of the combined 492 patients, Liao and his team then analyzed the structure and state of the white matter in their brains. After comparing the MDD patient scans to the controls, the researchers discovered a decrease in the amount of white matter in four areas of the brain: frontal lobe, right fusiform gyrus, left frontal lobe and right occipital lobe (Fig. 1) (Liao et al, 2013). Decreased amounts of white matter stem from the breakdown of the diseased myelin ("What Is White Matter Disease?", n.d). These findings support the hypothesis of brain chemistry as a cause of depression, as white matter loss can often lead to memory loss, slowed thinking, as well as other symptoms shared with depression.

Figure 1

MDD Patient Scans

"eta-analytic maps of fractional anisotropy reductions in patients with major depressive disorder; decreases in fractional anisotropy were localized in the (A) white matter of the right frontal lobe, (B) right fusiform gyrus, (C) left frontal gyrus and the (D) right occipital lobe"(3).



Currently many treatments exist for treating depression. While many are small scale holistic treatments, those most widely used are medication and cognitive therapy. Medical treatment for depression consists of putting patients on pills that suppress their depression, such as serotonin reuptake inhibitors (SSRIs) which help regulate the production and distribution of serotonin in the brain and body (Bouchez, nd). Serotonin serves as a neurotransmitter which helps different parts of the brain communicate (McIntosh, 2016). A popular hypothesis regarding the cause of depression stems from serotonin production and regulation going awry. However, medication and pills don't actually cure depression, but temporarily make the patients feel "better" (Derubeis et al, 2008). Cognitive therapy consists of patients talking about their depression with a therapist in order to find ways of recognizing when an episode (a period of time in which the patient feels depressed) is coming on, and how to best prepare for and mentally fight it. More specifically, cognitive therapy helps depressed individuals set positive goals for themselves as well as develop better, more individualized, coping mechanisms. Additionally, cognitive therapy helps patients prioritize more severe problems and stressful environments that need to be addressed over minor ones (Cherry, n.d). However, many people have started to criticize both cognitive therapy and medication due to the fact they don't always work and in some cases they make the patient's depression worse (Derubeis et al, 2008).

Due to the lack of successful cognitive and medical treatments, many doctors and researchers have started looking at exercise as a possible treatment for depression (Dime, 2001), since exercise has been shown to be linked with endorphin release. The 1994 study, *"Physical exercise stimulates marked concomitant release of β-endorphin and adrenocorticotropic hormone (ACTH) in peripheral blood in man"*, led by Dr. Franco Fraioli, of the University of Rome, tracked the release of β-endorphin and ACTH in men after exercise (Fraioli et al, 1994). Both β-endorphin and ACTH are hormones associated with stress relief. Dr. Fraioli and his team used 8 professional male athletes, aged 19-23, for the experiment. After inserting a catheter into to forearm of each subject and drawing a small sample of blood, the subjects ran on a treadmill at 3 different speeds for 2 minutes each: 6 km/h, 9 km/h, and 12 km/h. The subjects then ran at 15 km/h until they reached "maximum aerobic capacity" (maximum lung capacity as measured by a spirometer) (Fraioli et al, n.d). Blood was then drawn again from the subjects immediately after exercise, 15 minutes after exercise, and 30 minutes after exercise. After conducting blood tests on all the samples, Dr. Fraioli found boosted amounts of β-endorphin and ACTH at all 3 times; however, particularly immediately after exercise (Fig. 2) (Fraioli et al, n.d). In conclusion, both β-endorphin and ACTH were released from the pituitary gland (the master gland attached the bottom of the brain that regulates hormone release) (Fraioli et al, n.d) during and after exercise.

Figure 2

Data from "Physical exercise stimulates marked concomitant release of β-endorphin and adrenocorticotropic hormone (ACTH) in peripheral blood in man"

Condition	Basal Conditions	100% Maximal Effort	15 Minutes After Stopping	30 Minutes After Stopping
ACTH (pg/ml)	80±15	850±25	320±45	95±30
β-Endorphin (pg/ml)	320±70	1620±250	1080±120	420±65

While almost all forms of exercise can lead to positive uptakes in mood, certain forms have been shown to be more effective. The case study "Exercise and the treatment of depression: A review of the exercise program variables" led by Dr. Robert Stanton of Queensland University, compared the effectiveness of different types of exercise considering variables such as intensity and form (Stanton et al, 2014). Stanton and his team compared data of "adults aged 18–65 years with a diagnosis of depression" (Stanton et al, 2014) across a wide range of studies. The forms of exercise tested include resistance training, weightlifting, and aerobic. Different delivery methods (group vs individual) and frequency

Table 1

Comparison of Different Exercise Depression Treatment Studies

Summary of the Aerobic Exercise Prescription Guidelines for Healthy and Clinical Populations and for those with Depression

Author	Frequency (per week)	Intensity	Session Duration
Perraton et al.[23] (depression)	3	60–80%HR_{max}	30 min
NICE[18] (depression)	3	NR	45–60 min
Garber et al.[24] (healthy population)	≥5	Moderate	Min 30 min/session or ≥150 min/week
	≥3	Vigorous	Min 20 min/session or ≥75 min/week Or a combination to achieve ≥500–1000 kcal/week
Horden et al.[23] (type II diabetes and pre diabetes)	Min 3 with no more than 2 consecutive days without exercise	Moderate (55–69%HR_{max}) or Vigorous (70–89%HR_{max})	Min 210 min/week of moderate or 125 mins/week of vigorous exercise or a combination of both
Stanton and Reaburn (current review) (depression)	3–4	Low – moderate or patient preferred	30–40 min

Table 2

Effectiveness of Different Exercise Depression Treatment Studies

Program Variables of Aerobic Interventions

Author	Frequency (per week)	Intensity	Session Duration
Chu et al.[5]	4–5	High (65–75%$VO_{2reserve}$) Low (40–55%$VO_{2reserve}$)	30–40 min
Callaghan et al.[6]	3	Patient preferred	NR
		Prescribed (RPE = 12)	NR
Mota-Pereira et al.[28]	5 (1 supervised, 4 unsupervised)	Moderate	30–45 min
Schuch et al.[30]	3	Variable – duration and intensity aimed to expend 16 kcal/kg/week	
Trivedi et al.[29]	3 (median)	Variable – High dose (16 kcal/kg/week	
	2 (median)	Variable – Low dose (4 kcal/kg/week)	

NR – not reported

(how often) of exercise were also observed and compared. Stanton and his team concluded that all types of exercise were shown to benefit patients to differing degrees (Table 1-2). The most interesting finding amongst the case studies was how patients differing in age, gender, diagnosis, etc responded to different types of treatment. For example, older patients responded best to low intensity workouts while younger patients responded better to a high intensity workout. However, the most substantial results came from "supervised aerobic exercise, undertaken three times weekly at moderate intensity for a minimum of nine weeks in the treatment of depression" (Stanton et al, 2014).

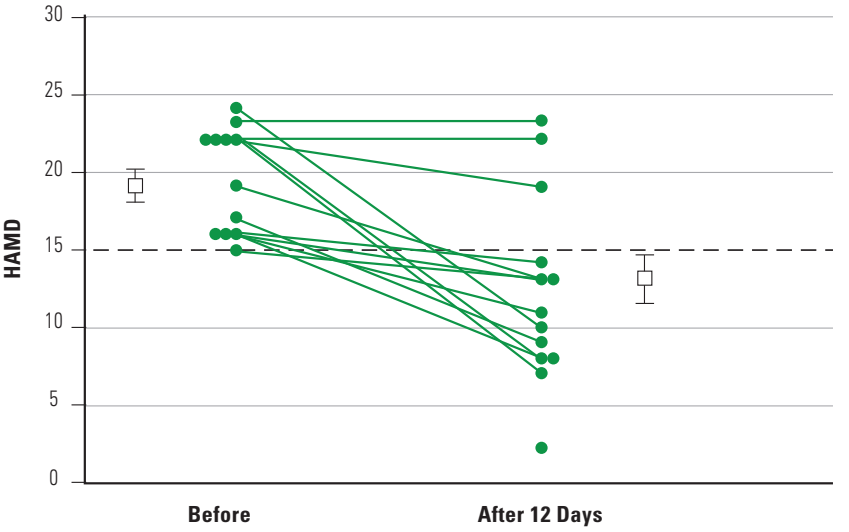
Mode of Exercise	Intervention Duration	Individual or Group	Level of Supervision
Individualized according to preference	8 weeks	Group or individual	Recommended. Qualifications and experience unspecified
NR	10–14 weeks	Group	Competent practitioner
Individualized according to preference	Ongoing	NR	Experienced fitness instructor
Walking, running cycling or swimming	Ongoing	NR	Appropriately trained and qualified personnel
Any aerobic activity	9 weeks	Group or individual	Appropriately trained and qualified personnel

Mode of Exercise	Intervention Duration	Individual or Group	Level of Supervision	Compliance
Treadmill (supervised), personal preference when unsupervised	10 weeks	Individual	Once weekly by investigator	80–100% (supervised session)
Treadmill	4 weeks	Group	Qualified exercise therapist	66% 50%
Treadmill (supervised), outdoor walking (unsupervised)	12 weeks	Both	Physical training teacher	91%
Stationary cycle, treadmill or cross trainer	Variable – final assessment at hospital discharge	Individual	Trained researcher	NR
Treadmill and/or cycle ergometer	12 weeks	NR	Cooper Institute staff	63.8% 99.4%

The study "Benefits from aerobic exercise in patients with major depression: a pilot study" led by Dr. F. Dimeo, published by the British Journal of Medicine, compares the diagnosis of 12 patients (5 men 7 women with a mean age of 49) suffering from clinical depression, before and after a 12 day workout regimen (Dimeo, 2001). Subjects were diagnosed by the Scale for Self Assessment of Depression (DS) test, as well as the Hamilton Rating Scale for Depression (HAMD) test. After the 12 days of walking on a treadmill for 30 minutes, the patients retook the tests. Dr. Dimeo and and his team discovered that the mood score decreased after the 12 day workout plan indicating a positive mood improvement following the exercise (graph 1-2) (Dimeo, 2001).

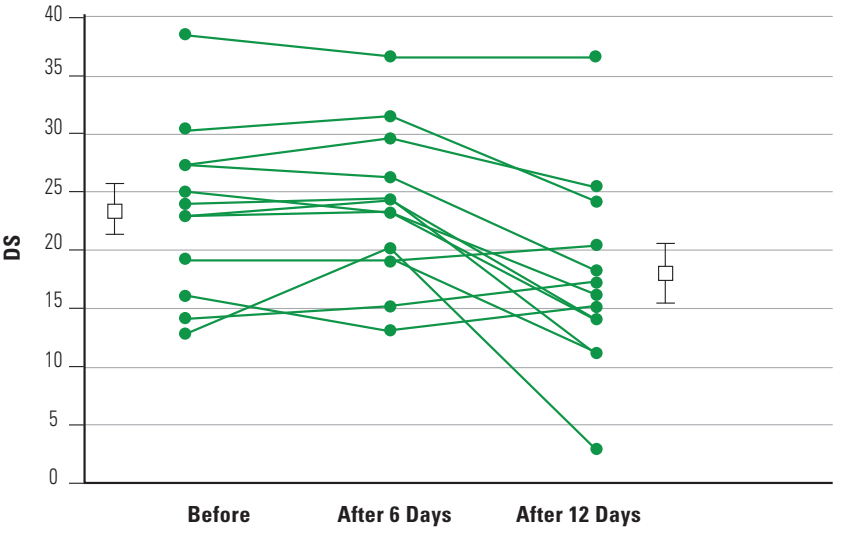
Graph 1

HAMD Test Score Before and After Study



Graph 2

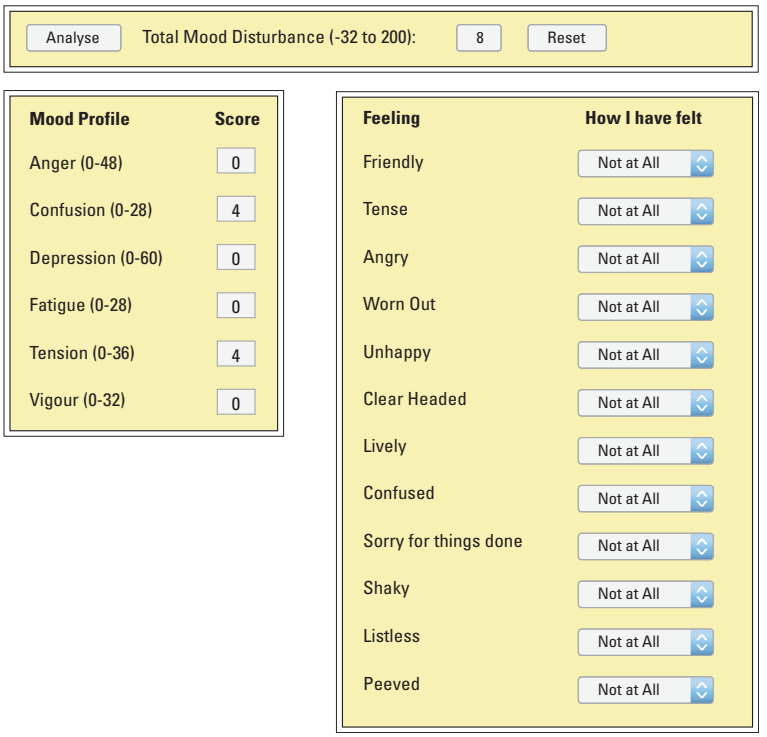
DS Test Score Before, Halfway, and After the Study



Lastly, the University of Wolverhampton's study, *"The effects of exercise on mood changes: The moderating effect of depressed mood"*, compared the mood test scores of 80 males, age 23-32, before and after a 1 hour aerobic dance class (Lane et al. n.d). First, the males were split into a depressed group and a non-depressed group. Before the class began, all the subjects received a mood profile as measured by the Profile of Mood States (POMS) test (Fig. 3). The subjects then took the POMS test for a second time directly after the class. Primary researcher Andrew Lane and his colleagues discovered that the decrease in "anger, confusion, fatigue, and tension, and increase in vigor was significantly greater in the depressed mood group" vs the non depressed group (Lane et al. n.d). These findings helped strengthen the link between exercise and mood, but also showed a more direct correlation between exercise and mood in people suffering from depression.

Figure 3

POMS Questionnaire, Mood Profile and Mood Score



All of these studies were applied to patients suffering from clinical depression, and did not consider how these findings may apply to people not suffering from depression. Using these papers as models, I was able to design my own research project. Rather than testing the effects of exercise as a form of treatment for depression, my study tracks the mood of BC students by using a mood survey in order to answer the question; how do varying types and intensities of exercise affect the mood of students over the span of 24 hours? According to Merriam-Webster, the definition of mood is a conscious state of mind or predominant emotion (Webster, n.d). A mood score is a quantification of emotions. When comparing a mood score (dependent variable) of a subject before, immediately after, 5 hours after, and 24 hours after exercise (independent variable) I will be able to track whether the exercise has an immediate effect on the subject's mood, if it takes time to kick in, or if there is an effect at all.

Materials

- Treadmills
- Mood Survey
- Dumbbells
- Pull up bar
- Yoga mats
- Weight bench

Methods

Alternative Hypothesis: There is a difference in BC student's mood before, directly after, and 24 hours after aerobic exercise, calisthenics, or weight training.
Null Hypothesis: There is no difference in BC student's mood before, directly after, and 24 hours after aerobic exercise, calisthenics, or weight training.
Experimental Hypothesis: Alternative Hypothesis

Eleven subjects, all of whom were seniors, completed 3 separate workout routines each lasting a week. The first workout routine consisted of only aerobic exercise: running. More specifically, subjects ran for 25 minutes at a reasonable pace. The second workout routine consisted of pure calisthenics: push ups, dips, crunches, and other bodyweight exercises. The last workout the subjects completed was a resistance training routine for their arms. This routine was made up of dumbbell curls, shoulder press, tricep extensions, and many other similar exercises. Every day before exercising, subjects would fill out a mood survey. The survey consisted of 20 "emotions" or "feelings" such as "happy," "tired," or "anxious". The subjects rated on a 1-5 scale (1 being "very little," 5 being "a lot") how much they were feeling that emotion. The "bad" emotions (tired, unhappy, etc) resulted in a negative score, while the

Figure 4

Calisthenics Workout Plan ("Max Reps", n.d)

positive emotions (happy, lively, etc.) resulted in a positive score. Once all emotions were filled out the numbers were added. The final score was between -40 to 40. The lower the score the worse mood they were in. The last question on the mood survey asked the subject to state whether any specific event had impacted their mood that day. This was to determine whether the mood increase or decrease was directly related to the exercise or outside factors. If there was some substantial event, that day was omitted in the data. The subjects then filled out another mood survey both immediately after exercising and 24 hours after exercising. After each 1 week workout routine was completed, subjects' responses were averaged to make a standard mood profile, which can be seen in the data.

Figure 5

Weight Training Workout Plan (Morris, n.d)

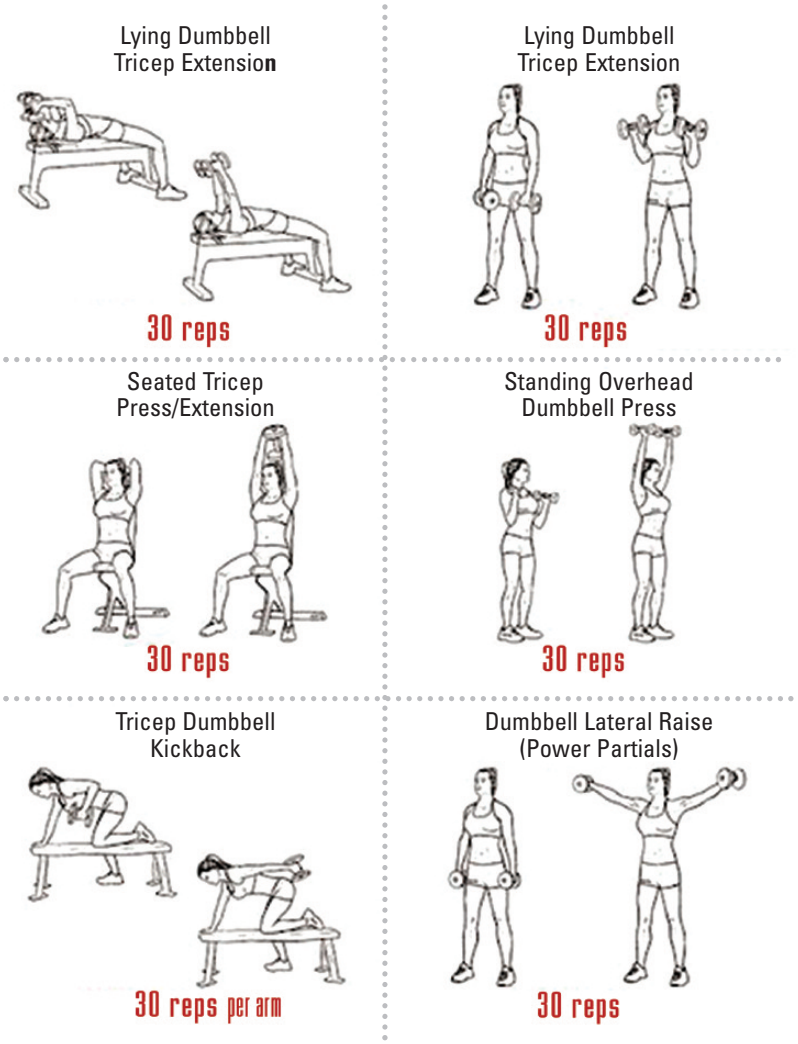


Figure 6

Questions from Personal Mood Tracker Survey

Friendly						
	1	2	3	4	5	
Not at all	○	○	○	○	○	Very
Tense						
	1	2	3	4	5	
Not at all	○	○	○	○	○	Very

Data

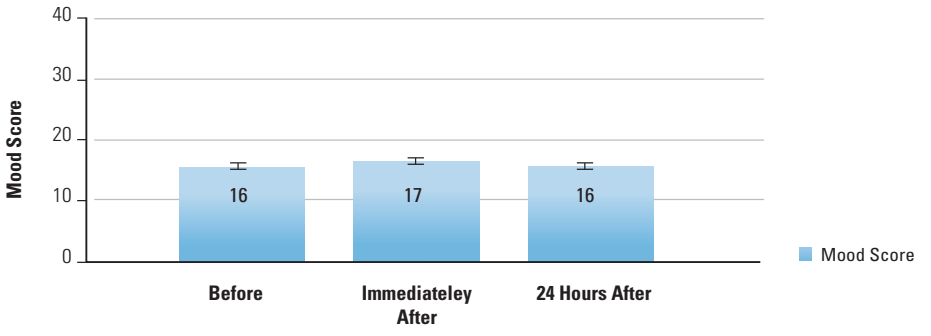
CALISTHENICS MOOD SCORES

Table 3

Table Showing Mood Scores of Participants Before, Immediately After, and 24 Hours after Calisthenics

Subjects	Before	Immediately After	24 Hours After After
1	19	21	18
2	10	15	13
3	13	17	19
4	17	20	16
5	16	19	15
6	12	17	12
7	15	13	16
8	14	16	15
9	17	17	19
10	15	16	14
11	17	16	19

Average Mood Score Calisthenics

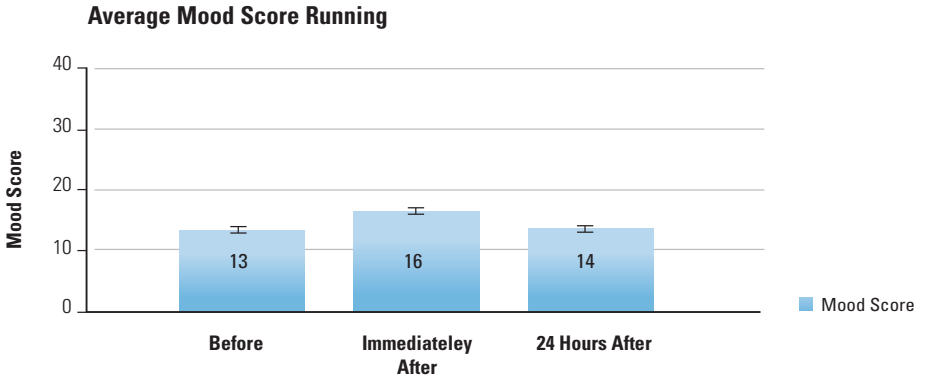


RUNNING MOOD SCORES

Table 4

Table Showing Mood Scores of Participants Before, Immediately After, and 24 Hours after Running

Subjects	Before	Immediately After	24 Hours After After
1	16	19	14
2	14	20	16
3	11	15	12
4	17	18	18
5	13	16	13
6	16	18	15
7	15	17	11
8	9	14	13
9	11	12	16
10	11	14	12
11	10	13	14



WEIGHT TRAINING MOOD SCORES

Table 5

Table Showing Mood Scores of Participants Before, Immediately After, and 24 Hours After Weight Training

Subjects	Before	Immediately After	24 Hours After After
1	10	13	12
2	12	14	14
3	16	20	19
4	15	16	13
5	13	14	14
6	10	11	10
7	18	19	18
8	12	16	15
9	11	13	12
10	17	18	17
11	9	11	10

Average Mood Score Weight Training

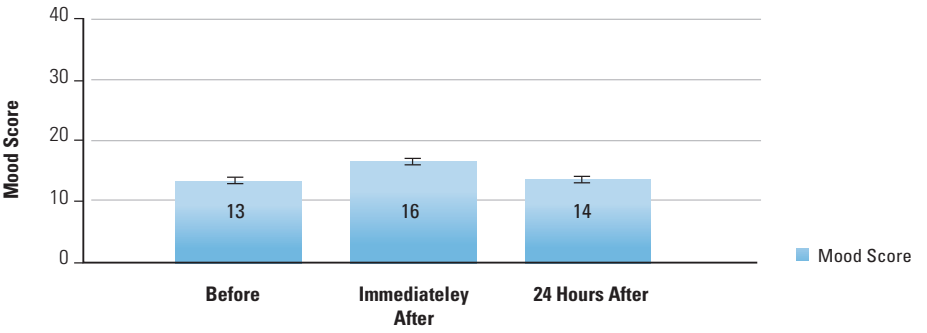


Table 6

Table Comparing the Average Mood Scores of All 3 Exercises at All 3 Different Times

	Before	Immediately After	24 Hours After After
Calisthenics	15	17	16
Running	13	16	14
Weight Training	13	15	14

Analysis

For all 3 types of exercise the subjects' average mood was highest immediately after exercising. The largest increase in mood score occurred immediately after running, when the subjects' average mood score increased by three from 13 to 16. Respectively, the subjects' average mood score increased by two immediately after both resistance and calisthenics, with increases of 13 to 15 and 15 to 17. In all three forms of exercise the subjects' average mood score increased by one 24 hours after exercising: 15 to 16 after running and 13 to 14 after resistance and calisthenics. Similar results can also be found on the individual subject scores. However, subjects 3, 9, and 11 experienced their highest mood score not immediately after exercise, but 24 hours later (table 3). The data suggests that all the forms of exercise had a positive effect on mood. However, after conducting an ANOVA test the change in mood score was only statistically significant when subjects were running which resulted in a p-value of .02. Weight training resulted in a p-value of .3, and calisthenics at .42.

Discussion

To better understand the connection between mood and exercise, this study tested how different types of exercise can affect both the short and long term mood of subjects. However, this study does not account for whether the time or type of exercise plays a larger role in determining the effectiveness of exercise. To further study this connection, research should be conducted on immediate and future dopamine release when partaking in aerobic exercise, calisthenics, and resistance exercise.

In addition, every subject responds differently to varying types of exercise for many reasons. For example, a 25 minute run may be easy for a trained runner, but quite challenging for a bodybuilder who is not accustomed to large amounts of cardiovascular exercise. Likewise, a runner may struggle at resistance exercise while it is easy for the bodybuilder. This is because as humans we get better at what our bodies grow accustomed to. This is a factor that quite possibly could have skewed data collection in this experiment because no research was done on these specific subjects' exercise history or knowledge. Another factor that may have affected the data collection is that running is the simplest form of workout routine to design and maintain, therefore it is easier for the subjects to get accustomed it. Lastly, subjects may not have been fully reliable when filling out surveys. For example, slate surveys would not be an accurate representation of the immediate or 24 hours effect. It is also possible subjects were not fully honest when filling out their surveys for personal reasons. However, this data is consistent with past research and resulted in a statistically significant p-value for mood over time as a result of aerobic exercise.

In conclusion, this study determined that the only form of exercise that has a statistically significant effect on mood is aerobic exercise. However, raw data still suggests that both resistance training and calisthenics may affect mood over the span of 24 hours, which should be further studied on larger scale experiment that accounts for variables such as age, exercise/medical history, and fitness.

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Memory Retention and Emotional Stimuli in Accordance to Race

by Alyssa Pierre

Abstract

In this study, forms of emotional stimulation are used to test the retention of emotions in people of different races to find if certain races retain specific emotions more than others. The experimental question is: "Does emotional arousal generate higher recall of specific details than basic stimulation in accordance to race?" To test this, Berkeley Carroll freshmen and sophomores completed a preliminary survey to collect their race and other factors (controls). Afterwards, they were given three stories, each corresponding to a certain emotion: anger, fear, and sadness. To test the different forms of stimulation, one group was given the stories alone (basic stimulation) while the other group was given the stories and a visual/auditory component (arousal) to heighten the three emotions. After two days, the subjects were administered a comprehension quiz on specific details from the stories, and it was found that the form of emotional stimulation does not affect the retention of specific details from different emotions in accordance to race. Arousal does not generate higher retention of specific details from different emotions in accordance to race, and basic stimulation does not generate higher retention of specific details from different emotions in accordance to race except when it comes to the scary story.

Background

Terms

- *Precise memory* - memory of details (NorthWestern, 2017)
- *Retention Score* - numerical value that represents memory recall of an individual
- *Autobiographical memory* - the "recollections of specific, personal events" (Holland, 2010)
- *Amygdala* - located in the frontal portion of the brain, near the hippocampus, and is responsible for "deciding what will have the emotional connection due to the beta-adrenergic system" (Gallagher, 1977)
- *Hippocampus* - located in the frontal portion of the brain, and decides "what is relevant and important enough to be encoded into [a] memory; for information to be deemed relevant it must either be continually rehearsed in the brain or have a strong emotional component" (Burnette, 2015).
- *B1 receptor* - "receives sensory input (vision, hearing, somatic sensation)" (Dartmouth, 2006). Located in beta-adrenergic system in the amygdala
- *Beta-adrenergic system* - a "neurochemical system" (Kuhar,1999)
- *Retention test* - test that assigns scores based on memory retention

Arousal

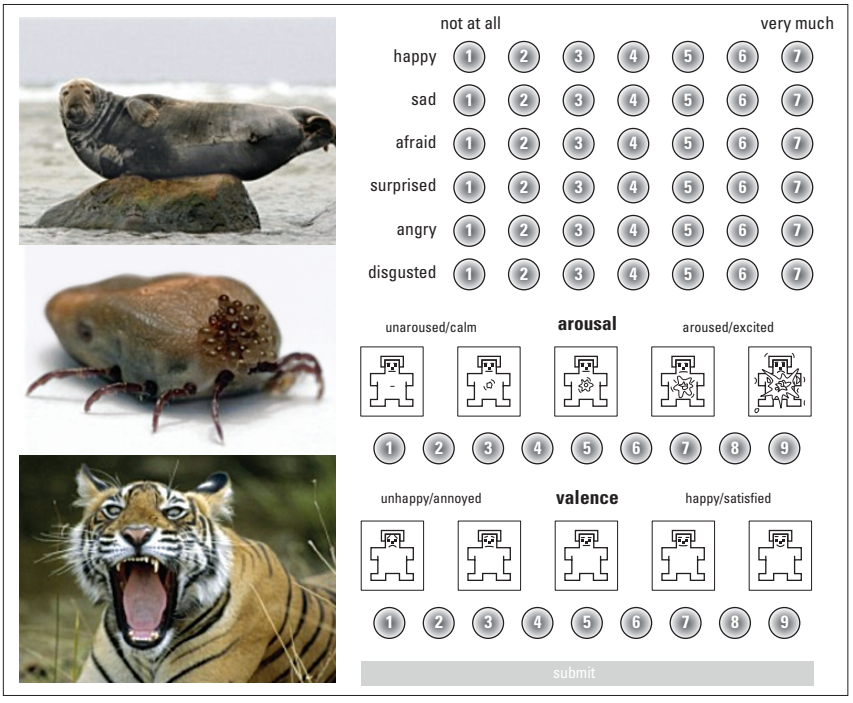
Emotions are "a conscious mental reaction (such as anger or fear) subjectively experienced" (Merriam-Webster) and can be divided into groups based on the intensity of a stimulus. If a stimulus results in a passive experience, we refer to these as feelings (basic stimulation). On the other hand, if the stimulus is greater, such that it induces the body into action or readiness for action, then this stimulus is said to have caused an arousal.

Emotion in the brain is first encoded through sensory input, then information from the senses is encoded in the orbitofrontal cortex, which is the area of the brain responsible for emotional processing and records individual subjective feeling (Chikazoe, 2014). Once the emotion has been processed, the orbitofrontal cortex sends it to the amygdala where an emotional significance is attached to it in correspondence with the individual's subjective feeling. The hypothalamus acts upon the emotional significance by determining the function that the body will do in response to the emotional significance and depending upon the intensity the function can either be low or high. A low response would suggest a passive experience (basic stimulation), a feeling, while a high response, or arousal, might induce a physiological response such as sweaty hands or shakes. (Mckay, 2016)

A study completed in 2016 tested simulation and sensitivity effect on long-term recognition. The study was a year-long experiment that consisted of rating images on a scale of arousal and valence *(relative capacity to unite, react or interact* (Merriam-Webster)). The participants were given 170 random images from three of the experiment's subsets: valence, emotions, and arousal. They were then asked to rate on a scale from 1-7 how happy, sad, afraid, surprised, angry, and disgusted the photo made them feel and rate on a scale from 1-9 on arousal and valence (Fig. 1). The study found that

Figure 1

Testing for Stimulation and Sensitivity Effects on Long-term Recognition



images rated highest on the arousal scale were retained more than those that scored lower on the scale due to arousal activating the brain more than basic emotion, and found no significant difference when it came to low stimulating emotions. From this, Marchewka indirectly concluded that stimulation enhances long-term recognition more than sensibility. (Marchewka, 2013)

Further supporting Marchewka's observations that arousal improves retention, Katherine Steinmetz at Boston University conducted a study with twenty-one subjects – nine females, and twelve males – who were exposed to three hundred arousal images and one hundred fifty neutral images. The images were deemed neutral or arousing by encoding memory retention processes, meaning that they analyzed how a subject encoded the information using an fMRI (functional Magnetic Resonance Imager). (Steinmetz, 2009). An hour and a half later, subjects were given a surprise retention test on the images, where they were shown each photo again for 2 seconds and asked to record whether or not they had seen it prior to the retention test. It was observed that the arousal images were retained more than the neutral images. This study concluded that "emotionally arousing information" is retained more than "neutral information" (Steinmetz, 2009).

A study completed by Gasbarri in 2005 also tested memory retention in accordance to emotional stimuli but stimulated subjects auditorily rather than visually with an additional inflection (greater emotional content) on stimulus level. The study consisted of two groups (neutral and emotionally stimulated), where both groups were told a story accompanied by slides and narration. The only difference was that the emotionally stimulated group had more emotional content in their slides and the narrator would emphasize the emotions more by crying during the sad story, laughing while telling the funny story or yelling the text during the angry story. The study concluded that those in the arousal group recalled more elements from the story than the neural group. (Gasbarri, 2005).

Emotions

Knowing that there is a direct correlation between memory retention and the intensity of emotional stimuli, is there a difference when it comes to autobiographical memory? A meta-study conducted by Alisha C. Holland at the Princeton University analyzed studies that tested emotion's effect on autobiographical memory (Holland, 2010) and found that emotional content of an experience influences the way the experience is remembered. Emotional content is further broken up into two sections: expected and cultural. Expected emotional content is known as the "rosy phenomenon" which was found in 1997 by Terence R. Mitchell. Mitchell tested if the way a subject expects the experience to be can affect the way the experience is later recalled and found that if a subject expected a highly positive experience, but experienced a slightly less positive event, the subject would nonetheless recall the experience as highly positive. An example Mitchell discusses is a vacation trip: if an individual expects a vacation to be fantastic, it leads that individual to have a slightly less fantastic trip because their expectations were too high but the individual will still remember the trip, years later, as fantastic. This study concluded that expected emotional content influences recall of an experience. (Mitchell et al., 1997).

Cultural emotional content refers to the idea that specific emotions that a culture values has a direct influence on how an experience is remembered. A study done in 2006 by associate professor Jeanne Tsai at Stanford University tested American students who valued the "cultural idea of high arousal positive affect" – a cultural value of more highly positive emotions – (Holland, 2010) and students who did not, and found that those who valued high arousal affect recalled more positive effects than those who did not and vice versa, concluding that cultural emotional content also has an effect on recall of experiences. (Tsai et al., 2006)

Another way emotions play a significant role in autobiographical memory retention is that emotions experienced when the autobiographical retrieval takes place influences the information recalled. (Holland, 2010). Feldman Barrett, a psychology professor at Northeastern University, conducted a study in 1999 that explored this role of emotions. The study consisted of subjects rating their emotions over the course of ninety days and then later recalling those emotions over that time period. The subjects were then given a score of extraversion (relating to higher positive levels of affectivity) and neuroticism (relating to higher levels of negative affectivity) to describe their personality, and it was found that subjects who had a higher extraversion score recalled feeling more positive than those who had a lower extraversion score, and subjects who had a higher neuroticism score recalled feeling more negative than those who had a lower neuroticism score, concluding that personality has a direct affect on information remembered. (Barrett et al., 1999)

These findings sparked discussions on the way emotions interact with memory retention and what neural mechanism supports it and how findings from literature, whether clinical or social psychology, can help support future explorations on the relationship between emotions and retrieval.

Race

Considering that there is an established direct relationship between the intensity of emotion and memory retention, racial groups which have historically suffered oppression for many years, may have a higher tendency to retain negative information than a race that has not had to deal with the same type of oppression (Monitor, 2016).

A study done in 2010 found that 70% of the students involved in a school-related crime were from a minority group, specifically Hispanic or Black. (Education Week, 2013). A survey of 72,000 students done in 2009-2010 concluded that "Black students were three and a half times more likely to be suspended or expelled than their White peers" (Lewin, 2012). The data from both studies confirm the idea that oppressed races receive harsher consequences in schools which can attest to the idea that those oppressed will retain negative emotions more than those who are non-oppressed, because, in some school settings, oppressed students are treated more negatively than non-oppressed students.

Inspired by Holland's work and considering Marchewka and Steinmetz's work on the increased level of retention due to arousal response, I decided to investigate the following: *Does emotional arousal generate higher recall of specific details than basic stimulation in accordance to race?* The independent variables are race and the way the subject is exposed to emotion (arousal or stimulation), and the dependent variable is retention scores. The goal of this study is to find more ways that can improve memory and explore the historical and social impact on a person's perception of emotional stimuli.

Methods

Emotions: Fear, Sadness, Happiness

1. Break subjects into two groups that correspond to type of stimulation
    a. Group 1: Control (basic stimulation) – read stories without visual/auditory aid
    b. Group 2: Experimental (arousal) – read stories with visual/auditory aid
2. Administer preliminary data collection survey two days prior to beginning of study
    a. Survey collects race, gender, grade and the subject's favorite color
        i. Organize subjects into race groups
            1. Minority
            2. Majority
        ii. Further divide race group, evenly, into groups based on type of stimulation. Groups 1 and 2 should have fifty percent minority and fifty percent majority
    b. Begin study two days later
3. Control Group
    a. Administer three emotionally stimulating stories at the end of the week
        i. Three stories are used, each one focuses on one of the primary emotions (Fear, Sadness, Happiness). See attached stories in supplemental materials
        ii. Subjects read each of the three stories
            1. Stimulation
    b. Administer a comprehension quiz at the beginning of the next week
    See attached Quiz in supplementary materials
        i. This quiz assesses the recall/retention of specific facts/items from the previous stories
        ii. Quizzes will be analyzed to determine the "strongest" type of emotional reaction/recall
            1. Quizzes will be analyzed according to specific scores on the retention test
                a. Higher score = higher retention
                b. Scores will be analyzed using one way ANOVA test to compare data sets
                c. p-values will assess marginal significance
        iii. Quizzes will be analyzed to determine emotional reaction/recall of specific details from stories by each race

4. Experimental Group
    a. Expose subjects to three emotionally stimulating stories but with an added visual and auditory component that correlates to each story at the end of the week. See attached files in supplementary information.
        i. Visual – Images
        ii. Auditory – background music
            a. Images are embedded into the story
            b. Music is played alongside the story
            1. Visual aid and auditory aid are intended to increase the intensity of the stimuli, further inducing a physiological response (arousal)
                a. Subject will read stories
                b. Corresponding background music will be playing alongside the reader
                c. Arousal
    b. Administer a comprehension quiz at the beginning of the next week
        i. Quizzes assess the recall/retention of specific facts /items from the previous movie clips
        ii. Quizzes will be analyzed to determine the "strongest" type of emotional reaction/recall
        iii. Quizzes will be analyzed to determine certain emotional reaction/recall in each race
            1. Quizzes will be analyzed according to specific scores on the retention test
                a. higher score = higher retention.
                    i. Each story had 10 questions (1/10 pts per question)
                        1. 10/10 = highest score
                        2. 1/10 = lowest score
                            a. In total, whole quiz was 30 pts
                b. Scores will be analyzed using one way ANOVA test to compare data sets
                c. p-values will assess marginal significance

Data/Graphs

Figure 2

HAPPY Retention Scores

	Minority	Majority
Group 1	9,9,8,10,9,8,5,7,7,9,2,10,9 *Average:* ***7.85***	7,4,5,9,7,7,7,8,10,8,9,7,10,8,4,5,7,8,9,5,8 *Average:* ***7.43***
Group 2	3,6,7,9,4,1,9,10,9 *Average:* ***6.44***	7,6,5,3,1,6,7,10,9,8,8,5,7,10 *Average:* ***6.57***

Figure 3

SAD Retention Scores

	Minority	Majority
Group 1	5,8,5,9,7,7,7,8,8,9,1,8,6 *Average:* ***6.77***	9,4,7,9,5,6,3,4,6,6,7,7,8,5,6,6,8 5,6,7,8,5,8 *Average:* ***6.30***
Group 2	4,10,6,8,3,0,9,6,7 *Average:* ***5.89***	9,6,5,3,4,4,5,8,8,7,8,7,6,9 *Average:* ***6.36***

Figure 4

SCARY Retention Scores

	Minority	Majority
Group 1	6,6,8,8,9,6,6,9,10,9,3,10,9 *Average:* ***7.62***	10,2,8,3,2,2,4,4,8,7,8,7,7,4,7,5,6 2,8,7,8,3,9 *Average:* ***5.70***
Group 2	3,6,8,6,2,4,8,5,9 *Average:* ***5.67***	8,8,2,1,3,3,5,5,7,8,7,9,8,7 *Average:* ***5.79***

Figure 5

Type of Stimulation v. Retention Score

Describes the relationship between the type of stimulation (either basic stimulation or arousal) and retention score in both groups. The relationship reveals that there is no significant difference in type of stimulation and retention; both groups retained a similar amount of information from all three stories. Averages can be seen in Figures 1-3.

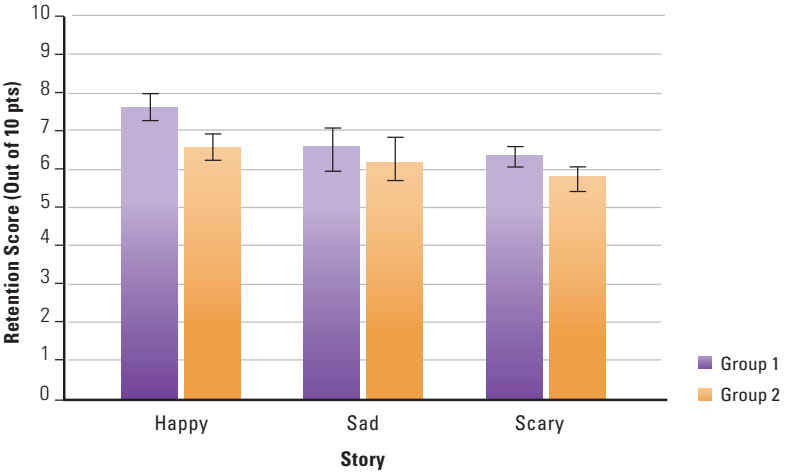


Figure 6

Race v. Retention in Group 1

Describes the relationship between the type of stimulation (either basic stimulation or arousal) and retention score in both groups. The relationship reveals that there is no significant difference in type of stimulation and retention; both groups retained a similar amount of information from all three stories. Averages can be seen in Figures 1-3.

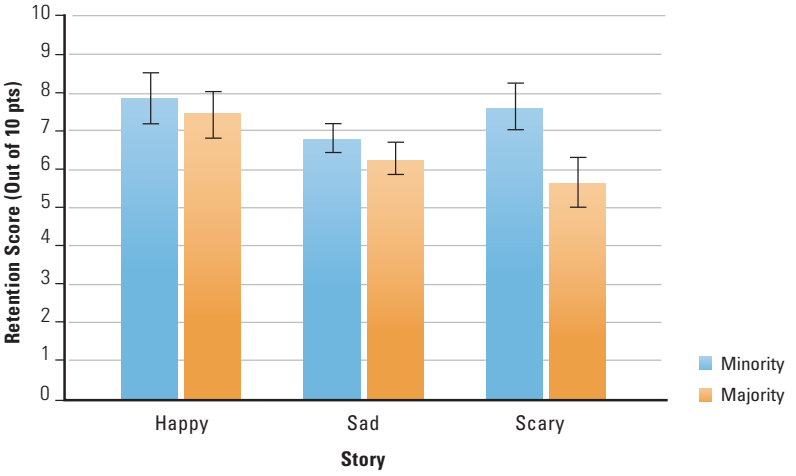
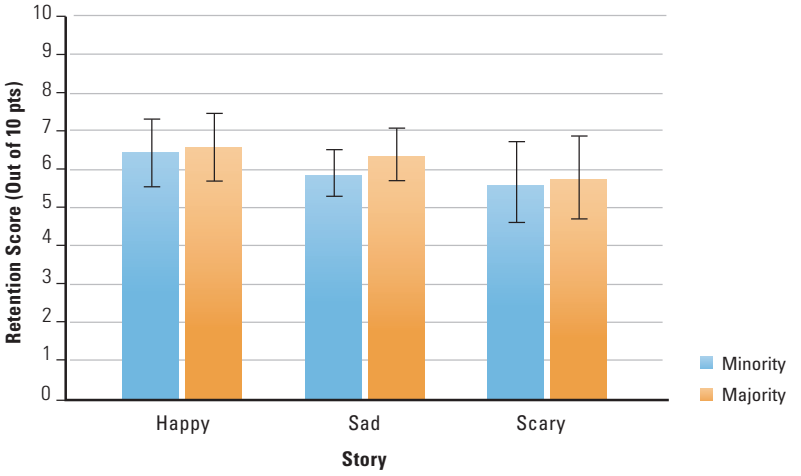


Figure 7

Race v. Retention Score in Group 2

Describes the relationship between retention and race in group 2 (arousal). The relationship reveals that there is no significant difference in race and retention score – when it comes to arousal – in the stories. Minorities and majorities retained a similar amount of information from the all stories. Averages can be seen in Figures 1-3.



Analysis

Using One-way ANOVA with Two Samples:

The data show that there is no significant difference between Berkeley Carroll sophomores and freshmen in retention score of groups with different emotional stimulation, basic emotional stimulation (reading stories) versus emotional arousal (reading plus an auditory/visual component) (Fig. 4). There is a .09 p-value in the happy story, 0.6 p-value in the sad story and a p-value of 0.3 in the scary story. Since my p values are greater than .05, the data suggests that there is no significant difference between retention of stories deemed happy, sad, or scary in different emotional stimulated groups. Both groups retained a similar amount of specific detail from the stories, further answering part a of my experimental question, that emotional arousal does not generate higher recall of specific details than basic stimulation.

Using One-way ANOVA with Two Samples:

When it comes to potential differences in retention scores between different races (minority versus majority) in the basic stimulation group (reading) (Fig. 5), data showed that there is no significant difference when it comes to happy or sad stories but there is a significant difference when it comes to scary stories. In the happy and sad story, data showed p-values of 0.6 and 0.5 respectively, while in the scary story data showed a p-value of .04. Since .04 is less than .05, the marginal probability ("the probability of finding the observed, or more extreme, results when the null hypothesis (H0) of a study question is true" (StatsDirect)), is deemed significant, while the marginal probability in the happy and sad story is deemed insignificant because the p-value is greater than .05. Basic stimulation in minorities and majorities generated a similar amount of specific detail recall from the happy and sad story, but when it came to the scary story, basic stimulation generated a significantly higher recall of specific detail in minorities.

Using One-way ANOVA with Two Samples:

When it comes to there being a potential difference in retention score in different races (minority versus majority) in the emotional arousal group (reading plus an auditory and visual component) (Fig. 6), the data suggest that there is no significant difference. There is a 0.7 p-value for the happy story, 0.7 p-value for the sad story and a p-value of 0.9 for the scary story, therefore, deeming all marginal probability insignificant. Minorities and majorities recalled a similar amount of specific detail from all stories.

Therefore, in answering part b of my experimental question, emotional arousal does not generate higher recall of specific detail in accordance to race but basic stimulation does, when it came to the scary story.

Discussion

As a result of past research, a reasonable explanation behind basic stimulation generating a higher recall of specific details is due to the nature of the stimulation and the political climate.

Basic stimulation corresponds to a feeling, which is "a conscious mental reaction (such as anger or fear) subjectively experienced" (Merriam-Webster). Minorities have faced the recurring struggle and pain of oppression for more than hundreds of years. From slavery to the latest Trump tweets, minorities have been subjected to objectification, oppression and limitation on all ranges which can account for recurring negative feelings such as pain, sadness, betrayal, and fright. These recurring negativities can be a reasonable explanation to a higher recall of the scary story in minorities. The absence of a significant difference when it came to sadness can imply that fright could be one of the more prominent emotions felt out of the recurring negative ones.

When discussing this research in the broad terms of science research, it can fit into the branch of political science, and should be taken as a call to eradicate the oppression of minorities because it affects not only one's self but also one's mental retention.

From here, more research should be done on the emotions of different races to see results on how environment can affect one's emotions and how being a certain race in an environment can affect emotions daily. This can bring new insight to the world and hopefully pave a way to a brighter future with no oppression.

Potential Errors

A potential error in my study is that I used data from both freshmen and sophomores, so the age difference could have affected emotions and retention score. Also, different grades (freshman and sophomore) could have different amounts of stress, and that would have affected my data as well.

Another potential error is that I had only sixty-three subjects, and having a greater sample size could change my results and make it more accurate.

Conclusion

In conclusion, emotional arousal does not generate higher recall of specific details from stories, and there is no significant difference in type of emotional stimulation in accordance to race except in group 1 (basic stimulation) when it came to the scary story, which could potentially be attributed to the recurring negative emotions felt by minorities due to past historical events and current political climate.

Supplementary Materials

STORIES

1. How Could You?

When I was a puppy, I entertained you with my antics and made you laugh. You called me your child and despite a number of chewed shoes and a couple of murdered throw pillows, I became your best friend. Whenever I was "bad," you'd shake your finger at me and ask "How could you?" – but then you'd relent and roll me over for a bellyrub.

My housetraining took a little longer than expected, because you were terribly busy, but we worked on that together. I remember those nights of nuzzling you in bed, listening to your confidences and secret dreams, and I believed that life could not be any more perfect. We went for long walks and runs in the park, car rides, stops for ice cream (I only got the cone because "ice cream is bad for dogs," you said), and I took long naps in the sun waiting for you to come home at the end of the day.

Gradually, you began spending more time at work and on your career, and more time searching for a human mate. I waited for you patiently, comforted you through heartbreaks and disappointments, never chided you about bad decisions, and romped with glee at your homecomings, and when you fell in love.

She, now your wife, is not a "dog person" – still I welcomed her into our home, tried to show her affection, and obeyed her. I was happy because you were happy. Then the human babies came along and I shared your excitement. I was fascinated by their pinkness, how they smelled, and I wanted to mother them, too. Only she and you worried that I might hurt them, and I spent most of my time banished to another room, or to a dog crate. Oh, how I wanted to love them, but I became a "prisoner of love."

As they began to grow, I became their friend. They clung to my fur and pulled themselves up on wobbly legs, poked fingers in my eyes, investigated my ears and gave me kisses on my nose. I loved everything about them, especially their touch – because your touch was now so infrequent – and I would have defended them with my life if need be.

I would sneak into their beds and listen to their worries and secret dreams. Together we waited for the sound of your car in the driveway. There had been a time, when others asked you if you had a dog, that you produced a photo of me from your wallet and told them stories about me. These past few years, you just answered "yes" and changed the subject. I had gone from being your dog to "just a dog," and you resented every expenditure on my behalf.

Now you have a new career opportunity in another city and you and they will be moving to an apartment that does not allow pets. You've made the right decision for your "family," but there was a time when I was your only family.

I was excited about the car ride until we arrived at the animal shelter. It smelled of dogs and cats, of fear, of hopelessness. You filled out the paperwork and said "I know you will find a good home for her." They shrugged and gave you a pained look. They understand the realities facing a middle-aged dog or cat, even one with "papers."

You had to pry your son's fingers loose from my collar as he screamed "No, Daddy! Please don't let them take my dog!" And I worried for him and what lessons you had just taught him about friendship and loyalty, about love and responsibility, and about respect for all life. You gave me a goodbye pat on the head, avoided my eyes, and politely refused to take my collar and leash with you. You had a deadline to meet and now I have one, too.

After you left, the two nice ladies said you probably knew about your upcoming move months ago and made no attempt to find me another good home. They shook their heads and asked "How could you?"

They are as attentive to us here in the shelter as their busy schedules allow. They feed us, of course, but I lost my appetite days ago. At first, whenever anyone passed my pen, I rushed to the front, hoping it was you - that you had changed your mind – that this was all a bad dream... or I hoped it would at least be someone who cared, anyone who might save me. When I realized I could not compete with the frolicking for attention of happy puppies, oblivious to their own fate, I retreated to a far corner and waited.

I heard her footsteps as she came for me at the end of the day and I padded along the aisle after her to a separate room. A blissfully quiet room. She placed me on the table, rubbed my ears and told me not to worry. My heart pounded in anticipation of what was to come, but there was also a sense of relief. The prisoner of love had run out of days. As is my nature, I was more concerned about her. The burden which she bears weighs heavily on her and I know that, the same way I knew your every mood.

She gently placed a tourniquet around my foreleg as a tear ran down her cheek. I licked her hand in the same way I used to comfort you so many years ago. She expertly slid the hypodermic needle into my vein. As I felt the sting and the cool liquid coursing through my body, I lay down sleepily, looked into her kind eyes and murmured "How could you?"

Perhaps because she understood my dogspeak, she said "I'm so sorry." She hugged me and hurriedly explained it was her job to make sure I went to a better place, where I wouldn't be ignored or abused or abandoned, or have to fend for myself – a place of love and light so very different from this earthly place.

With my last bit of energy, I tried to convey to her with a thump of my tail that my "How could you?" was not meant for her. It was you, My Beloved Master, I was thinking of. I will think of you and wait for you forever.

2. Reflections: Portrait of a serial killer (part 2)

The second one was just as easy. She was a forty-eight years old housewife and mother of three youngsters, only one of whom was with the guy she was married to at the time. Her name was Belinda Something-or-other. I can't remember her surname now. My knife took her life as painlessly as it had taken Jenny Slade's.

My reaction to Belinda's killing was almost exactly the same as it was to Jenny's: the almost-immediate incredible high followed by those awful tics and tremors and shakes when I got home. Some emotionless, callous killer I was turning out to be! Clearly my subconscious was trying to assert itself and make me feel some level of guilt for my actions, but I didn't take any notice. I wanted no part of thinking negatively about my murderous impulses. I was enjoying myself too much for that.

The local press picked-up on the fact that both murders were 'probably' caused by the same weapon, which didn't take much working out once the post mortem results were known. My 'weapon of choice' was a Sabatier six-inch long chef's knife I had liberated from the kitchen of a hotel I worked at briefly a couple of years previously. There was uproar when it went missing. Who knew chefs were so bloody attached to the tools of their trade? I mean, come on, bursting into tears because you'd lost a sodding knife? Pathetic.

In hindsight I must have already formed some kind of subconscious plan for the knife, otherwise why steal it? I wasn't a thief by nature and I sure as hell had no intentions of taking-up a career in catering! No, I was attracted to the knife for one reason and one reason only: its lethality, although I was not as clear-minded about it at the time I took illicit possession of the thing. It was only after I'd killed Lara and found myself pondering what it took to be a killer did I give serious consideration to the beautifully crafted weapon concealed in the false bottom of my old-fashioned dark wood wardrobe as a tool of destruction of life.

My third murder finally got me labelled as a serial killer. She was Georgina Templeton, a former model-turned-local-TV-presenter. At thirty-two, she was still stunningly beautiful and had a body to match. Her death caused outrage in the community, as though her beauty conferred on her special attention. It made me feel quite sick to think that people – the general public - were so bloody shallow. My other two kills had had their attractions, too, I'm sure their families would have said. Just because they were not as obvious as Georgina's didn't make their murders any less outrageous.

It was those thoughts that marked me out as different to all the other serial killers I had ever heard about. I don't recall any of them getting pissed at the media for giving more column inches to a former beauty-queen model than to a mum of three who had died at the hands of the same killer. Oh no, none of those cold-blooded bastards would have felt the sense of personal outrage and injustice that gripped me for my kills.

My twentieth birthday was still several months away yet I had already notched-up four murders. Not bad going for an ostensibly 'ordinary' teenager just starting out in life. The press do like to give names to serial killers. They tagged me as the 'Steel Blade Killer', which was about as boring and uno-riginal as you could get! Couldn't someone, somewhere, have come up with something just a little more imaginative? Christ. I thought journo's were intelligent.

It was more than two years since I'd killed Jenny Slade. The police were none-the-wiser two years down the line than they had been they day her body was found. I had not so much as been questioned about her, even though she and I were seen together that evening. As for my other kills? Same story.

Unlike many other serial killers I had no wish to be caught. If you believed all the bullshit psychiatrists and psychologists and their like spouted, all serial killers had sexual motives, had mother issues and secretly wanted to be stopped if not caught. That was why, apparently, so many of them involved themselves in the investigation of their crimes. Some even contacted the police directly to taunt them (think Jack the Ripper as the most famous taunter). Not me.

As I said at the outset, I had a very healthy and very active sex life, thank you very much. From the age of thirteen I had never had to work very hard to get into the panties of any girl I fancied. I'm not bragging but they were virtually throwing themselves at me pretty much as soon as I hit puberty, so there was no sexual angle to my kills whatsoever.

As for mother issues? My mum is the single most important person in my life. She's my rock, my confidante and, yes, even as I look forward to entering my twenties, she is my best mate. I cannot imagine life without her, which probably sounds a bit stupid when you consider that if I ever get caught and convicted I'll more than likely spend the rest of my days behind bars. But that's not a possibility I can see happening. I just have this feeling that I won't get caught, simple as that.

You see, so many criminals are utterly stupid. They do stupid things: they open their big mouths when they should keep them shut, they behave differently and they do things that they would not normally do. In short, they draw attention to themselves. I, on the other hand, have behaved exactly as I always have. I do the same things I always do, go to the same places, see the same mates and generally live my life like any other guy of my age. I am, in that respect, unexceptional. It is, actually, what makes me so damn exceptionally exceptional!

For no particular reason at all, I didn't kill again for more than twelve months. It's not as if I wasn't tempted, because I saw several good potential candidates to fall victim to the kiss of my blade. I just couldn't be bothered, truth be told. For all of that period I was a model citizen going about my daily life just the same as everybody else was. It was almost fun – but never enjoyable.

It cannot be denied that I have an outsize ego. Heaven knows I could not have achieved all that I have achieved without so much as a smidge of suspicion being directed my way and it not having an effect on me. I am, though, extremely careful. I keep my smugness concealed behind my charming and friendly persona. I have a well-developed supercilious air that only surfaces when I am forced into dealing with genuine dullards and those mentally and emotionally unable to differentiate between my pleasant self and the nasty bastard laying just beneath the surface of my *bonhomie*.

Kill number five came exactly two weeks after I celebrated my twenty-second birthday. All I remember about her was that she was older than all the others, fat and ugly and had a really bad personal hygiene problem, which made me angry. There is no excuse whatsoever, in my book, for not washing and keeping oneself clean. Ugh!

She died less than an hour after I met her. I didn't even know her name until I read it in the newspaper the following day. I felt exactly nothing for her or about her. All she was to me was another notch in my murder campaign. She was 'Number Five'.

3. Piano

Ever since Bill was small, he could remember his grandmother playing the piano. He would go to her house and find her playing the classics, smiling, and enjoying life. But today was different. His grandmother invited him over to dinner to discuss something with him. He knew it had to be bad because she sounded so serious, and she was making his favorite meal: spicy fried chicken, garlic mashed potatoes, green bean casserole, and cornbread.

Upon arrival, he noticed his grandmother wasn't at the piano. She was always there. She loved that thing. She had said once before that it gave her a sense of peace to allow her emotions to come out in the music. He walks out to the kitchen to look for her. She was there whipping up the garlic smashed potatoes. When he entered the kitchen, he was immediately greeted by her. She puts the mixer down to extend her arms to him, and says "I'm so glad to see you. I missed you."

They hug as he said "I've missed you too. It smells great in here. I can't wait to eat."

"Me either."

"What can I help you with?"

"Help me put all this stuff on plates and in bowls, and bring it to the table."

"Done."

Within minutes, they dig in. Bill said, "It tastes just like I remembered. It's so good."

"It is, isn't it? I made dark chocolate brownies with peanut butter chips for dessert."

"You always knew the way to a man's heart. So grandma, what is it you wanted to talk about?"

"Well, since you asked, I need you to help me pack. I'm ninety years old, my husband died and Edna wants me to move in with her, and I said yes. She's my best friend, might as well. But I want you to have my piano. I know how much it meant to you. I want to give it to someone who will love it as I did. You're the only person worthy of receiving my cherished treasure."

Taking a bite of his fried chicken, he said "Of course I will help you. Are you sure you want to move in with her?

"Yeah, Without my Edgar around, what's the point of living here alone? My grandkids and children have their own lives. It's time for me to have one too. Believe me, it will be fun. Edna and I are gonna party at the bingo every night! So will you take the piano"

"I will grandma."

While lying in bed that night, he was given a lot to think about. He was glad he could help his grandmother move but he was going to miss her.

He couldn't believe how generous his grandmother was with the moving process. Just for helping she gave him forty thousand dollars and the piano, while the remaining one hundred thousand dollars and her house was given to her three children who also helped. He took the piano home. It was a beautiful Steinway. It looked great in the living room.

Soon after bringing it home, he sat at the bench and began to play the songs his grandmother and him would play every weekend. His favorite was "Angel of Music" from "The Phantom of the Opera". Whenever, he had time he would try to teach his children the song, and when Grandma came back to visit, Bill and his kids played it for her. A new generation completing her tradition.

Images: https://docs.google.com/document/d/1OaweogYgKmLm8l9XcudQSu911bs0XJZD_RyY3VFkFLA/edit?usp=sharing

Preliminary data collection survey: https://goo.gl/forms/IATASdIn9UUmEqGJ3

Comprehension quiz: https://goo.gl/forms/jgfcUReJlFGsPH9Y2

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Ant Tunneling Behavior in Agar Environments by the Red Harvester Ant

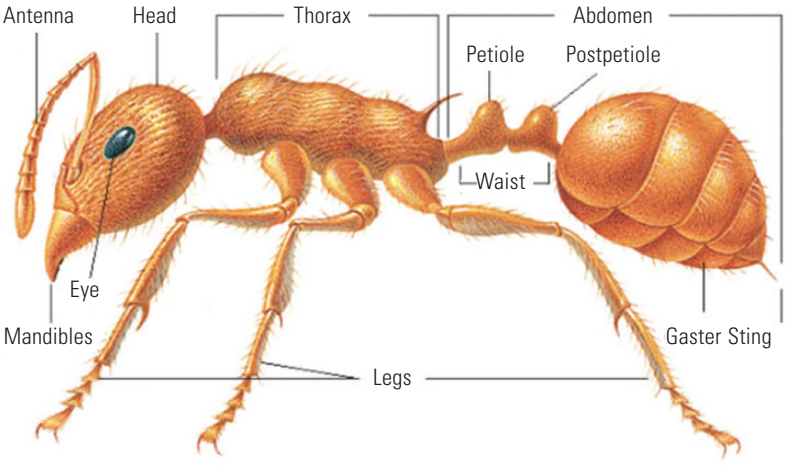
by Ellie Pike

Abstract

The advanced behavior and sociality of ants has been a topic of extensive research for decades. In the following study we focused on finding the ideal tunneling conditions for ants in an agar environment. We first investigated agarose concentration and observed that 4% agarose was the optimal concentration for effective tunneling. In addition we determined that a 2% sugar concentration was also ideal in successful architecture. Now knowing the comfortable concentration of sugar for tunneling, we then explored the minimum sucrose amount that led to viable ants. After two trials, we deduced that a 0g sucrose environment allows for viable ants. We then investigated the role of a chemoattractant (sucrose) in tunneling behavior, questioning whether it would impact architecture. After one trial, we concluded that an attractant did not seem to have an impact on tunneling behavior, however this experiment will undergo a second trial in the near future due to the apparent impact of food coloring.

Figure 1

External Anatomy of an Ant



Background/Introduction

It has been well documented that ants are so prosperous as a species because of their effective ability to "talk" [Holldobler, B., & Wilson, E. O., 1994]. In other words, ant communication is one of the keys to a successful ant colony. Nearly every action colonies undertake is calculated and intentional, maintaining a tight organizational (and social) structure. This is made possible through "local interaction", or the interactions between colony members that are physically close to one another (or, in a more general sense, interaction within the colony as opposed to species).

Although the topic has been debated, many scientists still divide ants into castes: notably, the worker caste, male caste, and the queen [Holldobler, B., & Wilson, E. O., 1994]. The worker caste, made up of the queen's daughters, is the heart of the colony as they raise young, and perhaps most importantly, locate food for the colony. It is during these foraging missions that ants' primary means of communication, pheromone signals, becomes crucial. Pheromones are essentially chemical cues that are laid (or "marked") to form trails. Such trails can draw other members of the colony to desired food sources and other attractions.

Figure 2

Internal Anatomy of an Ant

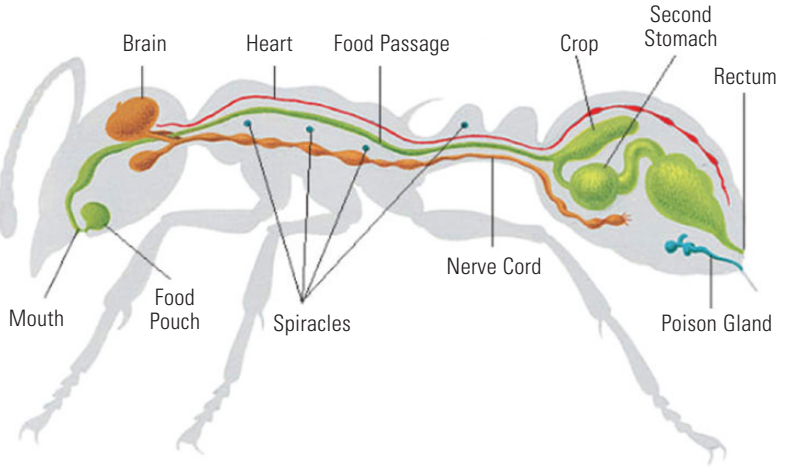
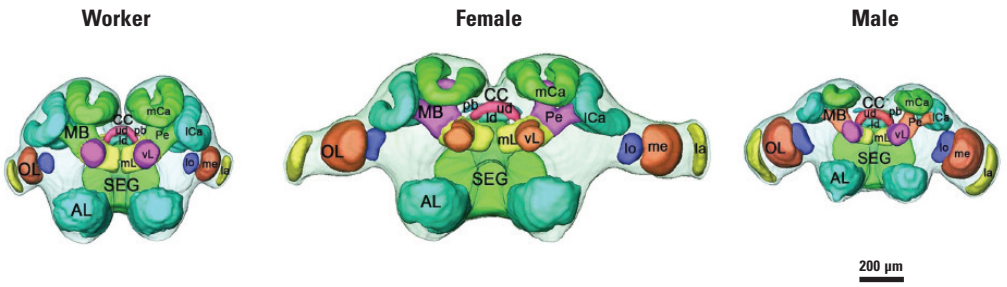


Figure 3

Brain Anatomy of Ants



Recently, researchers at the Kobe University and the National Institute for Basic Biology have studied the chemical profiles of ants, specifically focusing on their "chemosensory organs", most often found in their antennae, and the ways in which these contribute to their communication abilities [Hojo, 2015]. They investigated the gene expression of certain chemosensory proteins (CSPs) and found that because certain CSPs, are "known to bind pheromonal cuticular hydrocarbons", it is plausible that they contribute to ants' pheromone communication [Hojo, 2015]. Evidently, pheromone communication and chemical awareness in general is vital for an ant's success.

In an article entitled "Structure and Function of Ant (Hymenoptera: Formicidae) Brains: Strength in Numbers" authors note that worker ants have poor vision as they are missing "functional ocelli", organs that enable smaller creatures to see. Therefore, ant pheromone communication is even more crucial [Gronenberg, W., 2007]. Ants' sense of smell (specifically olfactory neurons) plays a similarly vital role, aiding in functions such as awareness of their surroundings. Antennae also contribute to the olfactory system and help orient an ant. The "expensive" nature of every neural component of an ants' brain is frequently referenced, seeing as there is little room in an ants' skull, and therefore everything must serve a purpose [Gronenberg, W., 2007]. In general, an ant's body is finely attuned to its needs and activities. This is clear in the case of mushroom bodies, described as, "central brain structures involved in learning and memory" [Gronenberg W., 2007]. Mushroom bodies are larger in workers, as they need to be more flexible and able to take in new information than other, higher up, castes. This neural phenomenon, whereby neural organs exist in different sizes in accordance with the specific duties an ant carries out, is common in ant societies. For instance, male ants work little, and therefore have smaller brains and limited capabilities [Gronenberg, W., 2007].

Foraging seems to be one of the processes in which the complexities and advanced sociality of an ant colony is most apparent. A paper entitled "Chaos – Order Transition in Foraging Behavior of Ants" describes the intricacies of foraging, creating a mathematical model to predict and simulate foraging behaviors [Li, L., 2014]. Though this model is highly sophisticated, aspects of it aid in further understanding patterns in ants' tendencies when it comes to food gathering. Researchers outline three factors that they believe are most important when it comes to foraging: hunting, homing, and path building [Li, L., 2014]. Hunting describes the process of searching for food: ants referred to as scouts search the surrounding area, traveling in a random path, until one of them stumbles upon something and begins to lay a trail [Li, L., 2014]. Homing highlights the role of a nest in foraging, in that when ants have a set place to return to, they are able to know their limits, and turn back if they become too exhausted. Path building denotes the "optimal path between the nest and the food source" [Li, L., 2014]. Researchers focused on the actual process of building this path, namely, as the title suggests, the transition from chaos to order.

As scout ants search for a possible food source, they engage in what is called "chaotic walking": walking randomly laying down pheromones with no particular intentions. Eventually an ant will locate food, and begin to draw a path, allowing other ants to catch on. However, this does take some time, and as an ant is drawing an intentional path, other colony members still engage in the "chaotic" phase of foraging. Though this may seem to detract from the actual pheromone path, it actually *increases* the density of the pheromone simply by making more of the substance, making it more likely to eventually be followed [Li, L., 2014]. A transition then occurs, and the "reinforcement of pheromone continues to weaken the chaotic behavior of the ants," eventually leading to an organized structure [Li, L., 2014]. Authors refer to the contribution of individual chaotic behavior to eventual mass-organization as "swarm intelligence", a phenomenon common in many social insect societies. Swarm intelligence refers to the way smaller scale action contributes to a larger, organized structure (whether that be in an

ant colony or elsewhere). The authors also note that, because pheromones evaporate, they are denser in shorter trails than longer ones [Li, L., 2014].

Foraging is closely interconnected to tunneling, and the way a colony organizes its nest. Ant tunneling behavior further exemplifies the advanced organizational abilities and flexibilities of ants. In a study focused on "granular media", the material being tunneled and dug in, researchers noted the adaptability of ants and their awareness when tunneling [Espinoza, 2010]. They highlighted the importance of local information, as previously discussed, and how it impacts the architecture of nests. The species of harvester ants used in this study was capable of thriving in many different environments, specifically highlighted here because of the variety in acceptable water amounts in their nests [Espinoza, 2010]. Mandible (see Fig. 1) size was also mentioned in impacting digging ability, further demonstrating that ant tunneling is dependent on many variables [Espinoza, 2010].

Another study, published in *Proceedings of the National Academy of Sciences,* focused on shape transition in ant nests, referencing a common trend in nest development. Specifically, researchers found that activity is rapid at first, and then transitions to "a saturation phase" where digging and tunneling peters out [Toffin, 2009]. In this study, two groups of ants, one with 300 workers and another with 50 workers, were used in order to "assess the influence of group size on morphogenesis as well as on shape transition during the building process" [Toffin, 2009]. Researchers deduced that shape transitions were more common in the group of 300 workers as opposed to that of 50, demonstrating how colony size has a lasting impact on the nature of a nest [Toffin, 2009]. Colony size in general is a crucial variable in many studies focused on ant digging and tunneling. In one such experiment, focused on "self organized digging activity", a model was developed centered around "recruitment" and "chemical communication" [Buhl, 2005]. After accounting for all these variables, it concluded that volume is related linearly to the quantity of ants in the nest [Buhl, 2005] . In other words, the more ants, the more extensive the nest.

As this study used the species *Pogonomyrmex barbatus* (Red Harvester Ants), it is critical to understand more in depth nest characteristics and general information about this species. In general P. barbatus live on a diet consisting mainly of seeds, and, at times, dead insects [Red]. Their nests are fairly widespread and are most often in spots with little plant life [Red]. *P. Barbatus* is known for its large body, and is very common in Texas [Davis, J.M.]. One important component of the nest is a region known as the midden, where ants leave anything they want to dispose of (dead ants, other unnecessary materials obtained while foraging, etc.) [Davis, J.M.]. The midden increases in size as the colony ages. A normal sized *P. Barbatus* colony usually consists of around 10,000 ants, each with very particular allocations and duties [Davis, J.M.].

As the ants used for this study were independent workers, without a queen, their behavior and organization was different from a traditional *P. Barbatus* colony. This study consisted of several parts. The first was evaluating the preferred level of agar concentration for successful ant tunneling and overall survival. The second was deducing the minimum level of sucrose that lead to viable ants, and the third was evaluating how a chemoattractant influenced tunneling behavior. In all studies, a relatively uniform procedure was followed for making the agar, yet apart from that, procedure varied across the three studies.

STUDY #1: Agar Concentration

Description

This was a foundational study with the goal being to glean the most suitable concentration of agarose for the ant-agar environment. In the early stages of this experiment, all containers were of the same concentration (1% agarose) and change in ingredients (sugar, albumin, or control) was the independent variable. Three distinct conditions were created: one simply made out of the agar solution, another with agar and sugar, and a third with agar, sugar and albumin (protein source). The albumin was discarded early on in the process as it did not dissolve correctly. Over the course of the experiment it became apparent, for reasons outlined below, that finding the most suitable concentration of agar was a more significant investigation. Therefore concentration/consistency of the agarose became the primary independent variable.

Materials/Methods

Construction of Boxes:

Materials

- Plexiglass (or other transparent, sturdy material)
- Hot glue

Building Boxes

The boxes were constructed in a relatively simple manner. The initial dimensions of the tanks were 6 x 6.5 x 1". Two 6 x 6.5" pieces, two 1 x 6" pieces, and two 1 x 6.5" pieces were cut out of a larger sheet of plexiglass and sanded down when necessary to be even with each other. The two larger pieces were set up facing each other, with the 1" slabs lining the sides and bottom of the tank. All slabs were hot-glued together. Once the general box was constructed, the top was built. The last 1" piece was used for the first layer of the top and another, smaller piece of plexiglass was cut for the second layer. This bottom layer was slightly smaller than the top of the box and therefore fit into the tank, whereas the other layer rested on top. Both layers were hot-glued together. Three boxes of this kind were constructed for Study #1.

Once constructed, the boxes were tested for leaks by pouring water into them and seeing if there were any holes or gaps in hot glue lining. More layers of glue were added accordingly.

General Agar Preparation:

Materials

- Water (380mL x 3)
- Agar/agar powder (8g x 3)
- Sugar (8g x 3)

  **Above amounts were based off a recipe for the Bhaktar diet – a common diet for ants in captivity [Penick, 2018]. Amounts were adjusted due to the size of our containers.*
- Beaker (1L)
- Hot plate
- Magnetic stirrer
- Glass stirrer
- Sharpie (or other permanent writing utensil)

Methods

*The below procedure describes the general process for making the agar ant farms. A similar procedure was followed for every trial/study in this paper.

1) Label containers in accordance with various testing conditions.
2) Place containers in refrigerator or freezer to cool off in preparation for agar.
3) Place 1 L beaker of water on hot plate and heat on high until boiling.
4) Add agar powder and drop magnetic stirrer into beaker to quicken transition into solution.
5) Once all agar has been mixed in, set aside 380 mL of agar (enough to fill one container), and pour into isolated tank. *throughout this time keep remaining agar solution on hot plate molten so that it does not harden.
6) After agar solution appears to have set enough to be moved without potential for spillage, transfer container into refrigerator to dry overnight.
7) Keep remaining agar solution on hot plate and add any additional ingredients (for our purposes, this ingredient would be sugar), repeating steps 5-6 when temporally appropriate.
8) Once all tanks have been poured, and have dried completely (overnight), remove from cold storage and poke three starter holes (per container) in the agar to give the ants a way to start tunneling. Holes should be approximately one inch deep, and separated by about two inches. There should be one in the middle of the container and two on each side.
9) Once holes have been made, transfer ants into tanks. It may be helpful to seal tops of tanks with tape, as ants often attempt to escape.

Note Transfer of Ants: It is helpful to use a metal scooper, or other relatively flat utensil, to move the ants from container to container. Protective gloves may be helpful as P. Barbatus has the capability to sting.

Procedural Note for Study #1:

In the initial stages of this study, as previously mentioned, we created three separate living conditions in the tanks (one with simply agarose, one with agarose and sugar, and one with agarose, sugar, and albumin). Therefore, in terms of step six, after the container with only agarose was poured, sugar was added to the mixture, and after the sugar + agar container was poured, albumin was added. However, the albumin did not mix well with the agarose and was quickly removed from the experimental design (as discussed below).

Data/Analysis

Concentration Alteration (I)

Before concrete data collection began, it became apparent that the texture of the agar was too moist. Moisture had condensed at the top of the tanks, making the ants look like they were walking in liquid, and the starter holes had closed in. The albumin mixture had a clotted texture (due to the albumin powder), and therefore was the most difficult for the ants to tunnel in. Overall, the ants were unable to effectively tunnel, and as this was true even in the control tank (whose only ingredient was agar) we deduced that this difficulty was due to the concentration of agarose. Up to this point, all tanks had a concentration of 1% agar.

Therefore, new 2% agar and 3% agar containers had been created and the ants were transferred out of their 1% solutions into these containers. The 3% agar contained sugar, and the 2% agar contained only agar (albumin was not included in either tank). At this point, we began to record concrete observations.

Tank Orientation Note:
Observations/analysis involving direction (i.e, "right" or "left" side of a tank) are made assuming the labeled side of the tank is facing the viewer (or in many cases, the camera).

Figure 4

Observations - Day 1

	2% agar	3% agar
Tunneling	1 notable trail on side of container	2 tunnels on left side of container
Mound	Discarded agar granules filled with dead ants and ant limbs	Discarded agar granules filled with dead ants and ant limbs, mound more prevalent in this container
Behavior	Not as many ants trying to escape via sides of container → overall seem less frantic	Ants trying to climb up sides of container and escape, seemingly more frantic
Additional Notes		

Figure 5

Observations - Day 2

	2% agar	3% agar
Tunneling	Similar progress as previous days' observations	Progress in tunneling → tunnels a bit more developed
Mound	Similar size to previous day	Bigger compared to 2% tank, suggests more tunneling has taken place
Behavior	Ants still displaying "escaping behavior" (climbing up sides of box)	Still displaying "escaping behavior"
Additional Notes*	Approx. 17 ants counted	Approx. 15 ants counted**

**Although noted specifically on this day, throughout the majority of this study, the ants appeared to be eating each other (or at the very least ripping apart dead ants limbs and moving them to specific areas in the tanks).*

*** These numbers are very rough as it is difficult to count ants when they are constantly in motion.*

Concentration Alteration (II)
On Day 2, after my observations were recorded, a new agar solution, of 4% agar, with sucrose, was made. The remaining ants in the 2% agar were transferred to the 4% container.

Figure 6

Observations - Day 3

	3% agar	4% agar
Tunneling	No record on tunneling progress for this day (we can assume tunnels looked similar to previous day).	Many trails dug → similar amount of trail activity to 3% tank
Mound	No record on the mound for this day (we can assume the mound looked similar to previous day).	Unlike other containers there is no large mound in side/middle of container; instead agar granules are spread throughout container.
Behavior	Ants are huddled, and seem to still be eating each other	Ants appear to be active, and no dead ones as of yet. Still climbing up the sides ("escaping behavior"), but appear less manic/frantic.
Additional Notes*	Approximately 14 ants	

At this point, I stopped making daily observations due to the fact that the 4% agar seemed to be the environment in which the ants most effectively tunneled.

Figure 7

3% Agar Container

Photograph taken on Day 1 of the 3% agar container. Note the mound of agar granules and ant limbs on the surface of the tank as well as the closed-in tunnel underneath.

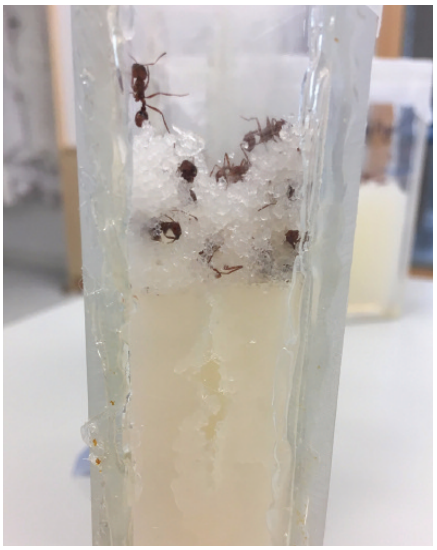
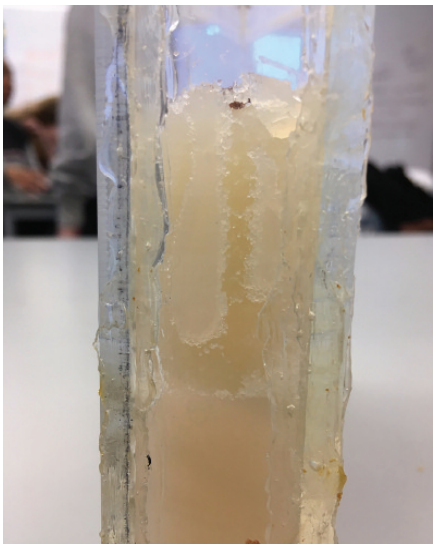


Figure 8

4% Agar Container

Photograph taken on Day 4 of the 4% agar container. Note the sturdiness of the tunnel as compared to Fig. 3 as well as its increased width.



Discussion/Conclusion

The main conclusion made from the above process was that the 4% agar solution resulted in an environment that was most effective for the ants to tunnel in. This conclusion came from a variety of observations. Primarily, that ants in the 4% environment made as much tunneling progress in under twenty four hours than the 3% ants had in double that time. In other words, the ants in the 4% tank tunneled much more rapidly and seemed more at ease (in that they weren't eating each other as they'd previously done). We also noted more excavated agar, another sign of increased tunneling. Although early on, one tunnel in the 4% container *did* close in, on the whole, the tunnels remained sturdy. In summary, we concluded that 4% agarose was the most successful concentration for ant tunneling. This conclusion was necessary in laying the groundwork for experiments that followed.

In addition to gleaning the ideal concentration of agar, many other conclusions about the experimental process itself were made. In terms of making the agar, we concluded that it was best to let the containers dry overnight so that there was no condensation present in the solution when the ants were transferred into their tanks as it is likely that this condensation also played a role in the difficulty ants had foraging. Additionally, because the ants in the 4% agar solution thrived as they did, we determined that 8g of sugar (the amount used in each tank where sugar was present) was a stable amount for the ants to survive on.

Because of the trial and error nature of this study, the ants were moved around quite a bit. They started in a temporary container filled with commercial feeding gel, were then moved to the 1% tanks, then the 2% tanks, and then the 3% and 4% tanks. All of this movement made the ants seem very agitated, which we observed through the multiple cases of "ant cannibalism". Therefore because of this agitation, we knew in the future to leave the ants in a temporary holding for, at maximum, two days and then transfer them to a permanent "home".

Lastly, because of the large width of the containers, visibility in the center of the tanks was limited. Therefore, for our own observational abilities and in order for the ants to be able to explore more widely as opposed to just focusing on one section of the container, we decided to shrink the general dimensions of the tanks to be 5.5 x 6 x 0.5" (see Fig. 5). This would give the tanks a new volume of 200mL with the agar filling approximately 180-170 mL of the container.

STUDY # 2: Sucrose Amounts and Viability

Question: What is the minimum sucrose level to allow for viable ants?

Description

Now that the ideal concentration of agar had been discovered, the next step in the experimental process was to determine what amount of sucrose led to viable ants (viable, in this case, meaning the ants are both alive and able to forage effectively). As 8g of sugar in a 400mL solution was determined to be a comfortable amount for the ants, we established this as our highest level of sucrose (in this trial cutting this in half to be 4g, based on the size of the holding tank). We knew that our lowest sucrose level would be 0g sugar, and for our middle amount we chose a fairly arbitrary value of 0.4g. There were approximately twenty ants per container, and photographs were taken periodically throughout the experimental period.

TRIAL 1: Materials/Methods

Materials

- 3 clear containers (dimensions: 5.5 x 6 x 0.5")
- Water (200mL x 3)
- Agar/agar powder (8g x 3)
- Sugar (4.4 g)
- Beaker (1L)
- Hot plate
- Magnetic stirrer
- Glass stirrer
- Sharpie (or other permanent writing utensil)

Procedure

The procedure for Study #2 was very similar to the "General Agar Preparation" procedure followed in Study #1, with the main alteration being the different sucrose amounts in the various containers. Therefore, in steps 5-7 the first container to be poured was the 0g container. Next, 0.4g sugar was added to the 1L beaker, and once dissolved, the appropriate amount of agar was poured into the second container. Lastly, 3.6g sugar was added to the agar solution and dissolved. This container (the 4g tank) was the last to be poured. Another difference in this procedure was the amount of agar per container. As the dimensions of these containers were much smaller than the previous trial, only about 170mL agar needed to be poured into each tank. Apart from these differences, other aspects of the procedure (i.e, drying of agar, poking of starter holes) remained the same as in Study #1.

Figure 9

Permanent Tank

Photograph depicts the new 5.5 x 6 x 0.5" tanks used for both trials of Study # 2.

Data/ Analysis

The following photographs are representative figures pooled from a larger collection of photographs taken Over a 33 day period(specific dates are displayed). Tanks are lined up in order of increasing sucrose level (ex: 0g, 0.4g, 4g). As discussed below, photographs from early December (soon after the trial began) do not show much progress in tunneling and therefore are not included in the data below.

Figure 10

Photographs taken on Day 13

Figure 11

Photographs taken on Day 18

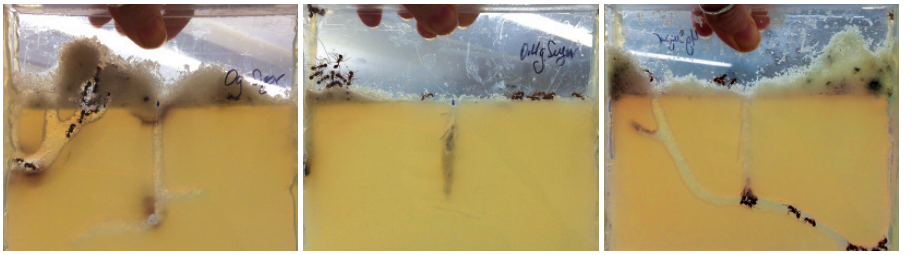
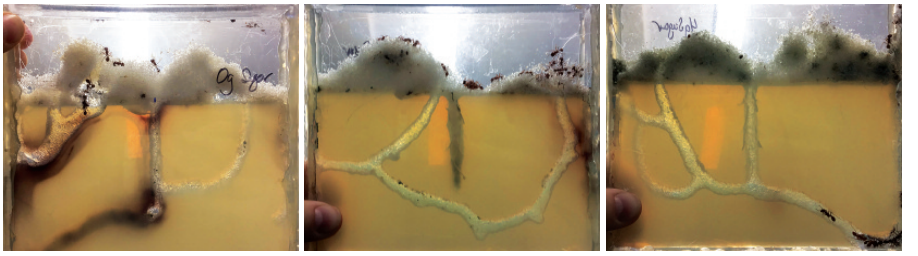


Figure 12

Photographs taken on Day 33



Analysis

The data gathered in Trial 1 *does* inform our experimental question (what is the minimum sucrose level to allow for viable ants?). Since all containers were eventually tunneled in, and by the end of the trial there were living ants in all tanks, all environments maintained viable ants. However, the ants in the 0g sucrose tank were viable *and* living in an environment with no sucrose – suggesting that 0g sucrose is the minimum amount to allow for viable ants.

Many observations can be noted from the above data. First, it took the ants many days to make significant progress in tunneling. Photos taken close to day 10 (not represented above) show outlines of tunnels that appear to be extensions of the starter holes. And, while there is a bit of a gap in the data gathering (from Day 5 to Day 12), it is not until Day 13 that we begin to see the first signs of significant tunneling – that is not just building off of starter holes. However, by the end of the month-long data collection, all tanks had been fairly well-tunneled, and many trends can be observed throughout this period.

For most of the experiment, the 4g and 0g containers stayed fairly consistent in their appearance in terms of tunnels. The 4g container was, for much of the experiment, the most widely explored – with tunnels stretching across the container and up the sides and bottom (evident as early as the Day 13 photograph). Tunnels consistently grew throughout the experimental period and were connected through a sort of web shape. As compared to other containers the 4g tunnels were relatively thin, therefore implying directional/intentional tunneling on the part of these ants. In terms of the 0g tank, ants seemed to focus most of their efforts on the left side of the tank – accounting for the wide and heavily excavated y-shaped tunnel. Although there were other tunnels, by the day 33 observation a good portion of them, specifically the tunnel in the bottom left hand corner, had been plugged up.

Unlike the other two tanks, the ants in the 0.4g tank were very inconsistent in their tunneling habits. For much of the experimental period these ants did not tunnel, and even plugged up their center starter hole. As can be observed all the way through day 19, there were very few discarded granules of agar (as compared to the other tanks), which suggest along with the lack of tunnels that tunneling activity was very low. However, between day 19 and day 27 there was a drastic shift and extensive tunnels were made in the 0.4g container.

Discussion/Conclusion

Much of the data collected in this trial merits questions. Most pressingly: what might explain the shift in tunneling behavior in the 0.4g sucrose tank? Before this shift, we hypothesized that there was perhaps a mix-up in tank labeling, and the 0.4g tank was actually the 0g tank and vice versa. This might have explained the lack of motivation in the 0.4g tank, if in actuality these ants had no sugar and were starving. However, after the sudden increase in foraging activity in this tank, such a hypothesis seemed less likely. Perhaps the shift had something to do with the ants being brought back to school (maybe they preferred the light/temperature in school as opposed to where they were kept over winter break), although if this were the case one would expect all ants to react and not just the 0.4g ants. Perhaps it was something about the moisture in the 0.4 g container, or the cooperation of the ants inside the container, but, regardless, the general tunneling behavior of the 0.4 g tank ants was far less predictable than the other two groups.

The general behavior of the ants was also very telling in terms of their general state and well being. The ants in the 0g container were often very crowded together in the top left region of their tank, and seemed to create an enclosure-like structure out of discarded agar granules. It is possible that this was an architectural choice made to increase their protection (as a sort of shelter), which would make sense for ants with the least amount of food. The 0.4 g ants were less patterned in their behavior although for a majority of the data collection period they resided near the surface of the agar (mainly because they had yet to tunnel). In contrast, the 4 g ants spent much of their time gathered at the bottom of their tank (see Figs. 7- 8) suggesting that they were the most willing to venture into their tunnels and treat their surroundings as a more permanent "home" than the other two groups.

There were quite a few variables that we were unable to control in this trial - the most significant being the presence of mold in the tanks. The earliest signs of mold became apparent in the 0g tank towards the start of the trial, around day 15. By the end of the study mold had worsened in the 0g tank and had become apparent in the both the 0.4g tank (on the side) and the 4g tank (in the mound of discarded agar at the top of the container). It is odd that the mold was most rampant in the tank with no sugar, as such a high presence would seem more plausible in the high-sugar tank. Regardless, this was a variable that likely had an effect on tunneling behavior, and was not controlled.

Additionally, there were significant gaps in data collection in terms of photograph-taking, resulting in certain shifts in tunneling behavior (such as in the 0.4 g tank) going un-documented. Therefore, for reasons such as these, another trial was performed.

TRIAL 2

Procedure for this trial was the same as the previous. Agar was poured on January 25th (day 1) and photographs were taken from Jan. 29 - Feb. 14 (day 4 - day 20). Once again, tanks were organized in terms of increasing sugar content.

Analysis

In this trial, we observed that the most widely explored tank seems to have been that of the 0g container. These tunnels filled up a majority of the tank and stretched up the sides, and the ants appeared more widespread, and therefore content, than in the previous trial. Instead of a mound of agar granules with a bit of space for ants to reside, this 0g container's discarded agar seemed intentionally organized in circular/ovular sections. Unlike Trial 1's ants, Trial 2 ants in the 0g container appeared much more comfortable spending time at the bottom of their container, exploring their trails (similar to the behavior of the 4g container ants in Trial 1).

Similarly to Trial 1, the 0.4g container seemed the least active at the start of the trial, with the central tunnel being the main architectural structure. However, the sides of the 0.4g container, as of day 7 (not pictured above), had been heavily excavated. Despite the apparent lack of tunneling, there was a substantial amount of discarded agar at the top of the 0.4g container, as of day 7, suggesting that, unlike in the majority of Trial 1, the 0.4g ants did not completely neglect tunnel building at the start of the trial. However, as observed in the day 14 image (see Fig. 10), the tunnels in this tank continued to develop, stretching across the tank both to the left and right.

In terms of the 4g container, the tunnels, were, in general, a bit wider than in Trial 1 and slightly less far-reaching. The ovular-shaped tunnel on the right side of the tank was a shape we hadn't seen before, but mirrored the connectedness we'd observed in past high sucrose tanks. In general, the ants in Trial 2 worked faster (in building sophisticated tunnels) than those in Trial 1. Tanks were put together on day 1 and we observed significant tunneling only four days later. Additionally, the tunnels appeared generally wider in this trial, as opposed to the disparities in width (some thin, some very wide) than we observed in the tunnels in Trial 1.

Discussion/Conclusion

Once again, we saw that a 0g sucrose environment *did* allow for viable ants. On top of this conclusion, many other findings in this trial should be noted. First of all, a pattern emerged over both trials in the middle-sucrose tank (that of 0.4g), although that was the least explored/heavily tunneled environment, at least at the beginning of trials. However, in Trial 2 many of the 0.4g ants escaped their tank which could account for the disparity in tunneling and rule out the possibility of a pattern. Escaped ants was

Figure 13

Photographs taken on Day 4

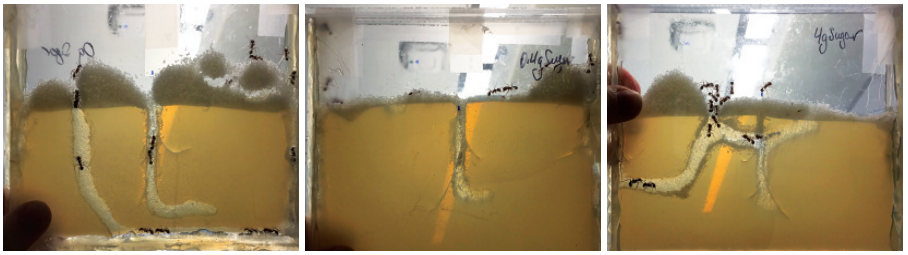


Figure 14

Photographs taken on Day 14

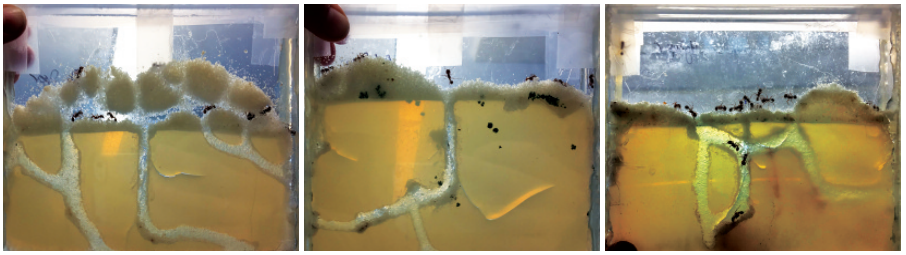
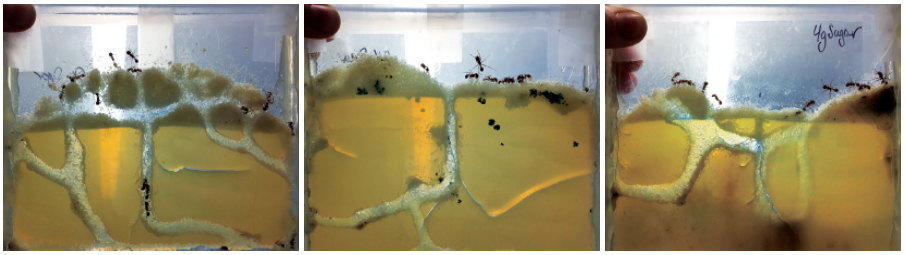


Figure 15

Photographs taken on Day 20



another variable that we were unable to control. This was a problem in both trials, but was more prevalent in trial 2. Additionally, there was some leakage in these tanks as they weren't quite cold enough when the agar was poured in resulting in a smaller amount of agar in each container as compared to Trial 1. Therefore this could have also contributed to the more rapid tunnel building and sophistication of architecture in Trial 2 as compared to Trial 1.

Once again, mold was a variable we were unable to control in this trial. We debated using tea tree oil as a mold resistor but decided against it as it, too, would be an additional variable. There were two distinct kinds of mold observed: the green, spotted type present in the 0.4g container and the darker cloudy type present in the 4g container, begging the question what different conditions in the tank led to these separate forms of bacteria?

STUDY #3: Impact of Chemoattractant on Tunneling Behavior

Question: How Does a Chemoattractant of Varying Concentration Affect Tunneling Behavior?

Description

At this point in the experimental process we knew the ideal concentration of agarose (4%) as well as the minimum sucrose amount that led to viable ants (0g). This study investigated the role of a chemoattractant (which, in this case was sucrose) in tunneling. There were three separate tanks all with a concentration of 4% agarose and a general sucrose level of 0g. Each container had a blue, triangular section in the bottom left hand corner that marked off where the chemoattractant began. The first tank was a control with a concentration of 0g sugar (and therefore no attractant), the next had an attractant with a concentration of 1g sugar, and the last had an attractant with a concentration of 10g sugar. Again, as with the sucrose viability experiment, these amounts were relatively arbitrary in that we wanted a high concentration of sucrose, a control, and something in between. As it has been found that ants are drawn to "sucrose baits" of higher concentration [Blüthgen, Fiedler, 2004], we hypothesized that the ants in the tank with a highly concentrated attractant would have an intentional and direct tunneling route towards that attractant. Furthermore, we hypothesized that the ants in the 1g concentration would not have as targeted a route, and the ants in the 0g tank would not alter their tunneling behavior at all.

Materials/Methods

Materials

- 3 clear containers (dimensions: 5.5 x 6 x 0.5")
- Water (160 mL x 3)
- Agar/agar powder (6.5g x 3)
- Sugar (11g)
- Blue food coloring (~6 droplets)
- Beakers (1L, 50 mL)
- Flask (25 mL)
- Hot plate
- Magnetic stirrer
- Glass stirrer
- Sharpie (or other permanent writing utensil)

Change in Tanks

As this study was being performed at the same time as Trial 2 of Study #2, more boxes needed to be constructed to house all the ants. The three new boxes constructed for this study were intended to have the same dimensions (5.5 x 6 x 0.5") as those in Trial 2 of Study # 2 but due to human error, ended up being slightly smaller, and therefore having a smaller volume than the other set of containers.

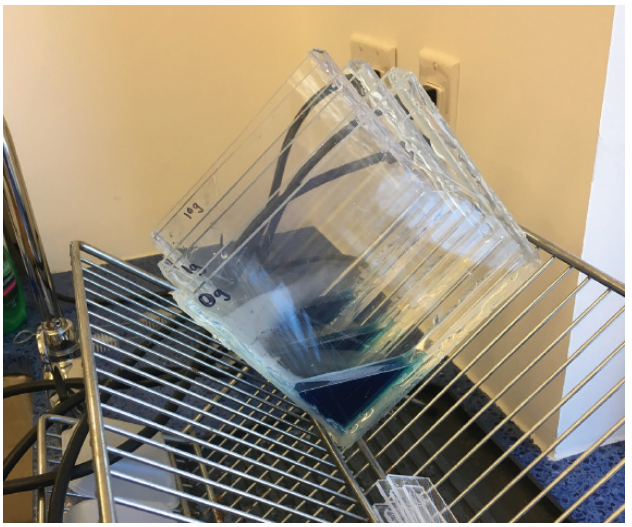
Procedure

*Though similar to previous methods in many ways, this particular procedure had many new facets and therefore was completely written out.

1. Place containers in refrigerator/freezer to cool off in preparation for agar.
2. Place 1 L beaker with water on hot plate and heat until boiling.
3. Add agar powder and drop magnetic stirrer into beaker to quicken transition into solution.
4. While solution is forming measure out 1g sugar and place into 50 mL beaker with 2 drops of blue food coloring.
5. Once agar has dissolved, measure out 20 mL solution (in 25 mL flask) and then pour into 50 mL beaker (with 1g sugar and food coloring).
6. Put mixture back on hot plate and stir with utensil until sugar has dissolved.
7. Pour newly dissolved mixture into the corner of tank. The container should be dried at an angle (see Fig. 12).
8. Repeat steps 4-7 with 10g sugar and then no sugar – simply food coloring (the order in which the various chemoattractants are made does not matter, so long as the procedure stays uniform). Let all containers dry at an angle until they are sufficiently hardened.
9. Pour remaining agar solution (140 mL per container) into tanks and transfer containers into refrigerator to dry overnight.
10. Once agar is dry poke three starter holes in the tanks. Starter holes should be closer to the center so as not to favor the chemoattractant section of the container.

Figure 16

Photograph displays the containers with their chemoattractants drying at an angle.



Data

*for all sets of images, tanks are in order of increasing sucrose level of attractant (ex: 0g, 1g, 10g)

Figure 17

Photographs taken on Day 4

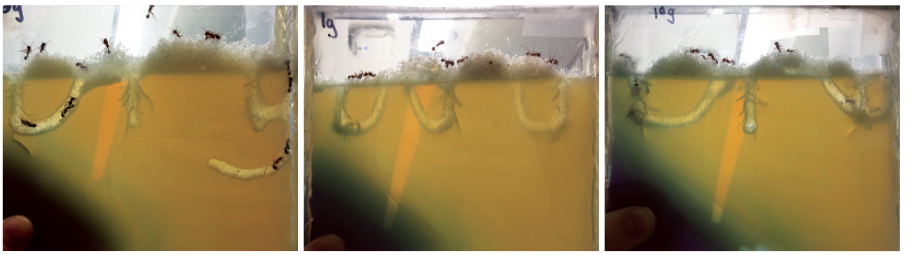


Figure 18

Photographs taken on Day 14

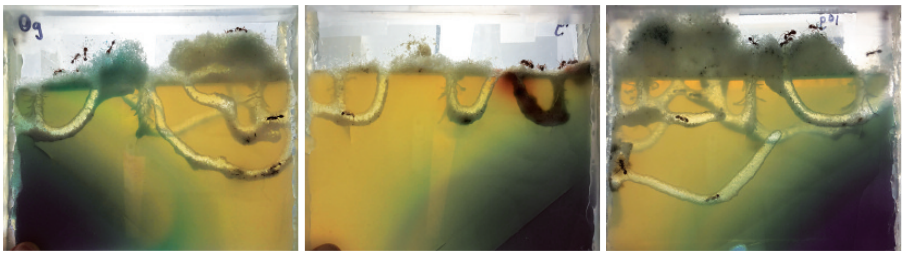
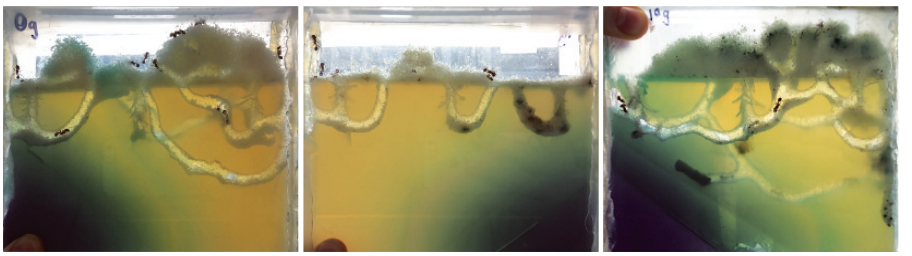


Figure 19

Photographs taken on Day 20



Analysis

In general, the ants did not alter their behavior in the way we hypothesized. Namely, the ants in the 10g container, or even the 1g container, did not direct their tunneling to the attractant. In general, the tunnels in this study were more circular and "loopy" than previous architecture, and appeared more concentrated on the top half of the holding tanks.

These tunnels were relatively thick (many were approximately 1 cm in some places), and because of their winding nature did not suggest any directional intent while tunneling. At the start of the trial, the ants that had ventured the farthest into their tank were the 0g ants, our control – with no attractant at all, and even these tunnels were made on the opposite side of the blue triangular section

marking off the attractant. The overall shape and organization of the 0g and 10g tanks were similar, and the set of tunnels produced in both environments were the most sophisticated so far. Specifically, in both cases there were multiple layers of tunnels – something we had not observed in previous trials.

Due to the similarities in the other two tanks, it seemed odd that tunnels in the 1g container were so visually different from their counterparts. These tunnels were of small size and u-shaped, and unlike in other tanks, were not interconnected. Over the course of the trial the ants did not appear to make much progress (apart from an extension on the right most u-shaped tunnel). As before, the discarded granules on the top of the container served as indicators for the amount of tunneling that took place, and the small pile suggested that there had not been very much. Additionally, one tunnel was completely plugged up, a behavior that was common in the sucrose viability experiment and seems to be closely connected with lack of tunneling and overall discontent.

Throughout the trial, the 0g ants were the only ones to have tunneled towards their food coloring section, and by the end of the data collection period had burrowed down the far left side of their tank. However, as this tank was the control, such behavior cannot be contributed to a chemoattractant. Ants in the 1g tank showed no interest in their attractant, and ants in the 10g container even seemed to avoid it – plugging the only tunnel that came close to the food coloring. Therefore, in this trial it seems that chemoattractants, no matter their concentration, deterred ants from tunneling in close proximity to them.

Discussion

In general, the observations in Trial 1 give rise to many questions. Among them, why were the ants so averse to tunneling near these chemoattractants? The most plausible answer to this question is that they were aware of the food coloring, most likely in a chemical sense as opposed to a visual sense, as their vision is generally poor [Gronenberg, W., 2007]. In future trials food coloring will not be used to mark where the attractant begins. Rather, the tank itself will be marked on the outside (with Sharpie or some other material). While the food coloring very likely played a role, it is also possible that the agar itself gave the ants enough sustenance that they were not attracted to the highly concentrated area of sugar.

Tunneling in this study was much more extensive than the other studies. This could be for a variety of reasons, among them that the tanks used in this trial were smaller and thinner in size than those used for both trials of the sucrose viability experiment. Once again, mold was present in these tanks which is a variable that must be controlled in the next trial of this experiment. Additionally, the number of ants that escaped was not recorded, and should be in the future to account for disparities in tunnel abundance and intricacy.

Additional Conclusions and Questions

As always, there was much more information gleaned from the above studies than could simply be represented in the Analysis and Discussion section. Firstly, over the course of several studies we determined that the most effective way to photograph the agar containers is to hold them up to a light source so that tunnels are illuminated. Keeping in the theme of light, it is important to consider whether the ants' holding environment, inside a shelf that was dark for a large majority of the day, impacted tunneling behavior. One study, investigating the impact of light on various behaviors (tunneling among them) of the western harvester ant, discovered that ants kept in an artificially lit environment at all times tunneled much more than ants kept in a dark environment at all times [Rearick]. Although this is just one study, it highlights the importance of light in holding environments, a variable that we should consider in future studies. Lastly, there were many behaviors observed across all studies that should be

noted. Among them were several previously described cases of ant cannibalism, as well as a tendency to place dead ants or dismembered limbs in specific places in the containers (often wedged inside the mound of agar granules at the top of the tanks). It is interesting to consider whether this behavior is a means of creating a makeshift midden in these artificial agar environments. Additionally, individual ants displayed repetitive behavior, the most notable being touching their gaster to the front of their body. Overall, there were many conclusions and observations noted from the above studies, and they will be taken into consideration in the experiments to come.

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CRISPR/Cas9 Editing

by Kian Sadeghi

Abstract

Streptomycin is toxic for bacterial cells because it binds to the ribosomes and RNA/protein complexes that assist the cell in protein synthesis. Utilizing the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 system we attempted to induce a point mutation in the rpsL gene which encodes the ribosomal s12 protein, thereby making bacteria resistant to the antibiotic. The point mutation was a substitution of an adenine (A) nucleotide for a cytosine (C) at location K87E of the rpsL gene of *E. coli.* To do this, *E.coli* was co-transformed with the Cas9 endonuclease, the gRNA with the spacer sequence, and an oligonucleotide which contained the desired A to C mutation. If CRISPR successfully mutated the gene, the bacterial cells would be resistant to streptomycin (Jiang, 2013). Considering that this experiment was conducted in a high school setting, the fact that the mutation was unsuccessful suggests that further tweaking of CRISPR/Cas9 procedure methods and procedure is necessary before the genome editing device can consistently work in a DIY setting.

Background

Organisms' genomes – the entire set of genetic code inside a cell – are made up of deoxyribonucleic acid or DNA. DNA is made up of genes. Each gene is made up of nucleotides A, G, T, and C that are responsible, generally, for coding for a certain protein with a certain function. (Doudna, 2017). Specifically, different genes code for polypeptides, which are polymers – repeating units – of amino acids. Proteins are made up of one or more polypeptides. The protein created yields a certain trait. This process of turning DNA into a trait is called gene expression. Gene expression consists of two parts: transcription and translation. In transcription, a gene is transcribed into an RNA molecule and then, in eukaryotes, changes to become messenger RNA (mRNA). During translation the mRNA's nucleotides (A, U, G, and C) are translated into amino acids from three nucleotides – called a codon – which code for a specific amino acid. The amino acids make up polypeptides and determine the shape of the protein and, since the structure of a protein determines its function, its specific trait. Genetic differences in the genome of an organism can be consequential as specific mutations can lead to diseases but, thanks to recent advances in DNA sequencing, scientists have the means to determine the genetic mutations that lead to many diseases. Knowing the mutation that can lead to a trait is helpful; however, for a long time, this information had been nothing more than a diagnostic. This was until the arrival of a gene therapy technique that used viruses (Doudna, 2017). Viruses are remarkable at inserting their DNA into the nucleus of their target cells. Thus, scientists engineered viruses with recombinant DNA – DNA engineered in a lab – in the hope of inserting a specific DNA sequence into the affected genome (Doudna, 2017).

Even though this kind of genetic therapy improved during the 1990's, the treatment was largely unsuccessful and even took a massive step back in 1999 when a patient died from an immune response to a high dose of a viral vector (Doudna, 2017). Unfortunately, this kind of gene therapy is ineffective on a wide variety of genetic conditions. For example, Huntington's disease is a disease where the mutated gene dominates the non mutated gene. Thus, the addition of another normal copy of the gene is ineffective. In search of a way to repair problematic genes, as opposed to just insert new ones, scientists turned to homologous recombination. This scientific phenomena is when genetic material is exchanged between two strands of DNA. It occurs in nature, during meiosis, when sections of DNA are exchanged between chromosomes to ensure genetic diversity. In bacteria, the process of homologous recombination can be used as a way to repair genes. By introducing recombinant DNA into a bacterial cell, an extra chromosome that may be paired with a matching gene could induce the bacterial cell DNA to combine with the recombinant DNA through homologous recombination (Doudna, 2017). Often, though, the recombinant DNA was inadequately introduced into the bacterial cell making the technique very ineffective (Doudna, 2017). In the early 2000s scientists adopted another gene therapy technique from nature: zinc fingers. Zinc fingers are naturally occurring proteins that recognize and bind DNA with fingerlike projections. Scientists engineered zinc fingers with the restriction endonuclease fokL to enable it to bind and cleave DNA. Using the two components, scientists created a functional tool for genetic engineering from a naturally occurring protein called Zinc Finger Nucleases or ZFNs (Dhanasekaran, 2003). After the binding of the complex and the subsequent double-strand break, homologous recombination would repair the DNA. Despite the initial success, ZFNs cost labs a significant amount of money since new zinc finger segments need to be created for every genome target. Also, the ZFNs frequently did not identify the targeted sequence or cut the target sequence (Doudna, 2017). Thus, ZFNs were not a reliable gene-editing tool. Similar to zinc fingers being engineered into ZFNs, DNA-binding proteins called transcription activator-like effectors (TALE) were engineered with a DNA cleavage domain to form another gene therapy device. In nature, TALEs bind to a certain DNA sequence. Combined with a DNA cleavage domain, a gene therapy device called transcription activator-like effector nucleases (TALEN) was created (Moore, 2014). TALENs were restriction enzymes – proteins that cut DNA – that could be customized to cut DNA at a specific sequence. TALENs, however, were short-lived as a genome-editing device called CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) was identified in bacteria, allowing scientists to easily, cheaply, quickly, and accurately edit the genome of any organism (Doudna, 2017).

Older, time-consuming, and costly methods of editing an organism's genome, like the ones noted above, were quickly dropped for this powerful gene-editing device. The CRISPR/Cas9 complex was found to be used by bacteria as a way to defend against repeat bacteriophage infection ("CRISPR/Cas9 Plasmids", 2017). There are three different bacterial CRISPR systems, but the type II system is the basis for the CRISPR/Cas9 system used today. ("CRISPR/Cas9 Plasmids", 2017) The immune response process works by recording viral genome – through a process that will later be described – which allows the adaptive immune system to destroy the next wave of the bacteriophage.

The adaptive immune response works as follows: first – as demonstrated in diagram A (in Figure 1) – within the genome of the bacteria, there is a CRISPR array which is made up of protospacers – DNA sequences from bacteriophages that have previously attacked the bacteria – and these are separated by short palindromic repeat sequences. From here, the CRISPR RNA's (crRNA) target genetic code is transcribed (copied) from a single protospacer (in red) to form the crRNA (the code that will direct the complex to the bacteriophage). This process of turning the protospacer into the target sequence is done with the help of a trans-activating crRNA that is homologous to the palindromic repeat that will become the crRNA. From here – as demonstrated by diagram D – the crRNA, tracrRNA,

Figure 1

Clustered Regularly Interpersed Short Palindromic Repeats Array

("CRISPR/Cas9 Plasmids and Resources", 2017)

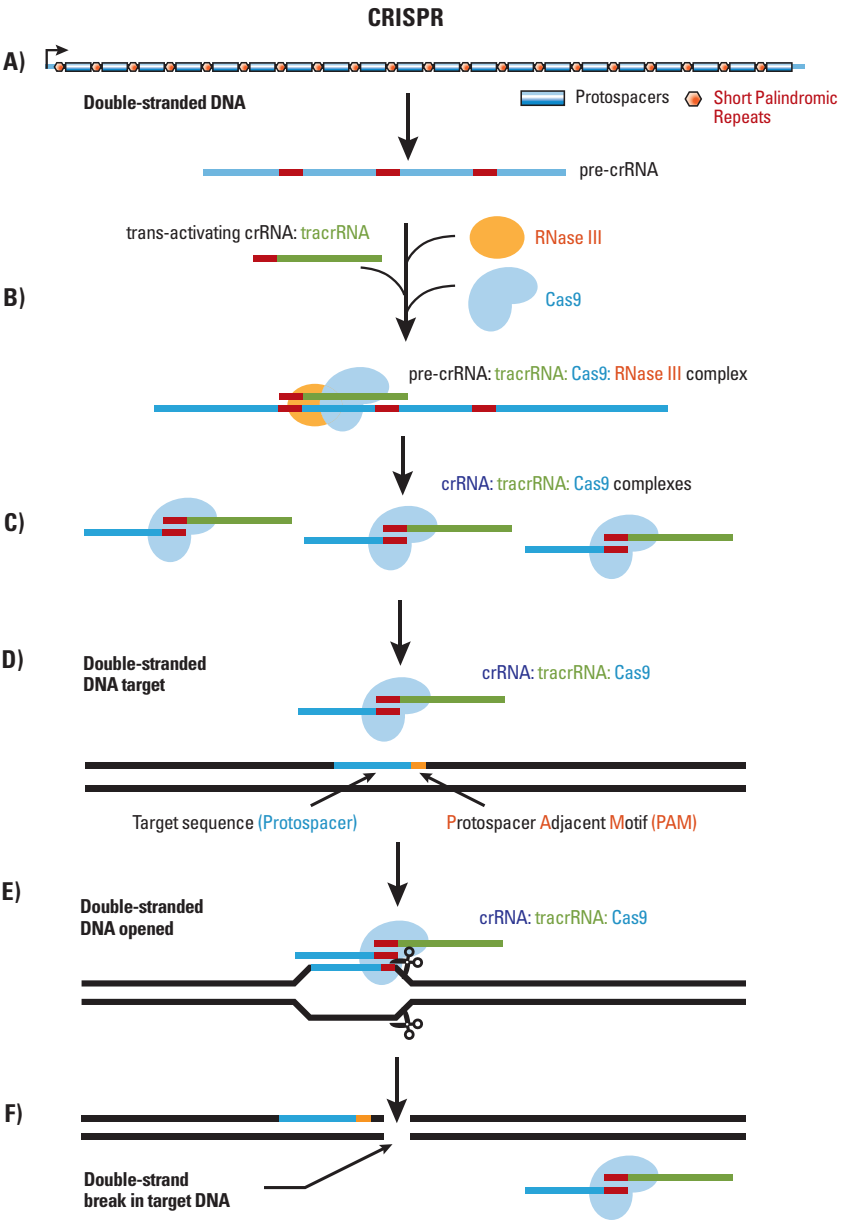
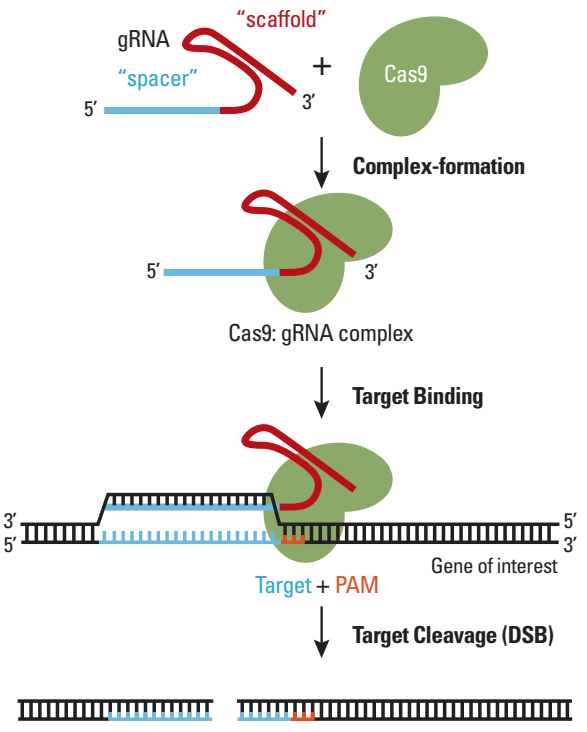


Figure 2

CRISPR/Cas9 Gene Editing System

("CRISPR/Cas9 Plasmids and Resources", 2017)



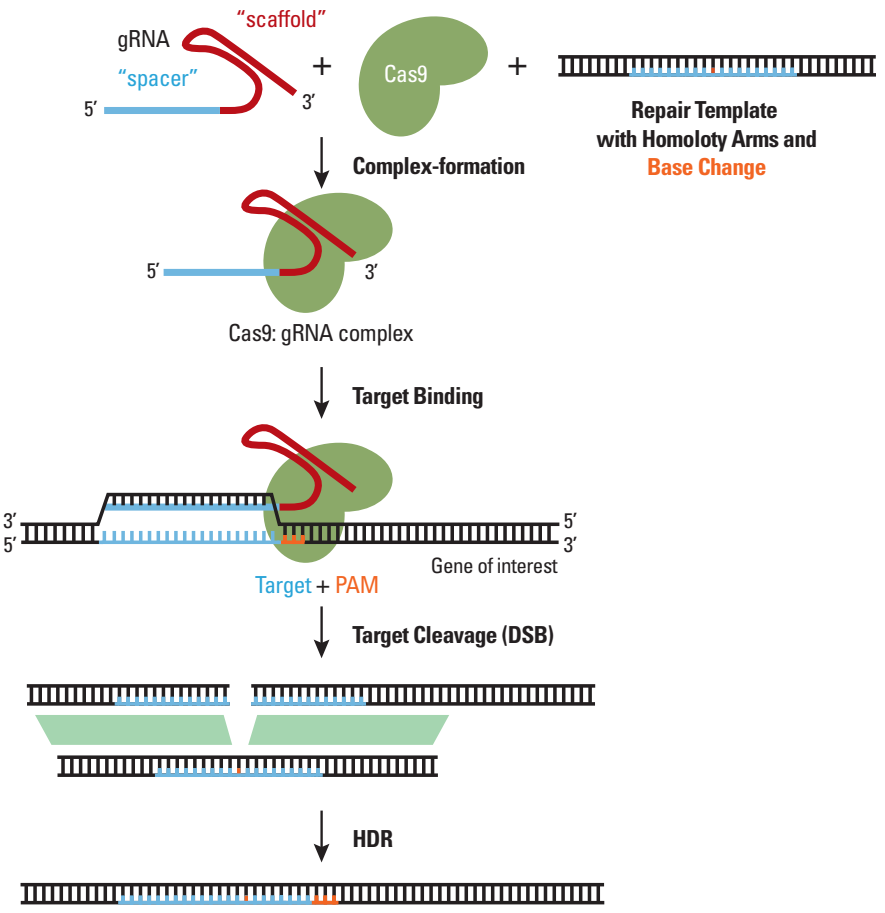
and Cas9 endonuclease form a complex that each seek out a sequence complementary to the specific crRNA. The complex can only bind if there's a protospacer adjacent motif (PAM) sequence directly after the targeted sequence or the crRNA. The viral DNA has a PAM sequence but the host sequence does not ensure that the complex will only cleave the target. Jennifer Doudna and George Churchill harnessed this bacteria's immune response as a new tool for genetic engineering (Doudna, 2017).

The current CRISPR/Cas9 gene editing system consists of two components, a "guide" RNA (gRNA) and a CRISPR associated endonuclease (Cas9). The gRNA combines both the crRNA and tracrRNA in a single synthetic RNA strand. As seen in Figure 2, the scaffold sequence on the gRNA is the tracrRNA which is responsible for binding CRISPR to the targeted DNA, and the spacer sequence on the gRNA is the crRNA which is responsible for taking the complex to the right part of the DNA. The Cas9 endonuclease creates a double-strand break on the DNA or, in other words, cleaves the DNA and either manipulates the genetic material with a knockout where you permanently disrupt gene function or edit the DNA with a specific change. (Doudna, 2017) When using the CRISPR/CAS9 system, the targeted sequence should be unique from the rest of the genome. In addition, the target sequence needs to be upstream of the PAM sequence, which, as previously mentioned, ensures CRISPR will only cleave the target since the viral DNA has a PAM sequence but the host sequence does not. The specific PAM sequence changes with the species of Cas9. ("CRISPR/Cas9 Plasmids and Resources", 2017)

Figure 3

Homology Directed Repair

("CRISPR/Cas9 Plasmids and Resources", 2017)

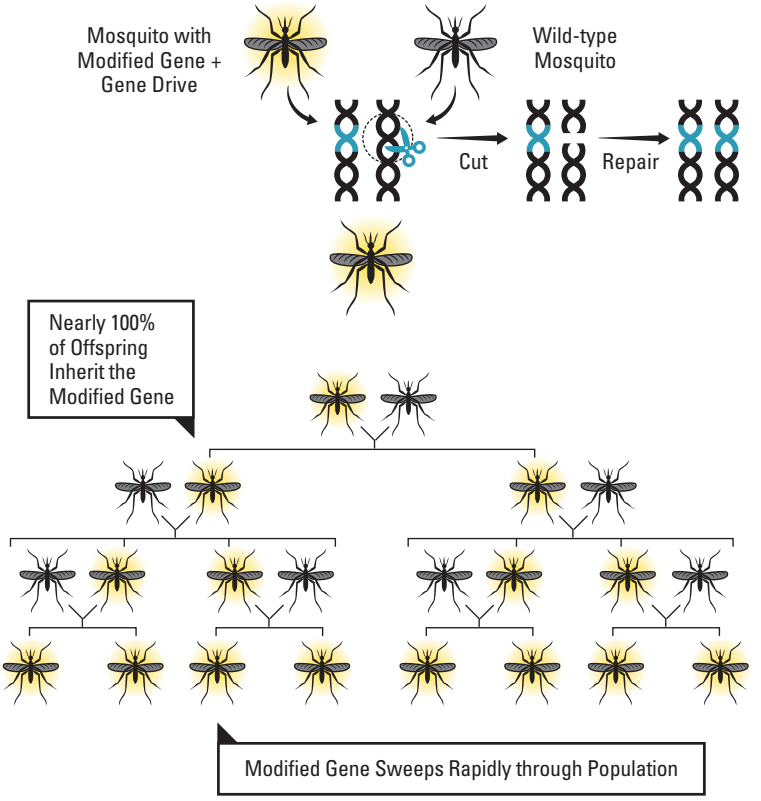


Once Cas9 cuts the target sequence, the DNA will repair itself by either non-homologous end joining (NHEJ) or through homology directed repair (HDR). ("CRISPR/Cas9 Plasmids and Resources", 2017) NHEJ is a faster but less efficient means to repair DNA. Non-homologous end joining utilizes overhangs on the ends of a double stranded break called microhomology to repair the DNA. If the overhangs are perfectly compatible NHEJ works well. However, even though NHEJ is more efficient than HDR, it is far more error prone with indels frequently occuring. As seen in Figure 3, HDR works with a user defined "repair template" that travels with the gRNA and Cas9 endonuclease. The repair template consists of "homology arms" that have homology to the nucleotides surrounding the target sequence and, also, the desired change in between the homology arms, which is illustrated in red in Figure 3. Once the double strand break occurs, the cell will use the template to repair the DNA. The template can be a single- or double-stranded oligonucleotide (short sequences of nucleotides) or a double-stranded DNA plasmid. The repair template cannot contain the PAM sequence otherwise the Cas9/gRNA complex may splice the template. ("CRISPR/Cas9 Plasmids and Resources", 2017)

Figure 4

CRISPR Gene-Drive Inheritance (Ledford, 2015)

The gene-drive system cuts the partner chromosome, then the repair process copies the modification to this chromosome.



With the rapid pace that CRISPR is being used, there has been little time to address the ethical and safety concerns that usually accompany the use of such a powerful tool. One of the safety issues includes the possibility of a gene drive. A gene drive is a situation in which a mutation made by CRISPR on one chromosome copies itself to the corresponding chromosome, so that nearly all offspring inherit the change (see Figure 4). If the scientist is looking to make a precise edit, the desired change is made with homologous recombination. The engineered change will spread through the entire population exponentially faster than if CRISPR was not used. As one can see in Figure 4, in just two generations every mosquito in the population contains the desired edit. A gene drive could have unprecedented effects on an ecosystem and could, potentially, dramatically affect entire populations if CRISPR's gRNA mutates and does not edit the correct part of the genome. In addition, CRISPR's off-target sites have mutation frequencies varying anywhere from 0.1% to 60%. (Ledford, 2015). An off-target site is when the gRNA accidentally binds to a sequence of DNA that closely parallels, or is exactly the same as, the targeted sequence.

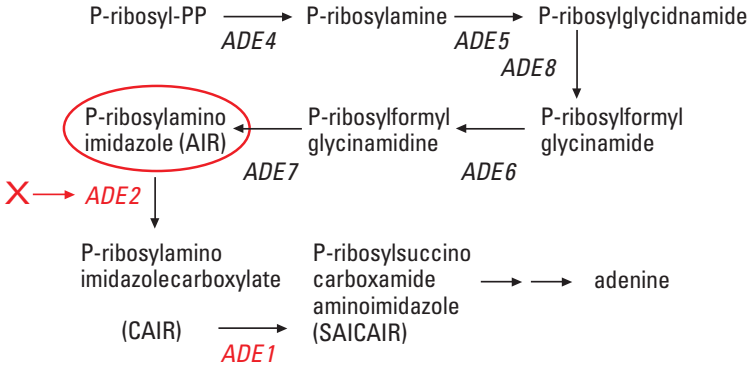
Even low-frequency events could be dangerous if they affect the cell's metabolism or accelerate its growth leading to cancer. However, in my opinion CRISPR's benefits surpass its risks. CRISPR can, in theory, be used on every human disease caused by a genetic mutation, including Retinitis Pigmentosa,

which is a disease that causes blindness due to abnormalities of the photoreceptors in the retina. (Bassuk, 2016). CRISPR could also be used to edit zygotes to cure mutation based diseases like the β-Globin gene in tripronuclear zygotes. (Ma, 2017). The β-Globin gene codes for hemoglobin so researchers have corrected the gene in zygotes. However these zygotes were unable to develop into a human embryo to ensure the experiment was ethical. More recently scientists have used CRISPR to correct the heterozygous MYBPC3 mutation in human preimplantation embryos. (Ma, 2017). The power of CRISPR is remarkable and the device can be used in ways that scientists have not yet explored.

Considering the potentially dramatic impact of CRISPR, a novice in genetic engineering should edit primitive and well understood species where off-target effects would not be consequential. Organisms like bacteria and yeast are the perfect model organisms. *Saccharomyces cerevisiae* (or *S. cerevisiae* – brewer's yeast) is a species of yeast that is used for baking, winemaking, and brewing beer. *S. cerevisiae* are ideal organisms since they are well understood, reproduce quickly, and can easily be cultured. *S. cerevisiae,* like human cells, are eukaryotic, which means they contain organelles and a nucleus. Lastly, *S. cerevisiae* is well priced considering its frequent use in molecular biology.

Figure 5

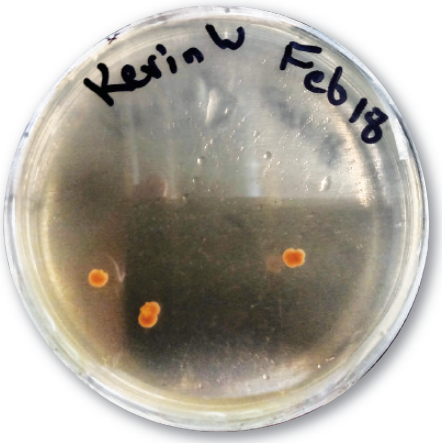
Utilizing CRISPR/Cas9, the ADE2 Gene was Disrupted and then Repaired



Considering these advantages, *S. cerevisiae* were used in an experiment conducted at Genspace – a community lab in Brooklyn, New York. The experiment conducted at Genspace was to expose us to the evolutionary CRISPR/Cas9 system and begin understanding and mapping out how CRISPR could be used in a high school setting. Utilizing CRISPR/Cas9, the ADE2 gene was disrupted and then repaired with the insertion of green fluorescent protein (GFP) coding sequence into the ADE2 ORF. The ADE2 gene is involved in the synthesis of the purine adenine. The gene produces an enzyme called Phosphoribosylaminoimidazole carboxylase which is responsible for converting P-ribosylaminoimidazole (AIR) to P-ribosylaminoimidazolecarboxylate (CAIR) as can be seen in Figure 5. As seen in Figure 6, yeast mutants of ADE2 results in precursor accumulation of AIR and a red pigmentation in yeast. The methodology was conducted in two parts: the "cut" workflow which involved cloning the pML 107 plasmid with the targeted ADE2 gene, and the "repair" workflow which created the repair template containing GFP. After the plasmid and repair template were transformed into the *S. cerevisiae,* a frameshift mutation occurred causing premature stop codons in the ADE2 gene and, thus, precursor accumulation of AIR and the red pigmentation in the yeast. Clearly, the CRISPR disruption was successful but it was unclear whether the GFP repair template was successfully transformed.

Figure 6

CRISPR successfully disrupting the ADE2 gene



After this experiment was conducted, we wanted to design an experiment involving the CRISPR/Cas9 complex in a high school setting. In one study titled "CRISPR-assisted editing of bacterial genomes" researchers used CRISPR to edit *Escherichia coli (E. coli). E. coli,* like *S. cerevisiae,* is a single celled organism that reproduces very rapidly and has a fully sequenced genetic code. The researchers wanted to induce a point mutation in the rpsL gene which encodes the ribosomal s12 protein. The point mutation was a substitution of an adenine (A) nucleotide for a cytosine (C) at location K87E of the rpsL gene of *E. coli.* To do this, *E.coli* was co-transformed with the Cas9 endonuclease, the gRNA with the spacer sequence, and an oligonucleotide which contained the desired A to C mutation. CRISPR successfully mutated the gene, resulting bacterial cell that was resistant to streptomycin. (Jiang, 2013) Streptomycin is toxic for bacterial cells because it binds to the ribosomes and RNA/protein complexes that assist the cell in protein synthesis. By using the CRISPR/Cas9 system to edit the rpsL gene and make the cell resistant to streptomycin, the power and usability of the CRISPR/Cas9 editing system will be demonstrated. Fortunately, conducting this experiment in a school was possible and nearly successful with the DIY gene editing kit from The ODIN (www.the-odin.com/).

Materials

- 1 - LB (Luria Bertani) Agar
- 1 - LB Strep/Kan/Arab Agar (Kan (25 µg/ml), Strep (50 µg/ml) and Arabinose (1mM))
- 1 - 250mL glass bottle for pouring plates
- 1 - 10-100µL variable volume adjustable pipette
- 1 - Box 1-200µL Pipette Tips
- 14 - Petri Plates
- 1 - Microcentrifuge tube rack
- Inoculation Loops / Plate spreader / Pairs of Nitrile Gloves in plastic bag
- 25~ - Microcentrifuge tubes
- 6 - 1.5mL Microfuge tubes containing LB broth
- 50mL Centrifuge tube for measuring liquid volume

- 1 mL Bacterial transformation buffer 25mM CaCl2, 10% PEG 8000
- Non-pathogenic *E. coli* bacteria or NEB® 5-alpha Competent *E. coli* (High Efficiency)
- 55µL of 100ng/µL - Cas9 plasmid Kanr
- 55µL of 100ng/µL - gRNA plasmid Ampr
- 55µL of 1mM- Template DNA
- Microwave
- Sharpie

Procedure 1: CRISPR/Cas9 Gene Editing with Non-pathogenic *E. coli* bacteria

Making Agar Plates

1. Take a tube labelled Agar media and empty its contents into the 250mL glass bottle. The type of media chosen first does not matter. Both LB Agar and LB Strep/Kan/Arab Agar will need to be used.
2. Using the 50mL conical tube labelled "For Measuring Water", measure and add 150mL of water to the glass bottle.
3. Heat the bottle in the microwave (or on a heat plate) for 30 seconds at a time, being careful not to let the bottle boil over. Do not screw the lid down tight. Gently place it on top and slightly turn it.
4. The media is ready when the liquid looks yellow. This should take about 2-3 minutes total of microwaving. Take the bottle out and let it cool until one is able to touch it without much discomfort. This will take 20-30 minutes.
5. While the bottle remains warm, pour the plates. One at a time, remove the lid of the 6-8 plates (depends whether there is a pGLO control. pGLO is a genetically engineered plasmid) and pour just enough of the LB agar from the bottle to cover the bottom half of the plates. Put the lid back on.
6. Let cool for at least one 1 hour before use. If possible let the plates sit out overnight to let the condensation evaporate. Then store in refrigerator at 4ºC upside down to avoid concentration dripping on plates ("CRISPR Bacterial").

Making Competent Bacteria

1. Use an inoculating loop to gently scrape out the bacteria and spread it onto a new LB Agar plate. Let the plate grow overnight for approximately 12-18 hours, or until white-ish bacteria begin are visible. Use an LB agar plate, not the LB Strep/Kan/Arab agar plate. Also, avoid placing the plate in areas that are cold or the bacteria will grow slowly. Consistent and warm temperature and locations are preferable.
2. Pipette 100µL of Transformation mix to a new microcentrifuge tube.
3. Using an inoculation loop, gently scrape some bacteria off the LB plate with the bacteria until the loop is filled, and mix it into the transformation mix. Mix until any big clumps have disappeared. This might require gently pipetting the mixture up and down. The transformation mix should be very cloudy. If it isn't, repeat the first step until the liquid turns hazy but not quite opaque in the tube. Make one tube for each CRISPR experiment and store them at 4ºC (39ºF) in the refrigerator if one is not immediately performing the experiment ("CRISPR Bacterial").

DNA Transformation and CRISPR

1. Find the DNA tube labelled "Cas9 and tracrRNA" and, using a pipette, add 10μL to the competent cell mixture. Change out the pipette tip for a new one.
2. Find the DNA tube labelled "gRNA" and, using a pipette, add 10μL to the same competent cell mixture that was added the Cas9 and tracrRNA to. Change pipette tips.
3. Find the DNA tube labelled "Template DNA" and, using a pipette, add 10μL to the same competent cell mixture that was added to the Cas9 and tracrRNA, and crRNA.
4. Incubate this tube in the refrigerator or on ice for 30 minutes. Do not allow it to freeze.
5. Incubate the tube for 30 seconds in 42ºC (108ºF) water. One can approximate this temperature by using water that is warm, but comfortable enough such that one can still keep one's hand in it.
6. Add 1.5mL of room temperature water to one of the LB media microcentrifuge tubes and shake to dissolve the LB.
7. Using the pipette, add 500μL of LB media to the competent cell mixture containing the DNA.
8. Incubate the tube at 30ºC (86ºF) for 2 hours or 4 hours at room temperature. This step allows the bacteria to recover and replicate the DNA and perform the CRISPR engineering process. Do not skimp on the time; this step is key for the experiment to work. If one is having trouble with the experiment, increasing this incubation time to 12 hours will increase the chances of experimental success. Take an LB/Strep/Kan/Arab plate out of the fridge and let it warm up to room temperature.
9. Using the pipette, add 200μL of the CRISPR transformation mixture on top of an LB Strep/Kan/Arab Agar plate.
10. Using an inoculation loop, gently spread the bacteria around the plate and let dry for 10 minutes before putting the lid back on.
11. Flip the plate upside down to prevent condensation from forming and dripping onto the bacteria.
12. Incubate the plate at 30ºC (86ºF) for 16-24 hours or room temperature for 24-48 hours.
13. If one begins to see little white round dots growing, then the CRISPR genome engineering experiment was a success ("CRISPR Bacterial").

Procedure 2: CRISPR/Cas9 Gene editing with NEB® 5-alpha Competent *E. coli:*

Making Plates

1. Empty the contents of a tube labelled Agar media into the 250mL glass bottle. The type of media chosen first does not matter. Both media will need to be used.
2. Using the 50mL conical tube labelled "For Measuring Water", measure and add 150mL of water to the glass bottle.
3. Heat the bottle in the microwave for 30 seconds at a time, being careful not to let the bottle boil over. Do not screw the lid down tight. Gently place it on top and slightly turn it.
4. The media is ready when the liquid looks yellow. This should take about 2 -3 minutes total of microwaving. Take the bottle out and let it cool until one is able to touch it without much discomfort. This will take 20-30 minutes.
5. While the bottle remains somewhat warm, pour the plates. One at a time, remove the lid of 6-8 plates and pour just enough of the LB agar from the bottle to cover the bottom half of the plates. Put the lid back on.
6. Let cool for at least one 1 hour before use. If possible let the plates sit out for a couple hours or overnight to let the condensation evaporate. Then store in refrigerator at 4ºC upside down so any condensation doesn't drip on the plates ("CRISPR Bacterial").

Transformation Using NEB® 5-alpha Competent *E. coli:*

1. For C2987H: Thaw a tube of NEB 5-alpha Competent *E. coli* cells on ice for 10 minutes.
2. Add 1-5 µl containing 1 pg-100 ng of plasmid DNA to the cell mixture. Carefully flick the tube 4-5 times to mix cells and DNA. Do not vortex.
3. Place the mixture on ice for 30 minutes. Do not mix.
4. Heat shock at exactly 42°C for exactly 30 seconds. Do not mix.
5. Place on ice for 5 minutes. Do not mix.
6. Pipette 950 µl of room temperature SOC into the mixture.
7. Place at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Mix the cells thoroughly by flicking the tube and inverting, then perform several 10-fold serial dilutions in SOC (super optimal broth (SOB) but with Catabolite repression).
10. Spread 50-100 µl of each dilution onto a selection plate and incubate overnight at 37°C. Alternatively, incubate at 30°C for 24-36 hours or 25°C for 48 hours.

Data and Analysis

Table 1

Expected Versus Actual Data Using ODIN Non-pathogenic *E.Coli*

Conditions	Bacteria/LB (200µl/Plate)	Bacteria/LB/ Strep/Kan (200µl/Plate)	Bacteria/LB + Cas9/tracrRNA (10µl)/ gRNA(10µl) (200µl/Plate)	Bacteria/LB/ Strep/Kan + Cas9/tracrRNA (10µl)/ gRNA(10µl) (200µl/Plate)	Bacteria/LB +Cas9/ TracrRNA(10µl)/ gRNA(10µl)/ Template DNA (10µl) (200µl/Plate)	Bacteria/LB/Kan +Cas9/TracrRNA (10µl)/gRNA(10µl)/ Template(10µl) (200µl/Plate)
Expected	Lawn	No growth	Lawn	No Growth	Lawn	Colonies
Actual	Lawn	No growth	Lawn	No Growth	Lawn	No Growth

Figure 7

Example of Lawn

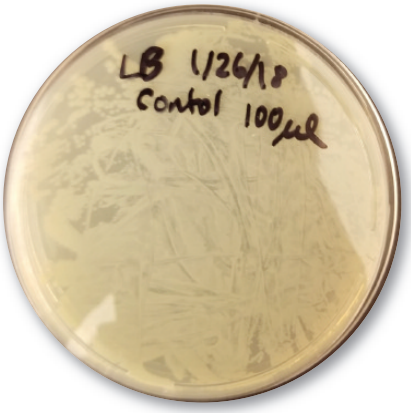


Figure 8

Example of No Growth

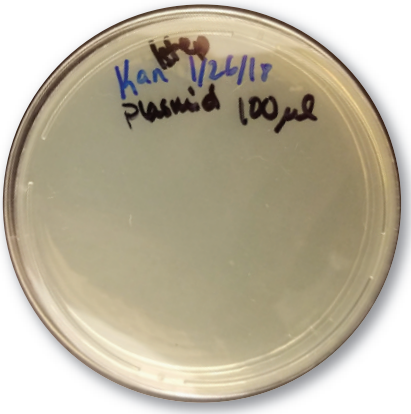
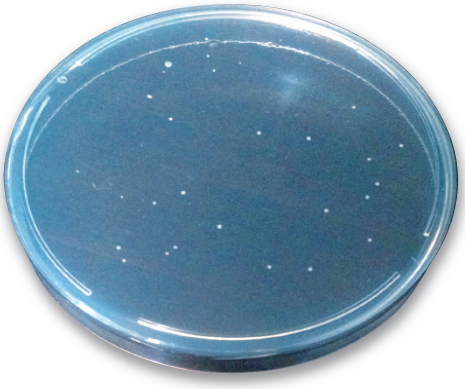


Figure 9

Successful Colonies From CRISPR/Cas9 Editing
(CRISPR Bacterial Guide, n.d.)



Trail One of CRISPR/Cas9 Editing of *E.Coli*

Using the ODIN Kit the following question was posed: "Can the CRISPR/Cas9 system, in a high school setting, be used to edit the rpsL gene in *E.Coli* thereby making the cells resistant to the antibiotic streptomycin?" The results of the first trial can be seen in "Data Table One." The LB and LB/Kan/Strep plate with just bacteria coupled with the two plates without the repair template served as negative controls. These plates served as negative controls since we wanted to see whether the growth of the bacteria on the experimental plate was a result of the CRISPR/Cas9 system and not another factor. If the expected result of a lawn in the LB plates and no growth in the LB/Strep/Kan differed from the actual result, we would then account for another factor that was impacting the bacterial growth besides the plasmids and template DNA. No growth was expected on the LB/Strep/Kan plates without the plasmids and repair template since streptomycin is an antibiotic that binds to the cells' ribosomes disallowing protein production and any growth of the *E. coli*. Colonies were expected for the bacteria transformed with Cas9/Tracr/gRNA and the template since the point mutation would induce the cytosine amino acid instead of an adenine nucleotide which would disallow the ribosomes to be binded by streptomycin, thereby allowing the bacteria to grow. The results were unsuccessful as colonies did not grow in the Bacteria/LB/Kan/Strep + Cas9/TracrRNA/gNA/Template plate. Every other plate satisfied the prediction. These plates were either a lawn, which looked similar to Figure 7, or had no growth as can be seen in Figure 8. This suggests that either the bacteria supplied by ODIN had a low transformation efficiency, so the bacteria simply did not intake the plasmids or repair template, the heat shock (or the recovery time) wasn't long enough also not allowing the bacteria to take in the plasmids, or the CRISPR plasmid supplied by ODIN simply did not work and the bacteria did in fact take in the plasmids and template.

Table 2

Expected Versus Actual Data Using ODIN Non-pathogenic *E.Coli* and Increased Concentrations of Plasmids and Template DNA

Conditions	Bacteria/LB (200µl/Plate)	Bacteria/LB/ Strep/Kan (200µl/Plate)	Bacteria/LB + Cas9/tracrRNA (15µl)/gRNA (15µl) (200µl/Plate)	Bacteria/LB/ Strep/Kan + Cas9/tracrRNA (15µl)/ gRNA(15µl) (200µl/Plate)	Bacteria/LB +Cas9/ TracrRNA(15µl)/ gRNA(15µl)/ Template DNA (15µl) (200µl/Plate)	Bacteria/LB/Kan + Cas9/TracrRNA (15µl)/gRNA(15µl)/ Template DNA (15µl) (200µl/Plate)	Bacteria/ LB/Amp	Bacteria/LB/ Amp/pGLO (15µl/Plate)
Expected	Lawn	No growth	Lawn	No Growth	Lawn	Colonies	No Growth	Colonies
Actual	Lawn	No growth	Lawn	No Growth	Lawn	No Growth	No Growth	No Growth

Trial Two of CRISPR/Cas9 Editing: Increased Concentrations and pGLO Control

To address the faults of the first trail, trial two had a few significant differences. For starters, the volume of the Cas9/TracrRNA, gRNA, and the template increased from 10 microliters to 15 microliters. This change was made in the hope that increasing the volume of the plasmid and template would increase the odds of a successful transformation. In addition, the heat shock of the bacteria was increased from 30 seconds to 40 seconds also to make sure the bacteria had enough time to take in for-

eign DNA. The bacteria were in a 37 °C environment during recovery instead of a 30 °C environment which also increased the odds of a successful transformation. Lastly, a positive pGLO control was added to the experiment. Two LB/Amp plates were made to see if the bacteria with the pGLO plasmid could successfully grow on the LB/Amp plate. If the bacteria was successfully transformed and, thus, pGLO made the bacteria resistant to Ampicillin, then the data would suggest something wrong with the ODIN kit's plasmid. If, however, the transformation did not work (no growth on the ampicillin plate with the bacteria) the data would suggest the bacteria has a low transformation efficiency and the *E. coli* would need to be replaced with lab grade transformation competent cells. The results were identical to the first trial and the bacteria did not grow on the ampicillin plate. This led us to purchase high efficiency bacteria and conduct the experiment with the new bacteria in trial three.

Table 3

Expected Versus Actual Data Using NEB 5-alpha Competent *E. coli* and Increased Concentrations of Plasmids and Template DNA

Conditions	Bacteria/LB (100µl/Plate)	Bacteria/LB/ Strep/Kan (100µl/Plate)	Bacteria/LB/ Strep/Kan + Cas9/tracrRNA (1µl)/gRNA(1µl) (10µl/Plate)	Bacteria/LB/ Strep/Kan + Cas9/tracrRNA (1µl)/gRNA(1µl) (100µl/Plate)	Bacteria/LB/ Kan +Cas9/ TracrRNA(1µl)/ gRNA(1µl)/ Template DNA (2µl) (100µl/Plate)	Bacteria/LB/Kan + Cas9/TracrRNA (1µl)/gRNA(1µl)/ Template(10µl) (100µl/Plate)	Bacteria/ LB/Amp	Bacteria/LB/ Amp/pGLO (10µlplate/ (100µl/Plate)
Expected	Lawn	No growth	No Growth	No Growth	Colonies	Colonies	No Growth	Colonies/ More Colonies
Actual	Lawn	No growth	No Growth	No Growth	No Growth	No Growth	No Growth	Colonies/ More Colonies

Figure 10

Colonies in 10µl pGLO Plate

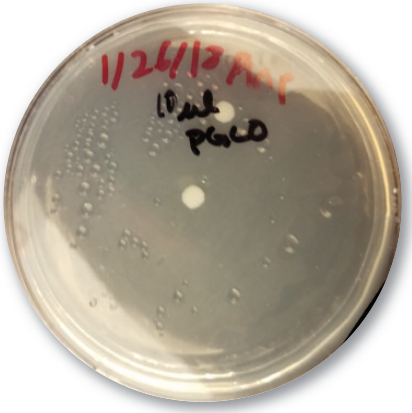
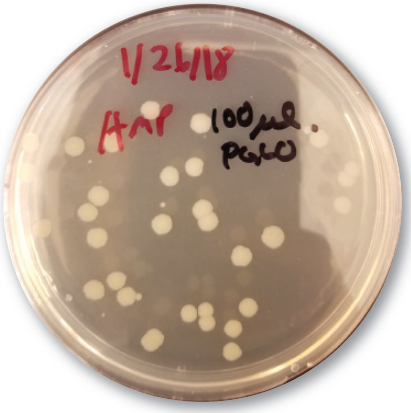


Figure 11

Colonies in 100µl pGLO Plate



Trial Three of CRISPR/Cas9 Editing Using High Efficiency Bacteria

The *E.coli* were a lot easier to deal with and a lot faster as no transformation solution was needed to make these cells competent. Due to the fact that there was a discrepancy between the concentration of Cas9/TracrRNA, gRNA, and template DNA called for in the ODIN kit and in New England Biolabs procedure for the competent cells, it was decided to only use 1µl of the Cas9/TracrRNA and gRNA plasmids (compared to the 10µl suggested in the ODIN kit) as the transformation efficiency of this bacteria is far greater than in the ODIN kit and, consequently, a smaller concentration of plasmids would not equate to a smaller chance of the bacteria successfully transforming. For template DNA, two different concentrations were used (2µl and 10µl) to ensure the bacteria wouldn't have too little or too much template DNA. In order to avoid possible overgrowth on the plates with the plasmids, two different volumes (one 10µl and one 100µl plate) were plated, as well as with the pGLO control (one 10µl and one 100µl plate). All the results turned out as expected except the experimental plate which, in the two plates with different template amount, yielded no colonies whatsoever. The pGLO plasmid was successfully incorporated into the bacteria. The greater the volume on the plates yielded a greater number of colonies. This can be seen when comparing Figure 10 and 11. Figure 10 (100µl) clearly has far more colonies than Figure 11 (10µl).

Conclusions/Discussions

Although the experiment was unsuccessful three times, it is possible the experiment will be successful after one or two more trials. In the next trial, a plate with just Kan resistance will be made. This would serve as another control, in addition to the pGLO plasmid, to check to see if the CRISPR plasmid – which is Kan resistant – allows the bacteria to grow on a plate with Kan.

A more likely explanation for why the kit did not work in the third trial is because the amount of template added to the experimental plates was too little. In addition to increasing template DNA, since the pGLO plate was not completely covered in colonies, the incubation period was probably not at the correct temperature of 37°C. In the next trial, we will be more aware of the temperature in the incubator. Even though the data suggests that CRISPR is not ready to be used in a high school setting, this conclusion would be premature as with any experiment conducted in a classroom, especially when the instrument being used is designed to work in a lab setting, there needs to be a significant amount of tweaking before consistent results can be obtained. Furthermore, the school lacks a refrigerator that can store competent bacteria at -80 ºC. This, however, most likely did not impact the bacteria's ability to transform as colonies did grow in the ampicillin plate.

Part of the appeal of CRISPR is that it is cheap and easy to use. Any time a news article uses these two words, though, they are making a gross-oversimplification. CRISPR is easy to use and cheap *compared to former gene-editing techniques* like TALEN or zinc fingers. CRISPR, is by no means cheap or easy to use for the average person. In fact, painting CRISPR as a technique that has the potential to be adopted by the widespread public is delusional. CRISPR is not – and will most likely never be – like the computer programming revolution where someone can merely build something as influential as Facebook while sitting in their dorm. Even though the revolution will be happening in labs, CRISPR/Cas9 has the potential to completely revolutionize medicine and the world as we know it. CRISPR will likely play a large role in precision medicine, which can be defined as tailoring treatments toward individuals that takes in account variability in genes, environment, and lifestyle. CRISPR may even be used one day to edit human embryos to rid them of any inherent disorders or make a child be a certain height or have a certain colored hair. Before we can get there, though, CRISPR needs to work consistently in correcting simple point mutations in organisms like *E.coli*.

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Factors Affecting Academic Dishonesty at High School

by Nysa Stiell

Abstract

In the present study, Berkeley Carroll 10th, 11th and 12th grade students were tested in order to answer the following questions: are older high school students (11th and 12th grade) more likely to admit to being academically dishonest on an academic survey than younger students (10th grade), and is there a direct correlation between an increase in heart rate and an increase in dishonesty? The experimental questions have a common theme of dishonesty. The first one focuses more on age and how it impacts which students are more dishonest while the second question strives to figure out why students, in general, may be more dishonest. The first part of my experiment consisted of administering anonymous surveys to students. The survey was comprised of nine (out of 20) specific academic honesty questions and eleven general questions about dishonesty such as "Is it okay to lie in order to gain something you want, as long as no one is harmed?" These general questions were a part of the survey so that students would focus on answering honestly rather than being too focused on writing the "correct" answer. The students answered yes/no or gave an open-ended response to 17 of the questions and, for the other three, picked a number on a scale of 1-5 that best represented their opinion. In the second part of my experiment, the students' heart rates were monitored as they answered a series of questions. First they received a survey asking them to initially answer all of the questions honestly, which I considered to be the "base level". They then answered 4 questions incorrectly, which I considered to be "round 1". The students then lied in response to all of the questions which I considered to be "round 2". Their base level heart rate results were compared to the rates during rounds 1 and 2. From this, the averages of the base beats per minute (bpm's) were compared with those of the bpm's in rounds 1 and 2. The resulting p-value, which is the marginal significance, was 0.53. Though this value was not scientifically significant, the visual measurement of each average was compared to the others. To find the results for my first experimental question, which tested which grade was more dishonest, I gathered the data from the students in each grade and divided by the total results of that grade. For the 12th grade the result was 53.44% of students who admitted to academic dishonesty while the 11th grade showed the lowest percentage of students with 30.86%. With this data, I was able to conclude that the 12th grade is most willing to admit to being academically dishonest, followed by the 10th grade and then the 11th grade.

Background

Scientists have done various studies to test the root of dishonesty as well as its impact. One common theme remains prevalent: reward. As Dan Ariely, a James B. Duke Professor of Psychology and Behavioral Economics at Duke University, was able to conclude from multiple studies, "The idea of cost-benefit analysis does not describe our personal experiences" (Association for Psychological Science,

2016). As people consider what they could gain from being dishonest, they tend to analyze whether or not it is worth it. Some small studies that Ariely did included asking participants to take a bribe in order to gain more money in a setup game. These studies showed that any attempt to gain a reward suppresses the dishonest thoughts that are carried along with it. In Ariely's analysis of how cheating and dishonesty are successful, it is necessary to rationalize dishonesty in order to promote the "slippery slope" that is created from continuous lying (Association for Psychological Science, 2016). The terms slippery slope refers to "what begins as small act of dishonesty and then escalates into larger transgressions" (Garrett, 2016). Apart from this discovery the researchers continued to work exploring dishonesty. Dan Ariely, along with Neil Garrett, a professor at the University of College in London, conducted a research plan to test how digressions from one's moral code grew over time and impacted the person in the long term, creating a slippery slope. This term means that as someone repeats dishonest behavior, the signals that are being sent to the amygdala, the part of the cerebral hemisphere responsible for emotion, aren't sent to the same extent (Garrett, 2016).

In the study, *The Brain Adapts To Dishonesty,* Neil Garrett concluded that it became easier for the participant to lie as the opportunity became more frequent. In this study, subjects were given opportunities to gain rewards for themselves by telling one lie. The study was conducted using two subjects and a jar of pennies which determined a person's willingness to lie for a monetary reward. The researchers asked the participants two types of questions: one that would determine their willingness to lie for self-reward and one that would reward them for also assisting their partner. By correctly estimating the amount of pennies in the jar, either the participant would receive a reward, their partner would receive a reward, they would both receive a reward, or neither of them would receive a reward. Each activity allowed the researchers to monitor the reactions of the brain through fMRI scans that measured blood flow. By assessing areas in the brain where there was more blood flow, the researchers could conclude there was more activity in the amygdala when the participant was being dishonest. The amygdala, located in the frontal portion of the temporal lobe, next to the hippocampus, is known to be responsible for emotions, emotional responses and behaviors as well as motivation. In addition to this, the amygdala is essential for decision making (Garrett, 2016). By pairing a stimulus and its emotional value the amygdala is able to create responses to emotional stimuli such as rewards or punishment. (Garrett, 2016). This section receives and gives off signals when dishonesty is registered in the brain. As the opportunity to lie became more evident and frequent there was less blood being transmitted from the brain to the amygdala after being tested by the fMRI (Garrett, 2016). The repetition of self-serving dishonesty led researchers to conclude that there is a significant signal reduction in the brain. As the levels of dishonesty increase, fewer signals were visible over time through an fMRI test. This concept is consistent with adaptations of the human brain; the more self-selected dishonesty occurs, the less stressful the brain and the amygdala registers it to be. As humans have adapted to self-serving dishonesty they register it less which leads to it occurring unconsciously more frequently. Through Garrett and Ariely's research, it is evident that the breach of one area of honesty can influence whether or not the person will continue to lie. As the range of dishonesty increases, the degree of the lies increases as well, over time (Garrett, 2016), (Engelman, 2016).

Another research study was done in 2013 by Heiko Rauhut, a researcher at the Institute of Sociology at the University of Zurich. Rauhut tested participants' honesty in relation to their counterparts. Each participant in the group study was given one die in a closed off, unobservable room, and had the chance to be paid whatever number the die landed on in Swiss Francs. Either the participant lied to gain more money or paid the price of honesty to receive less of a payoff. Each participant was then selected to either an "info treatment" or a "control belief" group. The "info treatment" group learned information about what percentage of the other participants told the truth while the "control

belief" group only gave their information without receiving any. This study demonstrated that participants in the information group who learned about others lying were then more likely to be dishonest to gain a larger monetary reward. Specifically, the participants who underestimated the dishonesty of their counterparts doubled their lying, while those who overestimated it decreased their lying by more than half (Rauhut, 2013). This study supports the concept of the slippery slope in showing that participants were willing to increase the extent to which they lied as the researchers conducted more trials. As they found out that their counterparts were also being dishonest their mindset changed to be more focused on the reward than the dishonesty that was required to acquire the money. Not only did their dishonesty continue as the trials went on, they also generally increased the magnitude of the lies.

Although these studies do adequately represent how the concept of the slippery slope impacts adults they don't show how it can impact adolescents. Researcher Xiao Pan Ding, from the Department of Psychology at the University of Singapore in August 2017 tested 59 right- handed students to examine their brain functions, such as blood flow, during honest and dishonest behavior. He concluded that as the students were dishonest their ability to learn and make sense of complex concepts decreased. Furthermore, he found that dishonesty can disrupt the brain developments that are supposed to develop as a child grows (Ding, 2017).

As students, we spend about 30% of our day at school, learning and making sense of complex information. Many of the key functions that need to be developed in our brains occur as we learn in school. It is important to ensure that our brains have the opportunity to fully develop and that this development is not shortened by academic dishonesty. For this reason, academic honesty is necessary in schools. In order to remove the concept of instant rewards through plagiarizing and copying, teachers should enforce integrity through honest test taking and homework skills. With a technological 21st century on our hands, students tend to believe that if they use technology to get the reward, they no longer consider it cheating (Piascik, 2010).

The research articles and studies about how the brain, specifically the amygdala, handles untruthful information were useful in creating a research study to assess the process of dishonesty. This led me to test academic dishonesty because it would ethically align with the values of the school as well as help to better understand the students in the community. However, it would be difficult to decipher those who admit to being dishonest and those who have been dishonest but do not admit it as well as monitor the reactions of the amygdala in high school students through brain scans, as I didn't have access to this technology. In order to test academic dishonesty in grades 9-12, I looked at plagiarism and cheating tendencies in Berkeley Carroll high school students.

Materials

- 15-20 students from the 10th, 11th and 12th grades at Berkeley Carroll
- Silent, controlled classroom setting
- Online anonymous survey (figure 1)
- Google sheets to track survey responses
- Paper copies of student quiz (figure 2)
- Heart Rate monitor on Apple Watch generation 2

Section 1 Procedure

1. Gather groups of students in the 10th-12th grades from SRD and physics classes who are willing to participate in the survey process.
2. To maintain anonymity, create a number system that aligns to each grade with 200+ being grade 10, 300+ being grade 11 and 400+ being grade 12.
3. Each student will take the survey, answering all 20 questions in a classroom setting with a teacher present. Once the student is given a number in accordance with their grade level, they can independently begin.
4. The 20 mixed general and academic honesty questions will be provided to each class through an anonymous link on their google classroom that will submit the data into a spreadsheet I will receive when it is completed by each student.

The survey is written below:

Figure 1

Honesty Questionnaire

a. Academic Questions

1. Have you ever plagiarized from an online source?
2. Have you ever plagiarized from a paper source ?
3. Have you ever copied ideas from websites like SparkNotes or Shmoop?
4. Have you ever turned in a piece of writing with work that wasn't original, without citing the source?
5. Have you ever copied a classmate's homework?
6. Have you ever given your homework to a classmate to copy?
7. Have you ever provided answers on an in-class assessment that were not your own (copied from someone near you, looked on your phone, brought in a cheat-sheet)?
8. Have you ever made up a story or excuse to a teacher in order to justify asking for an extension?
9. Have you ever stayed home from school on the day of an assessment in order to gain extra study time?

b. General Questions

1. What time did you get to school?
2. Who was the last person you lied to?
3. Is it okay to tell a lie in order to protect someone you care about?
4. Is it okay to tell a lie in order to protect yourself?
5. Is it ever okay to lie?
6. Have you asked anyone about any of these questions so far?
7. Is it okay to lie in order to gain something you want?
8. Is it okay to lie in order to gain something you want, as long as no one is harmed?
9. How often do you lie to your friends? (Scale 1-5)
10. How often do you lie to your parents? (Scale 1-5)
11. How often do you lie to your teachers? (Scale 1-5)

5. Analyze each response in order to determine if there are more "yes" responses to each question in the higher grades (11,12) than in the 10th grade.

Section 2 Procedure

1. Gather a group of students that are willing to participate in an experimental portion to test dishonesty.
2. Create a set-up quiz with 14 questions on topics from personal life, academics, sports and honesty. Have students come into a standardized classroom one at a time in order to take the quiz.

The survey is written below:

Figure 2

Honesty Questionnaire for BPM Determination

Questions
1. What is your shoe size?
2. What is your middle name?
3. What grade are you in?
4. What sport is played in Berkeley Carroll in the wintertime?
5. What is your favorite color?
6. Who is the school psychologist?
7. How many quarters are in a basketball game?
8. Why did the cow jump over the moon?
9. What is the chemical form of water?
10. Which is sweeter? Water or juice?
11. What neighborhood do you live in?
12. Where do plants grow from?
13. What is the current time?
14. What is your last name?

3. Have the student answer each question on their own time then retrieve the sheet of paper.
4. Once the students have all the correct answers, attach the Apple Watch to their left wrist with the Heart Monitor app upwards towards me.
5. Record the student's bpm before having them respond to the 14 questions. Monitor their response while they are answering the questions honestly and use this measurement as a basal level for this individual being 100% truthful.
6. The second time around, (round 1), have the student answer the same 14 questions but select 4 out of the 14 questions to have them willfully lie on while monitoring their heart rate. Verbally ask them the questions and have them respond but, on the selected 4 questions, have them look at a blank sheet with the questions circled.
7. Maintain the heart monitor on the student and monitor their bpm before the 4 selected questions, between questions 2 and 4 and then after the 4 selected questions.
8. The third time, (round 2), have the students lie on all 14 questions. Record their bpm before they begin answering the questions, and then when the subject is done record their final bpm.
9. After all students individually take all rounds of the quiz, analyze the collected data in order to determine whether or not the change in the subjects' bpm is significant.

Section 1
Data and Data Analysis

Experimental Question: Are upper class students (11th and 12th grade) more likely to admit to being academically dishonest on an academic survey than lower class students (10th grade)?

Independent Variable: yes or no responses to each question
Dependent Variable: number of lies on academic honesty questions

Figure 3

Students Admitting to Academic Dishonesty by Grade

Depicts the percentage of students by grade level who admitted to being academically dishonest. This chart was made by gathering raw data and calculating the percentage from each grade. Because each grade level is a percentage calculated out of 100 it could not be calculated with a p-value.

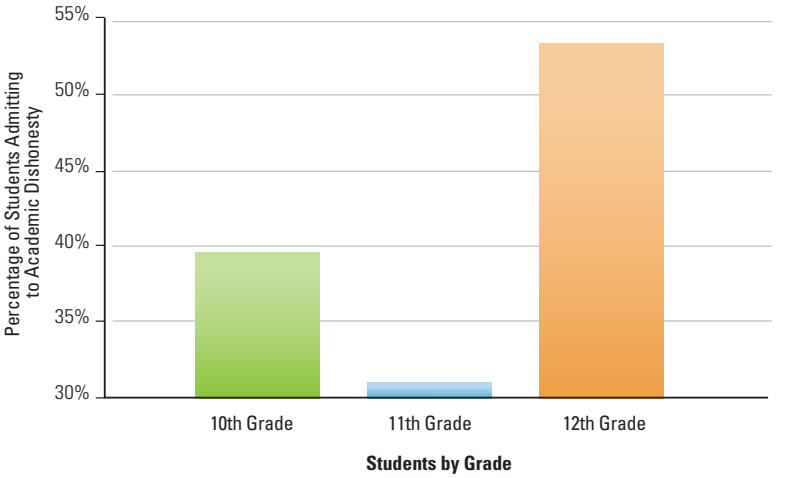


Figure 3a

Questions That Had the Most "YES" Responses

Each question that had the most popular yes answers along with how many students in each grade said yes.

Question	Have you ever copied a classmate's homework?	Have you ever given your homework to a classmate to copy?	Have you ever made up a story or excuse to a teacher in order to justify asking for an extension?
Number of Students that said "YES" in Grade 10	16	17	4
Number of Students that said "YES" in Grade 11	12	14	4
Number of Students that said "YES" in Grade 12	16	18	17

Analysis

Comparing the admitted academic dishonesty of the 10th grade test subjects to the 11th grade, the 10th grade had a slightly larger percentage of 39.60% compared to the 30.86% of the 11th grade. However, compared to the 12th grade's 53.44%, the 10th grade had about 13% less. When gathering students I attempted to use 20 from each grade so that looking at the the results we would be able to see a clear comparison. The differences in percentages between the 11th and 12th were the greatest. The 11th grade had the lowest percentage leading me to conclude that that grade has the least amount of students willing to admit to academic dishonesty while in the 12th grade that grade has the most students willing to admit to being academically dishonest. Using this sample size to reflect the greater population of the grades I can conclude that students in the 11th grade are less willing to admit to being academically dishonest while about 40% of the 10th grade is admittedly academically dishonest and almost 55% of the 12th grade is admittedly academically dishonest.

Section 2
Data and Data Analysis

Experimental Question: Does an individual's physical reaction (heart rate) to dishonesty decrease the more they are dishonest?

Independent Variable: Number of questions answered dishonestly
Dependent Variable: Beats per minute (bpm)

Figure 4

Beats Per Minute Readings

This table shows the raw data of each beats per minute (BPM) reading for each subject. The first row shows the basal reading before conducting the tests. The second row shows the fastest reading the subject had during round 1 of the test and the second row shows the fastest reading the subject had during round 2

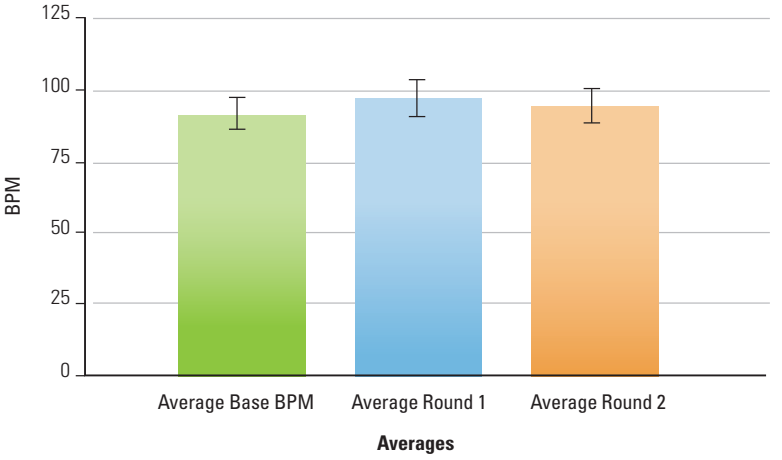
Subject Number	1	2	3	4	5	6	7	8	9	10
Base bpm	92	90	66	111	103	86	88	91	101	83
Peak During Round 1 (bpm)	94	123	77	108	104	88	78	93	105	86
Peak During Round 2 (bpm)	92	122	78	108	95	84	78	95	103	87

Experimental Data Analysis

Figure 5

The Average Number of Lies Compared to Average Heart Rate (BPM)

Shows the average heart rate in beats per minute for each round of the tests. For these results I divided the total BPM measurements by the number of participants and calculated the averages for each round.



Analysis

The results of a correlated t-test indicated whether there was a significant difference between average base beats per minute in the first round and average base beats in the second round for each subject. I calculated a p-value of 0.529849. This data suggests that there is no significant change in a subject's physical response when they lie more.

Discussions/ Conclusions

The substially larger percentage of 12th grade students admitting to academic dishonesty suggests that there is a lot that can be assumed about the academic integrity in Berkeley Carroll. For example, seniors are closer to leaving Berkeley Carroll so being academically honest may not matter to them as much. They also may simply be more willing to admit to academic dishonesty. For the juniors, the American Studies class they all take is based on the justice system and power. It also requires independent thinking and research which does not provide the same leeway for plagiarism and cheating that 10th grade classes do because their work is more class-based. In addition, looking at the data table to analyze the questions which had the most frequent 'yes' answers, there is a clear trend of copying homework. From the data, the largest number of students in each grade admitted to both copying another person's homework and giving their homework up to a friend to be copied. I have come to the conclusion that the 12th grade students are most willing to admit to being academically dishonest, followed by the 10th grade and then the 11th grade. There were areas which could have been improved to have more accurate

results. Firstly, the number of subjects I was able to test in the second study, 10, was minimal and definitely impacted the results I got. Another aspect of my procedure that could have been improved would be the device I used to test the subjects. Using my Apple Watch was efficient but, in terms of seeing real time data, the watch only measured and updated the beats per minute every 30-45 seconds. Overall, this experimental procedure can help to uncover the process and motive for students lying in high school.

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A Comparison of Vertical Illusions Between Knuckleball and Traditional Pitch Sequences

by Sara Tobias

Abstract

This study investigates the relationship between knuckleballs and vertical illusions by asking the question, "is there a difference in vertical illusions between batters facing knuckleball and traditional pitch sequences?" A knuckleball is a pitch that moves with minimal spin, resulting in random motion. My experimental question was created by thinking about how this random motion might affect a batter's ability to predict the ball's velocity and location – in other words, the batter's susceptibility to a vertical illusion. Vertical illusions were measured using both direct and indirect data. The indirect data is based on Major League Baseball (MLB) statistics of home runs per innings pitched (HR/IP), strikeouts per nine innings (K/9), line drive percentage (LD%), and soft hit percentage (Soft%). The direct data was gathered using video and a Zepp sensor to record vertical angle, attack angle, bat speed, and result of the swing. The indirect data suggested that there is no difference in vertical illusions between batters facing knuckleball and traditional pitchers; however, there is a difference in strikeouts per nine innings between knuckleball and traditional pitchers. The direct data shows that knuckleballs resulted in 70.6% swings and misses and only one instance of solid contact, which would suggest that there is a difference in vertical illusions between knuckleball and traditional pitches. However, because of limitations on the experimental procedure, this conclusion could not be statistically supported.

Key Vocabulary

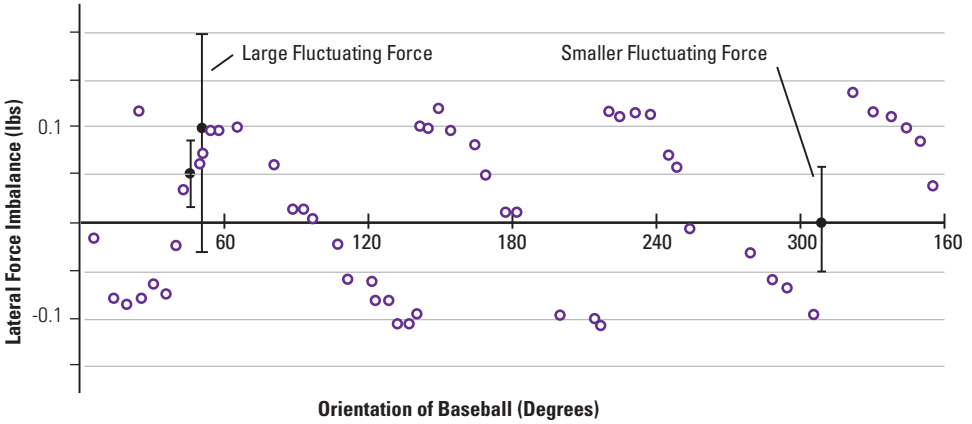
- *Attack angle:* direction that barrel of bat is moving at impact
- *Bat speed:* speed of bat at impact
- *Changeup:* a pitch thrown at a slower speed, usually moving downwards as it reaches the batter
- *Fastball:* a pitch thrown at a fast speed
- *Knuckleball:* a pitch with minimal spin which causes erratic movement
- *PITCHf/x:* system that tracks data of every pitch thrown in every MLB stadium
- *Traditional Pitcher:* Pitcher who throws conventional pitches, such as fastballs, changeups, and curveballs
- *Vertical Illusion:* a deceptive appearance caused by an incorrect prediction of the ball's velocity and location
- *Vertical Angle:* angle of barrel in relation to knob at impact
- *Zepp Sensor:* sensor attached to the knob of a bat that collects data when bat is swung

Background

In *Aerodynamics of a Knuckleball,* written in 1974 by Robert Watts and Eric Sawyer, the researchers addressed the question "What causes the erratic motion of a knuckleball?" From the batter's point of view, a knuckleball looks like it is "fluttering" or "dancing" because of this random movement. To test this, they used a wind tunnel that accurately represented the ball moving through the air. They were able to measure the forces of lift (force that acts at a right angle to the direction that the ball is moving) and drag (force that acts opposite to the direction that the ball is moving) to see what effect these had on the baseball. They found that as the position of the seams change, meaning as the ball rotates, the lateral forces (forces acting from the sides) on the ball change as well because the seams are raised above the baseball itself. Because the seams are asymmetrical, even if the ball only rotates a small amount, the lateral forces will still change. This property can be applied to any pitch thrown, not exclusively a knuckleball. Figure 1 below shows the lateral force on the ball in relation to its orientation (Watts and Sawyer, 1974).

Figure 1

Change in Lateral Force Based on Orientation of Baseball (Watts and Sawyer 1974)

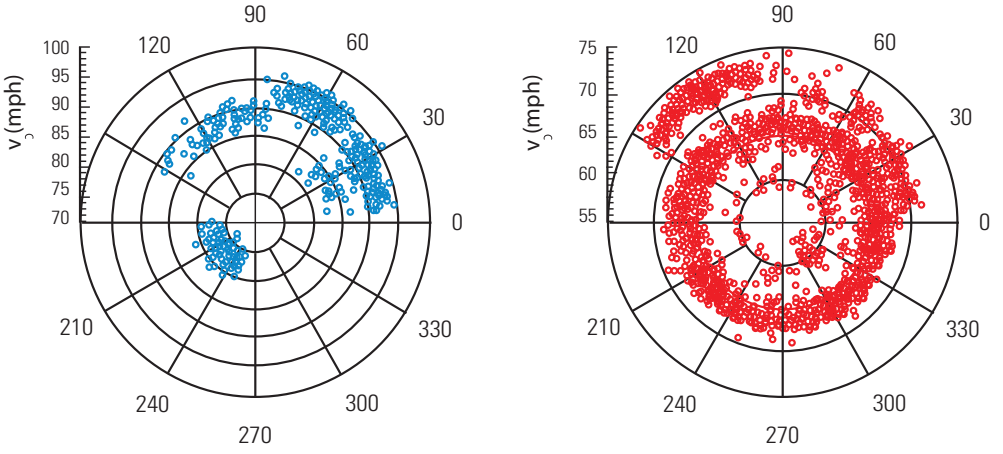


The change in force causes the direction of movement of the ball to change. The way in which the ball rotates is random because the asymmetrical seams lead to asymmetric lateral forces. Watts's and Sawyer's analysis of the trajectories of knuckleballs shows that, even though there is not a sudden curve in the trajectory, the ball is deflected from its smooth path due to lateral forces. However, batters witness the ball's lateral deflection changing at an accelerating rate as it moves towards them, which causes their view of the ball "fluttering" (Watts and Sawyer, 1974). Finally, Watts and Sawyer found that the lateral forces acting on the knuckleball are proportional to the velocity squared. They represent this in the equation F=v2, with F being the lateral force and v being velocity (Watts and Sawyer, 1974). The harder the ball is thrown, the larger the lateral force acting on it will be. Therefore, the harder the ball is thrown, the more erratic the movement will be because lateral forces dictate a knuckleball's movement. The ball must rotate fewer than one full rotation before reaching the plate to get the optimal movement needed to deceive the batter. If too much spin is put on the ball, it will not move much from its original path; however if zero spin is put on the ball it will move in a straight line because the seams are not changing position. Therefore, the optimal amount of spin is about one or less than one rotation before reaching the plate (Watts and Sawyer, 1974).

Alan Nathan published the paper *Analysis of Knuckleball Trajectories,* building on a lot of the same ideas that Watts and Sawyer had researched almost 40 years before. Beginning a few years before this paper was published in 2012, a system called PITCHf/x was installed in every MLB stadium as a way of measuring the movement of pitches. This system uses cameras placed at different angles that track the position of the ball throughout its flight. These points are compared and the intersection of the points shows where the ball is at each moment in time. In using PITCHf/x data of knuckleball pitchers, both past and present, Nathan found that the movement as well as the velocity (magnitude) of a knuckleball is random. The trajectory of a knuckleball, however, is much like the trajectory of any other baseball pitch. This is because the force needed to suddenly change the trajectory of a pitched ball would have to be much larger than any force that would typically affect the baseball. Since it is impossible for a pitcher to put this amount of force on the ball mid-flight, knuckleballs, just like any other baseball pitch, keep a smooth trajectory. Nathan notes that Watts and Sawyer did not come to this same conclusion because of their use of a wind tunnel in their study, which led them to believe that the trajectory was not smooth. Nathan's paper illustrates well the random angle and velocity of a knuckleball as seen in Figure 2 below.

Figure 2

Angle and Velocity of Pitches Thrown by a Fastball Pitcher (Blue Dots) and a Knuckleball Pitcher (Red Dots) (Nathan 2012)

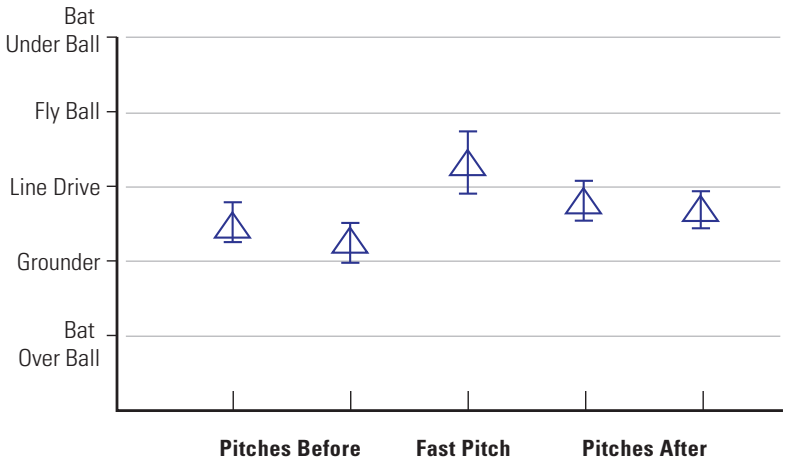


The pitches thrown by a fastball pitcher are clearly clustered based on angle and speed. If a fastball is thrown at 90 mph, it will travel at about a 65 degree angle. For a knuckleball pitcher, however, the angle and speed are random as one can observe from the red points being scattered in a ring shape around 66 mph. Nathan also notes that the lateral movement on a normal pitch is caused by the Magnus force from the spin on the ball. The Magnus force in conjunction with a pitched baseball suggests that a pitched ball spinning in a moving airstream will be affected by a force that causes it to curve away from its perceived path based on the angle of the spin. A ball with a lot of topspin, like a curveball, will be more susceptible to the Magnus force because of its large amount of spin and will therefore move downward as it travels to the plate. If a knuckleball does not spin, the Magnus force must have no effect, therefore creating no predictable movement (Nathan, 2012).

Terry Bahill and David Baldwin discuss how changes in velocity affect a batter's prediction of the location of the pitched baseball in the strike zone in The Vertical Illusions of Batters. The researchers observed that many batters claim to have seen a fastball with so much velocity that it rises as it gets close to the plate, or a curveball that dives down. Both these situations, however, are impossible due to the laws of physics and therefore must be caused by some kind of vertical illusion from the batter's point of view. This illusion is caused by an incorrect prediction of the ball's velocity which leads to an incorrect prediction of the location the ball will travel to. Batters base their predictions on the previous pitch (Bahill and Baldwin, 2003); therefore, if the batter sees a fastball, they will predict that the next pitch will end up in the same location as that fastball. If the next pitch is a curveball, since it has less velocity than a fastball, it will drop more than the fastball before it reaches the plate. Because of this, if the batter swings they will likely swing over the ball, resulting in a ground ball. The batter misinterprets their error in judgement of the velocity and location of the ball as a phenomenon in the ball's flight (Bahill and Baldwin 2003). In the example of a fastball followed by a curveball, the batter would believe that the curveball had a sudden dip down as it traveled to the plate. This, however, is impossible because it would have to follow a perfect parabolic path. Typically, if a batter expects a pitch to be faster than it is, they will swing over the ball and hit a ground ball, while if they expects the pitch to be slower than it is, they will swing under the ball and hit a fly ball or pop-up. Figure 3 shows that when the batter suddenly sees a faster pitch, they will hit a flyball because their bat will be under the ball (Bahill and Baldwin, 2003). Pitchers will vary the speed of their pitches and work fast off of the mound to ensure that the batter still has an image of the previous pitch in their mind. Bahill and Baldwin's main conclusion is that vertical illusions of the ball rising into the batter's eyes, or dipping down to their knees, is caused by an incorrect prediction of the ball's distance and speed which leads to the misreading of this error as the actual flight of the ball (Bahill and Baldwin 2003).

Figure 3

Angle of bat and result of swing based on pitch sequence (Bahill and Baldwin 2003)



While Bahill and Baldwin did not specifically do research concerning knuckleballs, their research begs us to question what we would find if we looked at the random movement and velocity of knuckleballs in the context of vertical illusions. In my research I will bring the concepts of knuckleball movement and vertical illusions together to try and understand the differences in vertical illusions between batters facing traditional pitchers and knuckleball pitchers. This leads to my experimental question – "Is there a difference in the occurrence of vertical illusions between batters facing knuckleball pitchers and batters facing traditional pitchers?" In this question, the independent variable is the different types of pitchers, either knuckleball or traditional. A knuckleball pitcher is defined as a pitcher who throws primarily knuckleballs, while a traditional pitcher is defined as a pitcher who throws primarily fastballs. The dependent variable is vertical illusions and will be measured both indirectly and directly. First, it will be measured indirectly by home runs per innings pitched (HR/IP), strikeouts per nine innings (K/9), line drive percentage (LD%), and percent softly-hit balls (Soft %). Then it will also be measured directly, using video of batters facing traditional and knuckleball pitchers and software which will measure the angle of the bat before impact and the location of the ball as it reaches the batter. The results of both the direct and indirect experiments will be combined to come to a conclusion about the differences in vertical illusions between knuckleball and traditional pitchers.

Procedure

The indirect data was collected by identifying eighteen knuckleball pitchers from 1976 to the present using simple random sampling. The sampling was done by assigning each team a number (1-30 alphabetically) and assigning each pitcher on these teams a number, also alphabetically. Two numbers were randomly generated which corresponded to a team and a pitcher on that team. For example if 1,1 were generated, that would mean the team is the Arizona Diamondbacks, and the pitcher is Jake Barrett. The PITCHf/x program with data published in FanGraphs was used to find data for HR/IP, K/9, LD%, and Soft% for each pitcher. The data was analyzed using a two-tailed t-test to calculate the p-value.

The direct data was collected using a live pitcher, who was a traditional pitcher at a Division 1 level, and 3 high school hitters. Since he was able to throw knuckleballs, he served as both the traditional and knuckleball pitcher for the live experiment. A batting cage was set up with home plate 55 feet from the pitcher's rubber[1] and a piece of tape indicating where the batter should stand. A Zepp sensor was attached to the knob of a 3 inch, 30 ounce bat to be used by all hitters. Batters warmed up by taking front toss (taking batting practice when the pitch is thrown underhanded from a short distance away from the batter) and the pitcher warmed up by throwing for 10 minutes. The pitcher threw three sequences of pitches to each hitter. Every hitter got sequence one, before moving on to sequence two, and then sequence three. The first sequence was 10 pitches of fastballs (F) and changeups (C) and went FFCFFCFFCF. The second sequence was 9 pitches of knuckleballs (K) and fastballs (F) and went KKFKKFKKF. The third and final sequence was 5 pitches of fastballs (F), changeups (C), and knuckleballs (K) and went FCKFK. Every sequence was recorded using a video camera set up behind the pitcher to capture the result of the pitch. Each swing was recorded by the sensor attached to its knob that measures vertical angle, attack angle, and bat speed.

[1] On a standard baseball diamond the plate is 60 feet 6 inches away from the rubber, but because of spatial restrictions the plate is 55 feet from the rubber.

Data

Figure 4

Round 1

Video results data tables.

The batted results of rounds 1, 2, and 3 for batters 1, 2, and 3 in relation to pitch type (fastball, knuckleball, or changeup).

Swing #	Pitch Type	Batter 1	Batter 2	Batter 3
1	Fastball	Take	Take	Check
2	Fastball	Foul pop right	Hard Ground Middle	Swing and Miss
3	Changeup	Swing and miss	Take	Take
4	Fastball	Swing and miss	Take	Swing and Miss
5	Fastball	Take	Ground Left	Take
6	Changeup	Take	Take	Take
7	Fastball	Take	Foul Left	Swing and Miss
8	Fastball	Foul Line Drive Left	Take	Swing and Miss
9	Changeup	Foul Ground Left	Swing and miss	Swing and Miss
10	Fastball	Swing and Miss	Take	Foul Right

Figure 5

Round 2

Swing #	Pitch Type	Batter 1	Batter 2	Batter 3
1	Knuckleball	Take	Ground Left	Take
2	Knuckleball	Take	Swing and Miss	Swing and Miss
3	Fastball	Take	Take	Swing and Miss
4	Knuckleball	Take	Pop Up Left	Check
5	Knuckleball	Swing and miss	Pop Up Middle	Check
6	Fastball	Take	Pop Up Middle	Swing and Miss
7	Knuckleball	Swing and miss	Take	Swing and Miss
8	Knuckleball	Take	Check	Swing and Miss
9	Fastball	Foul right	Swing and Miss	Swing and Miss

Figure 6

Round 3

Swing #	Pitch Type	Batter 1	Batter 2	Batter 3
1	Fastball	take	take	take
2	Changeup	take	ground left foul	Foul straight back
3	Knuckleball	Swing and miss	Swing and miss	take
4	Fastball	Line drive left	Foul tip right	take
5	Knuckleball	Hard ground middle	Swing and miss	take

Figure 7

Bat Speed, Vertical Angle and Attack Angle

The resulting bat speed, vertical angles, and attack angles at contact of each pitch sequence that was swung at by batter 1.

Traditional Pitchers (randomly sampled):

Figure 8a

Strikeouts Per 9 Innings for Knuckleball vs. Tradional Pitchers

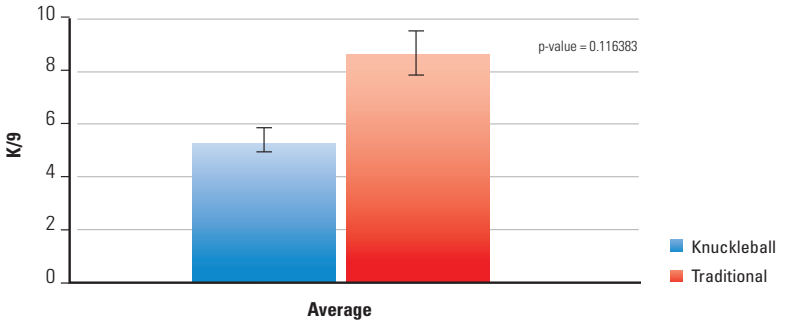


Figure 8b

Home Runs per Innings Pitched for Knuckleball vs. Tradional Pitchers

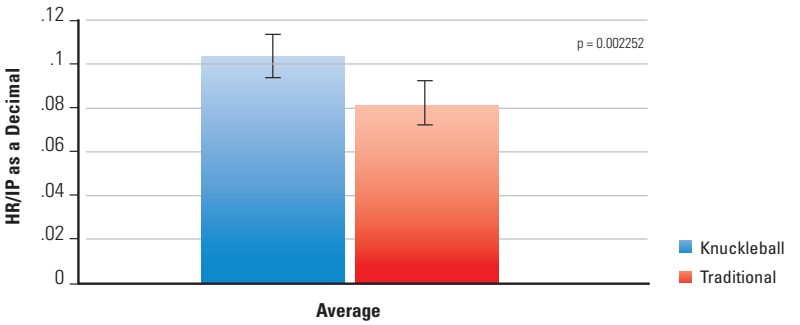


Figure 8c

Line Drive Percentage for Knuckleball vs. Traditional Pitchers

MLB indirect data. Comparisons of home runs per innings pitched (a), strikeouts per nine innings (b), line drive percentage (c), and soft hit percentage (d) between knuckleball and traditional pitchers.

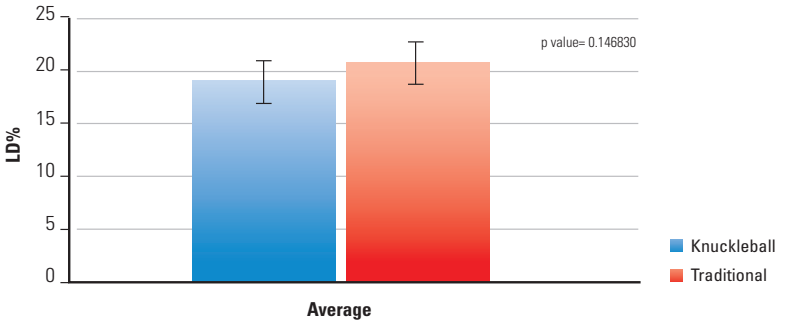
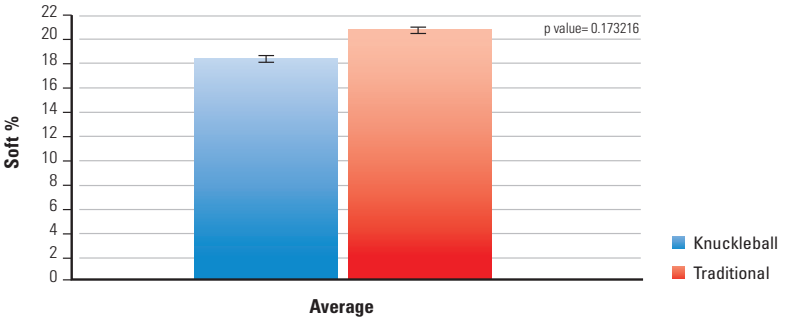


Figure 8d

Soft Percentage Between Knuckleball vs. Tradional Pitchers



Analysis

The initial data I collected looked at the differences in vertical illusions between batters facing knuckleball and traditional pitchers using home runs per innings pitched (HR/IP) and strikeouts per nine innings (K/9). I conducted two independent, two-tailed t-tests to find a p-value which will represent the significance level of the data. HR/IP yielded at p-value of 0.116383, and K/9 yielded a p-value of 0.002252. These graphs were made using the averages of K/9 and HR/IP for knuckleball and traditional pitchers. The knuckleball pitchers' average HR/IP was 0.10374, and average K/9 was 5.37. The traditional pitchers' average HR/IP was 0.174, and average K/9 was 8.779. The error bars were made using the standard error of the mean (SEM). The data presented suggests that there is a difference in vertical illusions as measured by K/9 innings, but there is not a difference in vertical illusions as measured by HR/IP. This being the case, there is not enough evidence to conclude that there is a difference in the occurrence of vertical illusions between batters facing knuckleball and traditional pitchers. However, because there was a significant p-value in K/9, there is a possibility that batters facing traditional pitchers are more susceptible to vertical illusions. For a greater understanding of vertical illusions at the Major League level, I gathered data using two other means of observing vertical illusions: line drive percentage (LD%)

and soft hit percentage (Soft%) for batters against the same knuckleball and traditional pitchers. The graphs and error bars were constructed in the same way as the previous graphs. Neither of these statistics lead to a significant p-value (0.146830 and 0.173216 respectively), though knuckleball pitchers had lower percentages of both line drives and soft hits. Traditional pitchers had an average LD% of 20.8 and an average Soft% of 20.9, while knuckleball pitchers had an average LD% of 17.7 and an average Soft% of 18.4. When measuring LD% to illustrate vertical illusions, a lower percentage of line drives indicates a lower percentage of vertical illusions. This statistic alone might suggest that knuckleball pitchers had a lower vertical illusion percentage than traditional pitchers. However, when measuring Soft% to illustrate vertical illusions, a lower percentage of soft hit balls indicates a higher percentage of vertical illusions. In this way, knuckleball pitchers would be considered to have a higher vertical illusion percentage. Therefore, no significant conclusions can be drawn from either LD% or Soft% because they do not match up with one another, and in fact, they oppose one another.

Since the indirect Major League Baseball data did not provide any significant values that could lead to a greater conclusion about vertical illusions between knuckleball and traditional pitchers, I went on to collect direct data using a live pitcher and high-school aged batters. A portion of the data was collected by video, and a portion was collected using a Zepp sensor. The Zepp sensor was only able to pick up the entirety of the data for Batter #1, while the video camera picked up data for all 3 batters. All the data collected by the Zepp sensor was gathered from Batter #1 through their 3 rounds of batting practice. According to Zepp, a goal bat speed is 45 mph, a goal vertical angle is -25 degrees, and a goal attack angle is 10 degrees. The greatest bat speed was 65 mph on a FF combination and resulted in a line drive to the 3rd base side. The lowest bat speed of 47 mph occured on a knuckleball that was thrown after a fastball, as did the second lowest bat speed (49 mph). Two knuckleballs thrown in a row resulted in a bat speed of 51 mph, while a knuckleball followed by a fastball resulted in a 52 mph bat speed. The two knuckleball combinations that resulted in a higher bat speed were KF (61 mph) and CK (59 mph). The closest to the goal angle of -25 degrees occurred on FC, FK, and KF combinations, all of which had lower bat speeds. The majority of vertical angles were around 0 degrees, showing no specific trend in changes in vertical angle based on pitch sequence. There was more variation in attack angle, ranging from -21 degrees to 18 degrees. Fastball-knuckleball (FK or KF) combinations yielded higher attack angles than KK or CK combinations. Four out of eight knuckleballs thrown were not swung at, and 3 out of the 4 that were swung at resulted in a swing and miss. The one knuckleball that was put in play resulted in a hard ground ball up the middle (the previous pitch had been a fastball which resulted in a line drive to the 3rd base side). The first two knuckleballs thrown resulted in takes, as well as two more takes on the next two pitches. In round 2, only 1 out of the 10 pitches thrown was connected with (foul 1st base side). This was the first round when knuckleballs were introduced. In round 3, both knuckleballs were swung at, one resulting in a hit. Because so few knuckleballs were swung at, it was difficult to come to a conclusion about vertical illusions using the results of batter #1.

Though data for batters 2 and 3 was not collected by the Zepp sensor, it was collected by video. After being thrown two knuckleballs (which resulted in a ground ball and a swing and miss) batter #2 took a fastball. The second time batter #2 was thrown two knuckleballs in a row they swung and missed at a fastball. Batter #3 swung and missed at the fastball in the KKF sequence both times it was thrown. When thrown a changeup followed by a knuckleball, batter #2 swung and missed at the knuckleball and batter #3 took the knuckleball. Batter #3 only made contact with one pitch (a changeup) after knuckleballs began to be mixed into the pitch sequence. When thrown a CKFK sequence, batter #2 grounded the changeup to the 3rd base side, swung and missed at the 1st knuckleball, fouled the fastball to the 1st base side, and swung and missed at the second knuckleball. Regardless of what pitch was thrown prior to it, a knuckleball resulted in a swing and miss the majority of the time when thrown to either batter #1, #2, or #3. Because knuckleballs resulted in so many swings and misses, one

might be able to assume that knuckleballs resulted in more vertical illusions than either fastballs or changeups. However, there were very many limitations, including the fact that these batters have seen very few knuckleballs in their baseball careers. With this being the case, one cannot come to the conclusion that there is a difference in vertical illusions between batters hitting knuckleballs and traditional pitches.

Conclusions and Discussions

Though the results of both the indirect and direct data were not explicitly conclusive, it is important to mention the limitations that restricted the data collection. First and most importantly, the three subjects were high school students hitting against a pitcher who had played for a Division 1 college. Not only that, but the hitters did not have experience hitting against knuckleballs and so it was difficult for them to make the adjustment to knuckleballs in the short amount of time that they were hitting. Also, because of spatial limitations, the pitcher was throwing from 55 feet instead of 60 feet 6 inches and was not throwing off of a raised mound. The hitters may have adjusted their normal bat angles because the pitches were not coming from 10 inches above the ground as they would have if they had been thrown from a pitcher's mound. This de-elevation may also have inhibited some of the movement of knuckleballs and changeups. In terms of the actual collection of the data, when video taping the pitches, the ball sometimes blended in with the white background making it difficult to pick up exactly where the ball was hit. The Zepp sensor also did not pick up some of batter #2's and #3's swings which made it impossible to compare the Zepp data to the video data for these batters.

Although there were slight patterns that arose between different sequences of pitches, not enough of these same sequences were thrown to allow us to presume that this pattern would be continually repeated. The indirect data suggested that there might be a difference in HR/IP between knuckleball and traditional pitchers, but I was not able to replicate this in my direct data collection to find whether there is a true connection between HR/IP and vertical illusions. In the future, the experiment might be more successful if I repeated the same pitch sequences many more times so that a clear pattern was visible. Not enough of the data gathered indirectly yielded significant results, making it impossible to accept my hypothesis that there is a difference in vertical illusions between knuckleball and traditional pitch sequences. Though the data gathered directly showed a lot of swings and misses on knuckleballs, this was not significant enough to come to a conclusions about vertical illusions. Therefore, taking into account both my directly and indirectly collected data, I am not able to come to a particular conclusion on whether there is a difference in vertical illusions between knuckleball and traditional pitch sequences.

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The Effects of Protein and Carbohydrate Supplementation on High School Varsity Basketball Players' Energy and Recovery Levels

by Lukas Yurasits

Abstract

The aim of my experiment was to figure out how the supplementation of carbohydrates (sugar), protein (amino acids) and the combination of the two affect the energy levels and recovery process differently for high school basketball players during and after practice. My independent variables were the different supplements I gave my subjects while my dependent variable was the qualitative answers of how my subjects felt turned into quantitative data through a survey I created. My alternative hypothesis was that there would be a difference in the subjects' energy levels and how they recovered between each supplement. My experiment was split up into two four-week trials with each trial involving two practice dates using water, sugar, protein, and Gu (a gel product marathon runners use). My results were not significant except for the comparison of the average energy survey answers between sugar and the Gu product with a p-value of .020262. Since that was the only comparison that was significant I had to accept my null hypothesis of seeing no difference in the subjects' energy levels and how they recovered between each supplement. In conclusion, different protein and carbohydrate supplementation did not affect the energy and recovery levels of high school basketball players.

Background

We all see professional athletes use their amazing athletic abilities on TV every night, but the important question to ask is how these athletes gain or maintain those abilities. One answer is sports nutrition. How the energy and recovery of these athletes are affected or enhanced by nutrients is at the center of this field. This industry is now a multibillion dollar business with sports teams wanting their athletes to perform at the highest levels possible. While this field of study is gaining more and more followers, there are a lot of complications that come with testing and figuring out what supplement affects which aspects of performance because there are many variables including weight, height, body fat, skill level, strength, etc. Sports nutrition is a combination of medicine, nutrition, physiology, kinesiology, biology, chemistry, and many more fields. It is an intersectionality of multiple fields of study that focuses on the individuality of how supplements affect athletes. The nutrition facet is one of the most complicated, comprised of so many nutrients which perform a wide variety of functions for the body. I have separated nutrition into four main pillars: water, carbohydrates, protein, and fats.

Water is of course the first and main pillar of nutrition because that's what comprises most of our body. Water transports nutrients through the body, flushes out all the waste, and is the major component of all bodily fluids. You can go weeks without food but only five to seven days without water (Saltman, Paul, et al, 1993). The energy, or fuel, for the body is measured in calories. Our body burns these calories and produces the energy the body needs through chemical reactions. This "energy" is the taking apart of complex molecules and then adding them into new ones in the body. Digestion, the formation of body cells and tissues, and the use of fuel all falls under the umbrella of metabolism. Metabolism converts this fuel into the energy the body needs for all functions. The first and most important source of fuel is glucose. Carbohydrates – grains, fruits, and vegetables – are not directly glucose, but become glucose after a transformation or two. Carbohydrates are made up of carbon, hydrogen, and oxygen, and they either come in single or double molecular structures or very complex ones called starches with 300 to 1000 molecular compounds. The one-molecular sugars are monosaccharides which consist of glucose, fructose, and galactose. Two-molecular sugars are disaccharides which consist of sucrose, lactose, and maltose. Glucose is found in nearly all fruits and vegetables and it moves rapidly from the digestive tract to the bloodstream. Fructose is different in structure than glucose and moves more slowly into the bloodstream. It isn't as effective until it is in the liver and converted into glucose. Galactose is rarely if ever seen by itself and exists primarily as a part of lactose. The disaccharide molecules are monosaccharides linked together and always contain at least one glucose. Sucrose is regular table sugar with a glucose and a fructose; it is sweeter than glucose but not as sweet as fructose. Lactose is milk sugar composed of glucose and galactose. Maltose is malt sugar which shows up when beer is brewed and in malt milkshakes; it is comprised of two glucose molecules. Starches are long chains of only glucose molecules but they take longer than simple sugars to be broken down in the digestive system. However, they are more beneficial because they have fiber and other important nutrients. Glucose is the primary source which feeds the nervous system. If the glucose stored in your body was depleted and your digestive system tried to move to fats and proteins it wouldn't be as effective or useful. Fatty acids and other fat molecules can't be used to fuel the nervous system, and when the body starts to break down proteins it will damage the tissues and muscles that the protein is taken from to keep the body functioning (Saltman, Paul, et al, 1993).

Proteins are used for more processes than any other nutrient in the body from fuel to hormones to building tissues and muscles. There are 50,000 forms of proteins all built from very long strands of 22 amino acids – 13 the body can produce on its own and 9 which are needed from foods. Proteins are present in every cell of the body and handle most of the body's "hard work": demolition, construction, self-defense, transportation, and emergency repairs. A form of proteins are enzymes which direct and accelerate certain chemical reactions such as demolition work of breaking down nutrients in the digestive system and construction work of putting compounds together from the small raw materials available in foods. Antibodies are proteins that protect the body from viruses. Proteins can also transport nutrients to other places while keeping the body's fluid balance, and a special protein, collagen, helps to form the clots and scar tissue to seal wounds and is also a major component in tendons, ligaments and reinforcing artery walls. Without proteins kids may not grow and develop in the correct manner becoming weak and more vulnerable to diseases and infections. There are nine essential amino acids: isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, and histidine. High quality proteins like eggs contain these essential amino acids. While these are essential for everyone, each body is different so one person may need more of one amino acid than someone else or may need more in general to develop a strong, healthy body (Saltman, Paul, et al, 1993).

Fat is an essential part of our foods because that's where most of the flavor resides. Yet another one of its major functions is keeping the body's demand for fuel consistent. Two thirds of the body's

energy needs are provided by fat, and the body's fat mass is a place to store all of that energy. Fatty acids help with the maintenance of cell membranes and blood vessels, synthesizing hormones, nerve impulses, and memory storage. While people always try to avoid fats, they are essential to the development of the body and human's health. A lack of essential fatty acids can damage kidneys, make blood vessels more fragile, and cause cholesterol build up in the lungs, liver, skin, adrenal glands, and may also cause enlarged heart (Saltman, Paul, et al, 1993).

These are essential elements of people's nutrition on a day to day basis. It follows that moderation is important because the body can be affected negatively by too much of one nutrient and not enough of another. When you add sports into this equation of nutrition, it is important to figure out when, why, and how these nutrients should be given to athletes. Figuring out how these four pillars of nutrition really affect an athlete's performance is an essential question in sports nutrition. How does each pillar affect the energy levels of these athletes during competition? What are the athletes eating and drinking to gain a full recovery after a workout or strenuous activity? Why is this supplement causing a change in performance numbers? When is this supplement at its full potential? I have read multiple papers to get a better understanding of how other scientists have formed and observed the effects of nutritional supplements.

In the study *The acute effects of multi-ingredient pre-workout ingestion on strength performance, lower body power, and anaerobic capacity,* researchers were testing the supplementation of multi-ingredient performance supplements (MIPS) on 12 division III college football players in the offseason. Participants needed to have participated in a resistance training program (workouts that utilize body weight, gravity, bands, or weighted bars as resistance against your limbs to improve muscle strength) utilizing bench press or back squats for more than two years. Subjects that consumed any other nutritional supplements like caffeine, creatine, or beta-alanine within the last 3 months were excluded. Also they were told to keep their regular eating and exercise habits with an exception of the subjects not being able to exercise 48 hours before the testing day. The subjects came in for a familiarization test for the exercises, a baseline test a week later, then the first of two experimental tests where they would eat the MIPS or the placebo. The experimental test consisted of a blood lactate test, a questionnaire to assess focus and energy, consumption of placebo or MIPS supplement, and a standard dynamic warm-up 20 minutes later. Following the warm up subjects began with a countermovement vertical jump test (CMVJ), then back squats (BS) for 5 sets of 5 repetitions, and bench press (BP) of 5 sets for 5 repetitions, and lastly a full out sprint test against resistance. The next experimental test occured a week later with the subjects taking the opposite treatment. The experiment didn't produce many significant results, with the CMVJ jump test having no significant differences in either lower body peak or mean powers, and no significant effects in the BP and BS workouts. The only real conclusion the study was able to find through the questionnaire on focus was that the subjects felt less fatigue and more alert after taking MIPS (Jagim et al, 2016).

The purpose of the next study I read was to see the effect of evening caffeine ingestion on knee extensor and knee flexor strength and power in females taking oral contraceptive steroids (OCS). The study had 10 female subjects who were team sports players (soccer, hockey, and netball). Some of the control variables were that participants had to control their diet 48 hours before the experiment day, and alcohol, caffeine, and exercise was prohibited 24 hours before the experiment. The subjects then had to keep a food diary for those first 48 hours before the experiment to replicate it for the next experimental day. The experimental process consisted of a familiarization test with the exercises used and then the subjects would return on days 5-8 for the first experimental test and days 18-22 for the second test. Once the subjects came to the lab – as well as during and after the experiment – they took a blood test to measure glucose, insulin, and free fatty acids, and then were given a whole gelatin

capsule with either the caffeine supplement or the placebo. Sixty minutes following the ingestion, the athletes carried out their exercises of six 15 minute blocks on running on a treadmill to simulate patterns of a soccer match, isokinetic dynamometer for assessing leg strength and power, and a countermovement jump (CMJ) using a jump mat. The results of this experiment were similar to the last with not many significant results. There were no effects on peak torque (rotational force) and isometric (a muscular action where tension is created without muscle contraction) strength in knee extensors, no effect on mean powers of both knee extensors and flexors, no effect on the jump test, and no effects on the heart rate through the treadmill test. The only statistically significant effect they saw was a trend in the increase of strength in the knee extensors for the caffeine group. The experimenters said that caffeine could help with sprinting and kicking activities, but would need more research to make concrete conclusions (Ali et al, 2016).

Another paper I read was about the effects of creatine supplementation on cardiovascular adaptation to submaximal exercise, maximal endurance, and body composition. Creatine plays a vital role in the release of energy for muscles because it directly turns into phosphocreatine (PCr) which are reservoirs for ATP. ATP (adenosine triphosphate) is the molecule used to produce contractions in muscles. Sixty endurance trained athletes were split up into two groups: one experimental and the other control. Like the other papers, diet was a big control variable. In this study there was no alcohol or caffeine one week preceding the experiment and throughout the whole experiment. The activities the subjects could perform were also controlled. Outside the experiment, subjects could not take part in strenuous activity two days before the exercise test. The experimental group was given 5 g of creatine monohydrate and the control group was given 5 g of maltodextrin – a placebo – per day for 4 weeks and the same training program was given to both groups. Body mass, body fat, and fat free mass were all measured before and after supplementation. One week before the experiment the subjects ran through a familiarization test on a treadmill. The test consisted of a treadmill test in which the experimenters measured heart rate responses, time until exhaustion, and resting, maximal, and recovery heart rates. Each group was tested only twice during the 4 weeks. This study however had many more significant results than the others with an improvement in heart rate responses in the submaximal exercise and an increase in maximal endurance after supplementation. There were no effects on body composition or changes in resting, maximal, and recovery heart rate responses (Manna and Khanna, 2016).

In the last study I read, the purpose of the experiment was to observe the effects of a pre-work supplement in conjunction with three week high-intensity interval training (HIIT) on aerobic and anaerobic running performance, training volume, and body composition. The study took twenty-four males and females who were moderately trained and tried to control for age, stature, body mass, VO2 max (a measure of oxygen use), and percent body fat. The participants visited the lab 18 separate times, the first 3 for a familiarization test, the next 3 for the baseline test, the next 9 for training days – 3 per week, all separated by 24-48 hours – and the last 3 for post-testing sessions. There was an experimental group of 13 test subjects who were given a supplementation of 100 mg of caffeine, 0.5g of creatine, 1g of BCAAs – branched chain amino acids that reduce protein deterioration during intense exercise – 9g of whey protein, 2.5g of cordyceps sinensis, and a .075 g of combined citrulline and rhodiola. The control group of 11 was given a placebo in powder form similar that given to the experimental group. Each group took their supplement without eating four hours prior, they weren't allowed intense exercise 24 hours prior, and they started the testing exactly 30 minutes following ingestion of supplement. The actual test was five sets of running exercises with the intensity of the test increasing each set, and a minute rest in between each set. The conclusions of the experiment was as follows: the supplementation significantly improved critical velocity and total training volume when compared to the placebo group, and a trend to improvement of lean body mass was observed. However, because of so many

different products in the supplement, the experimenters couldn't pinpoint which nutrient led to these results (Smith, Abbie E, et al, 2010).

These studies and their methodologies gave me a way to frame my own experiments. In my first experiment I wanted to see the difference in running times after a workout when drinking water, Powerade or Gatorade. I conducted a between subject test, similar to the first two experiments. The experiment took place over a few weeks so that my participants were put through one exercise, like the last two experiments, to really focus on one type of performance because testing multiple performances can create more variables like fatigue and can affect every other test. My data did not significantly support the conclusion that Gatorade or Powerade created a difference in running times. The next experiment I conducted dove more into the nutritional pillars of protein and carbohydrates. For this experiment I reduced my subject group to people I could rely on being committed and who worked out regularly. I wanted to figure out how the supplementation of carbohydrates (sugar), protein (amino acids) and the combination of the two affect the energy levels and recovery process for high school basketball players during and after practice. My independent variables were the different supplements I gave subjects while my dependent variable was the qualitative answers of how my subjects felt turned into quantitative data through a survey I created. My alternative hypothesis was that there will be a difference in the subjects' energy levels and how they recovered between each supplement.

Materials

I used 7 varsity basketball players in total for two rounds of my experiment. One student had to leave for a semester school so I had to replace him with another player, so I ended up with 6 subjects for both rounds of experimentation. I tested four different supplements on these subjects: water (baseline), cookies and cream protein powder usually used for after lifting, sugar, and GU© which is a supplement usually used by marathon runners to give them the most energy for the long distances.

Image 1

Supplement Facts of the Lemonade Energy Gel from GU

Supplement Facts

Serving Size 1 Packet (32g)
Servings Per Container 24

Amount Per Serving	% Daily Value
Calories 100	
Total Carbohydrate 21g	7%*
Sugars 6g	†
Calcium 35mg	3%
Sodium 180mg	8%
Potassium 55mg	2%
Roctane Amino Acid Blend 975mg Leucine, Valine, Beta-Alanine, Isoleucine	†
Ornithine Alpha-Ketoglutarate (OKG) 450mg	†

*Percent Daily Values are based on a 2,000 calorie diet.
†Daily Value not established.

Procedure/Methods

My experiment was split up into two different 4 week periods in which I conducted the same tests on my 6 subjects. Each week the subjects were given one of the four supplements: water, protein, sugar, and GU©. Because the GU© had 32 grams in total of a combination of amino acids and carbohydrates and a few other nutrients (Image 1) I had to make sure there was also about the same 30g of protein and sugar. I had to use about 43 grams of the protein powder because that gave me 30g of protein and also weigh out 30g of sugar, and then I dissolved both in 16 ounces of water. Throughout these 4 weeks I measured the energy and recovery levels of the subjects through surveys. These surveys were meant to turn the qualitative data of energy and recovery into something quantitative. I had five different questions for both the energy and recovery surveys all rated on a 1 to 7 scale. The energy survey questions were:

How energized did you feel on a scale of 1 to 7?
(7 being very and 1 not at all.)

1 2 3 4 5 6 7

How out of breath did you feel after practice (the first part) 1 to 7?
(1 being very and 7 being not at all.)

1 2 3 4 5 6 7

When did you feel your arms start to get tired during the shooting drill, give a starting time? (1 being early, 4 being a minute, and 7 being not at all.)

1 2 3 4 5 6 7

How sleepy do you feel on a scale of 1 to 7?
(1 very sleepy and 7 not sleepy at all.)

1 2 3 4 5 6 7

How much longer could you work out on a scale of 1 to 7?
(1 being not at all and 7 being another hour workout.)

1 2 3 4 5 6 7

With the questions on a rated scale of 1 to 7, when all of the answers were added up they were in a domain of 7 to 35; 35 being very energized and 7 being no energy at all. The scale worked the same way for the recovery questions; 35 being fully recovered and 7 being not recovered at all. The recovery questions were:

Does anything hurt on your lower body?
If so how much on a scale of 1 to 7?
(1 being a lot and 7 being nothing. Specifically where?)

1 2 3 4 5 6 7

Where: _____

Does anything hurt on your upper body?
If so how much on a scale of 1 to 7?
(1 being a lot and 7 being nothing. Specifically where?

1 2 3 4 5 6 7

Where: _____

Are you sore? How much on a scale of 1 to 7?
(1 being a lot and 7 being not at all.)

1 2 3 4 5 6 7

How much do you feel your energy levels changing since the end of practice?
(1 being a small amount and 7 being a lot.)

1 2 3 4 5 6 7

How strong does your body feel overall?
(1 being weak and 7 being as strong as possible)

1 2 3 4 5 6 7

Throughout the 4 week experimentation period, I picked two days of practice to administer my experiment. About 5 to 15 minutes before practice I gave each subject the supplement. The first week was water so no supplement was given because it was the baseline, the next week was protein powder, the week after was sugar, and the last week was GU©. Each practice would start out with the same activity to add an extra control. The chronology of the activities were: 7 minute warm-up of dynamic stretching, 6 minutes of layups and jump shots, 6 minutes of a drill called duke which consists of lays-ups and jump shots into one, 3 on 2, 2 on 1 drill of back and forth, defensive shell drill for 5 minutes, two minute shooting drill for each player, and then ending with four minutes of free throws. I then proceeded to give each subject the energy survey to fill out right after shooting. Also, during the shooting drill, I had each subject's partner count the number of shots taken and made for other data points. After practice I sent each subject the recovery survey through a Google form. This is recovery survey 1 – the short term survey. I then sent another survey two days later – recovery survey 2 – to see if the recovery was more long term or short term. I continued this process for each experiment date and supplement type.

Data and Results

Figure 1

Energy Survey Results Round 1

First round averages of each subject's energy survey scores for every supplement. Each color represents a different supplement.

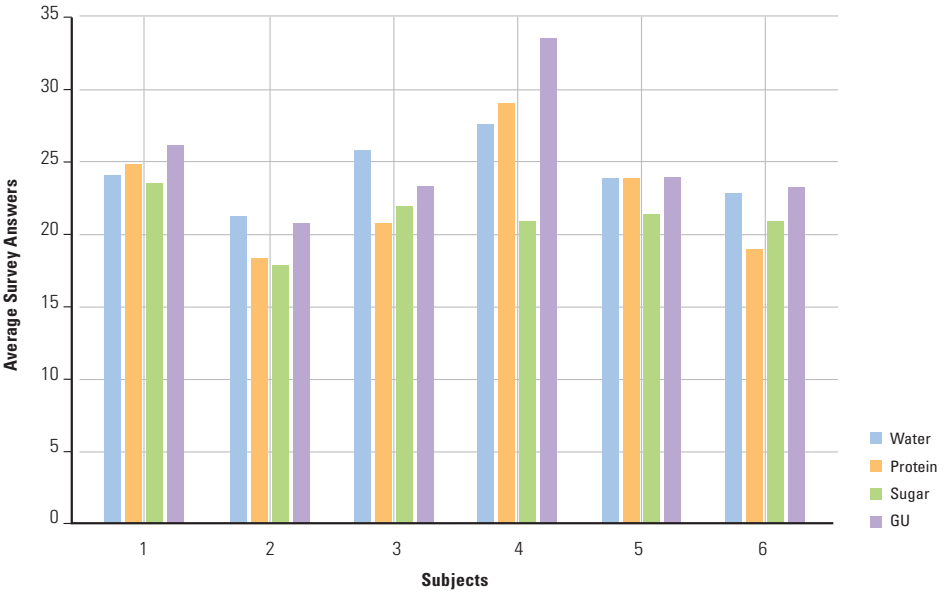

Table 1

This table is the numerical representation of Figure 2 with the actual averages of the survey answers for each supplement.

Subjects	Water	Protein	Sugar	GU
1	24.3	25.0	23.8	26.3
2	21.5	18.5	18.0	21.0
3	26.0	21.0	22.0	23.5
4	27.5	29.0	21.0	33.5
5	24.0	24.0	21.5	24.0
6	23.0	19.0	21.0	23.5

Figure 2

Shooting Percentages Round 1

First round averages of each subject's shooting percentage of the 2 minute shooting drill for each supplement.

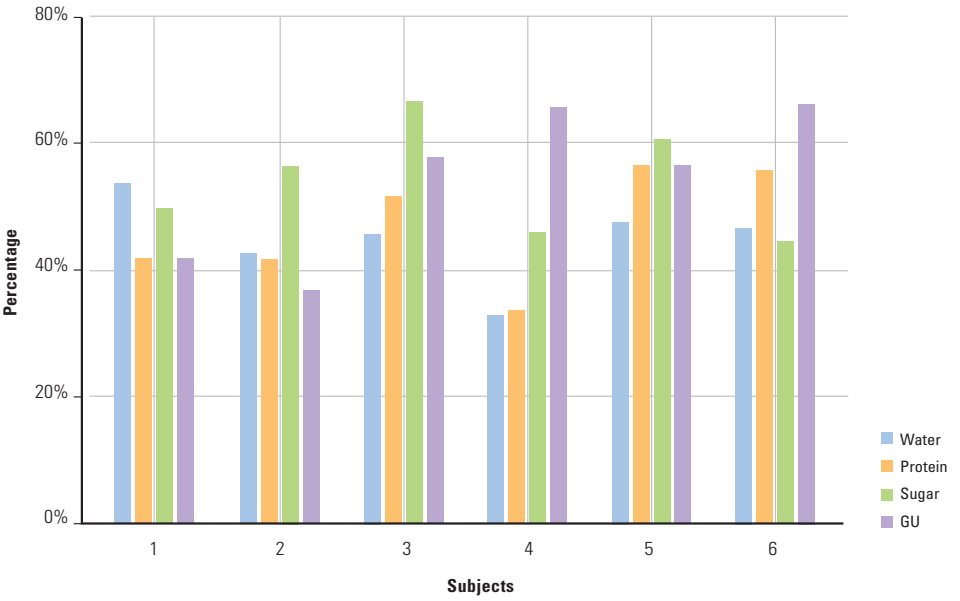

Table 2

The numerical representation of each subject's shooting percentage averages for each supplement.

Subjects	Water	Protein	Sugar	GU
1	54%	42%	50%	42%
2	43%	42%	57%	37%
3	46%	52%	67%	58%
4	33%	34%	46%	66%
5	48%	57%	61%	57%
6	47%	56%	45%	67%

Figure 3

Recovery Survey 1 Results Round 1

First round results of the average survey scores for each subject for the short term recovery survey for each supplement.

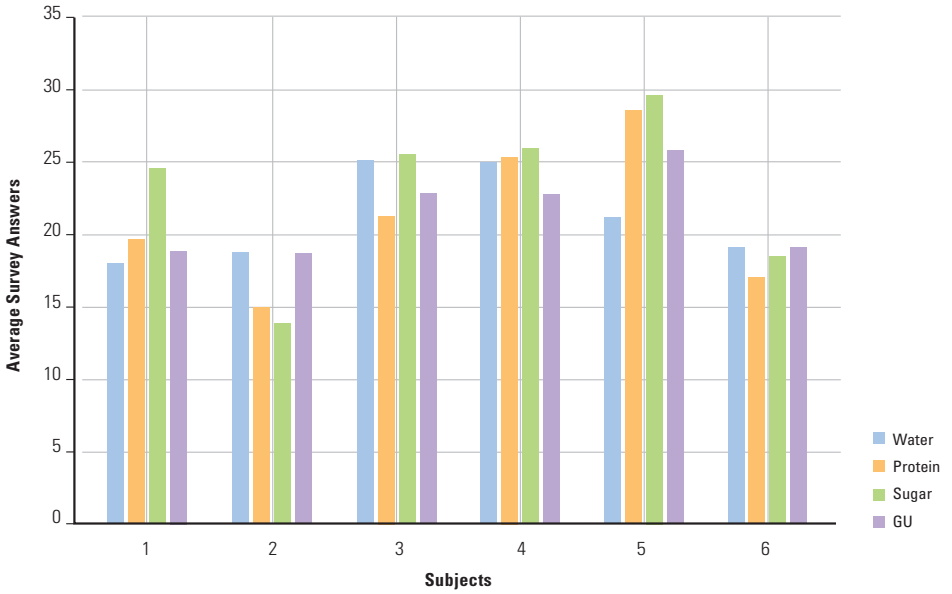

Table 3

This is the numerical representation of Figure 3.

Subjects	Water	Protein	Sugar	GU
1	17.5	19.5	24.0	18.0
2	18.0	15.0	13.0	18.0
3	25.0	22.0	26.0	23.0
4	25.0	25.5	26.5	23.0
5	21.5	28.5	29.5	26.0
6	19.0	17.0	18.5	19.0

Figure 4

Recovery 2 Survey Results Round 1

First round results of the average survey answers for each subject on the long term recovery survey for each supplement.

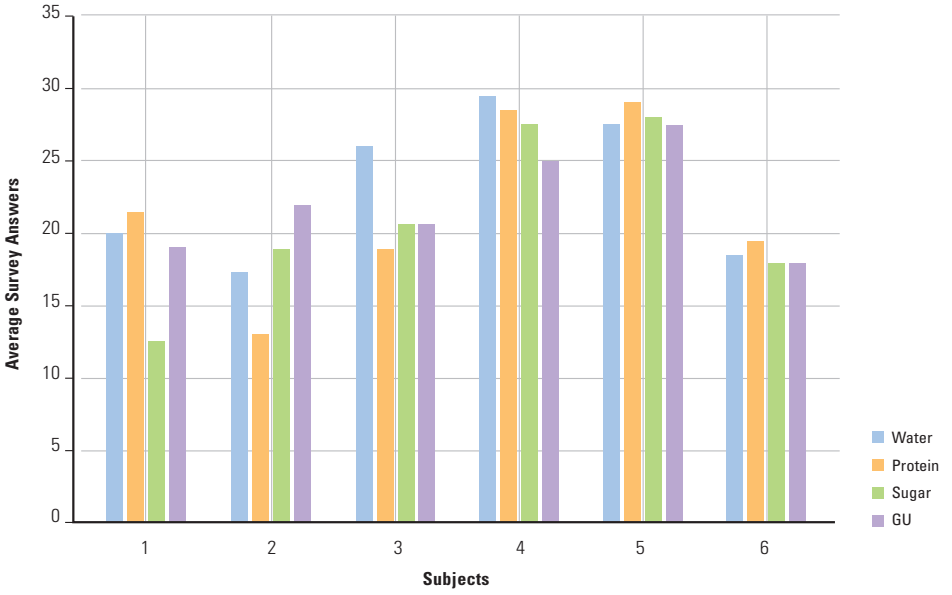

Table 4

The numerical representation of Figure 4.

Subjects	Water	Protein	Sugar	GU
1	20.0	21.5	12.5	19.0
2	17.5	13.0	19.0	22.0
3	26.0	19.0	20.5	20.5
4	29.5	28.5	27.5	25.0
5	27.5	29.0	28.0	27.5
6	18.5	19.5	18.0	18.0

Figure 5

Energy Survey Results Round 2

Second round results of the average energy survey scores for each subject, for each supplement.

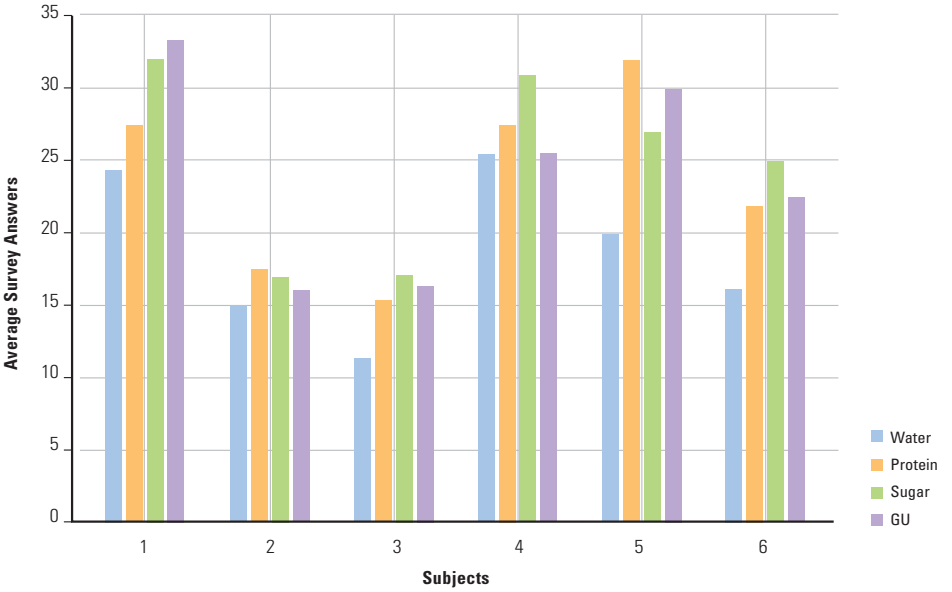

Table 5

The numerical representation of Figure 5.

Subjects	Water	Protein	Sugar	GU
1	24.5	27.5	32.0	33.5
2	15.0	17.5	17.0	16.0
3	11.5	15.5	17.0	16.5
4	25.5	27.5	31.0	25.5
5	20.0	32.0	27.0	30.0
6	16.5	22.0	25.0	22.5

Figure 6

Shooting Data Round 2

Second round results of the 2 minute shooting drill for each subject for each supplement.

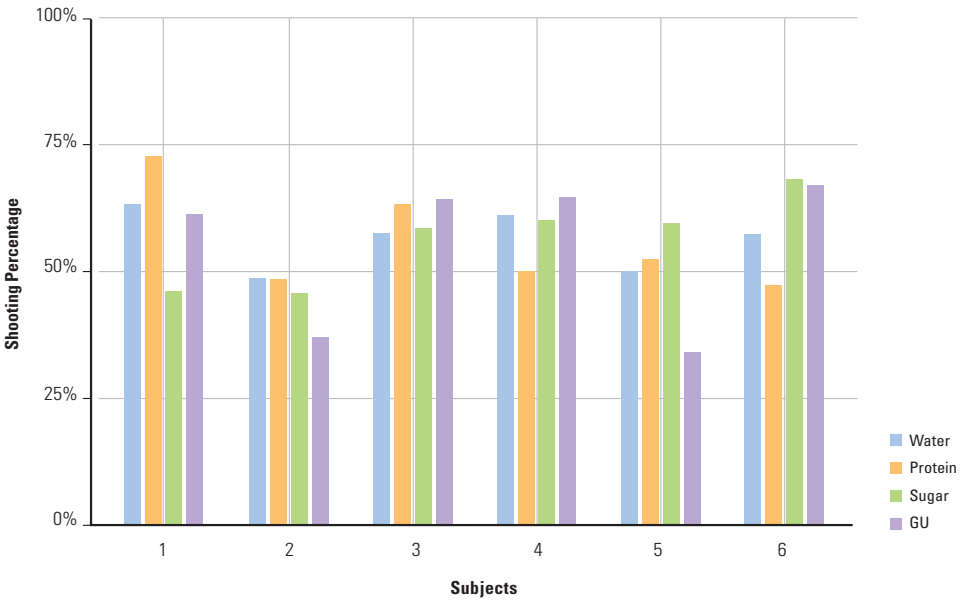

Table 6

The numerical representation of Figure 6.

Subjects	Water	Protein	Sugar	GU
1	63%	72%	46%	61%
2	48%	48%	45%	37%
3	57%	63%	58%	64%
4	61%	50%	60%	64%
5	50%	52%	59%	34%
6	57%	47%	68%	67%

Figure 7

Recovery Survey 1 Results Round 2

Second round results of the average survey answers from the short term recovery survey for each supplement.

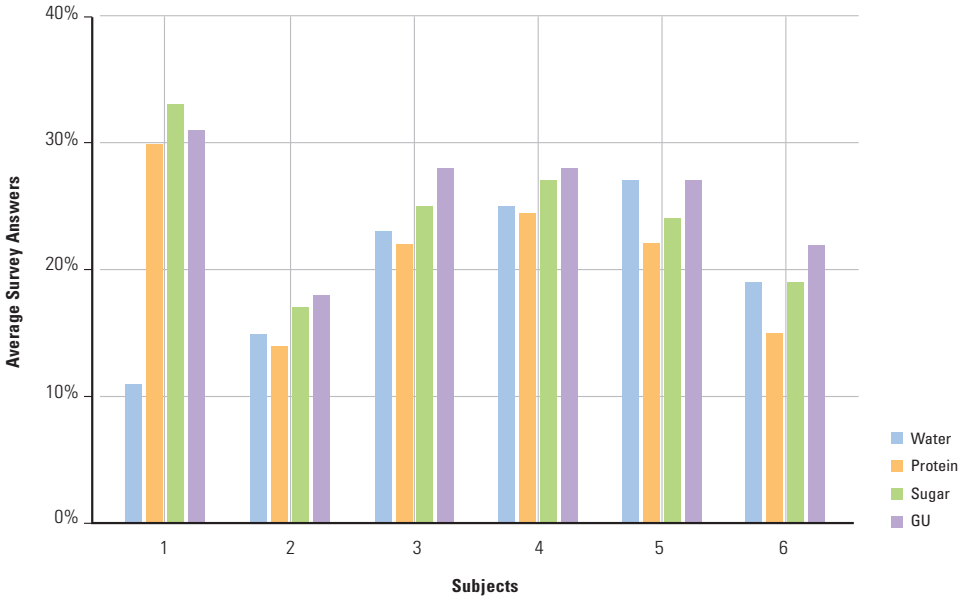

Table 7

This is the numerical representation of Figure 7.

Subjects	Water	Protein	Sugar	GU
1	11.0	30.0	33.0	31.0
2	15.0	14.0	17.0	18.0
3	23.0	22.0	25.0	28.0
4	25.0	24.5	27.0	28.0
5	27.0	22.0	24.0	27.0
6	19.0	15.0	19.0	22.0

Figure 8

Recovery 2 Survey Results Round 2

Second round results of the average survey answers for each subject for the long term recovery survey for each supplement.

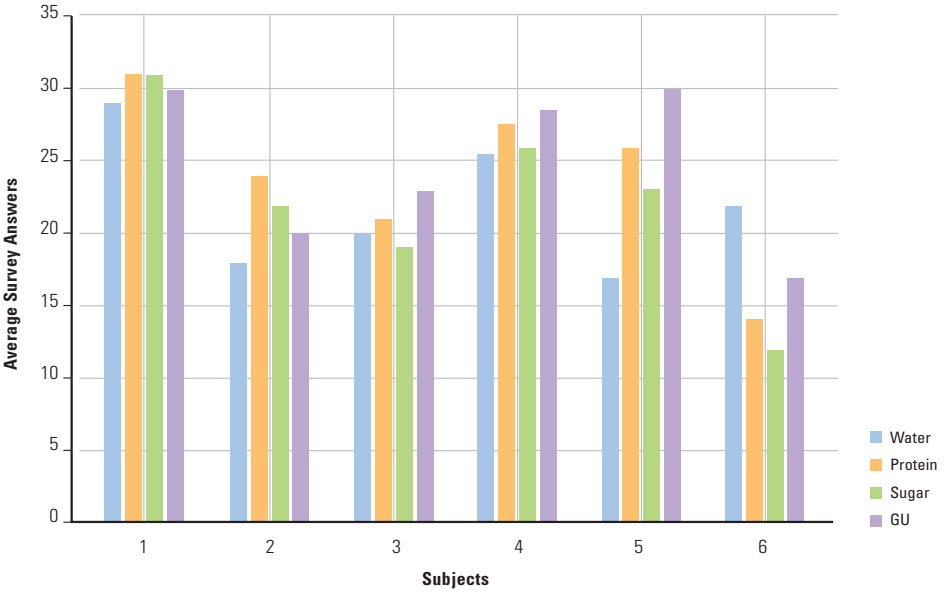

Table 8

The numerical representation of Figure 8.

Subjects	Water	Protein	Sugar	GU
1	29.0	31.0	31.0	30.0
2	18.0	24.0	22.0	20.0
3	20.0	21.0	19.0	23.0
4	25.5	27.5	26.0	28.5
5	17.0	26.0	23.0	30.0
6	22.0	14.0	12.0	17.0

Data Analysis

Figures and Tables 1, 2, 3, and 4 are from the first round, and figures and tables 5, 6, 7, and 8 are from round 2. I used a correlated analysis of a variance (ANOVA) test to analyze the results. Through this test I compared the averages of each subject's survey answers for energy, short term recovery, same night, and long term recovery, 48 hours later. I compared the water (baseline) survey answers to the protein answers, then the water averages to sugar averages, water to GU, protein to sugar, protein to GU, and last sugar to GU. The ANOVA test allowed me to compare surveys of all four supplements together. A p-value of greater than 0.05 indicated no significant difference between any of the surveys. For figure 1 the p-value was .020262, but the only significant difference was between sugar and the GU. Figure 3 had a p-value of 0.497849, which was not significant. Figure 4 had a p-value of 0.583522, which was also not significant. Moving onto round 2, figure 5 had a p-value of 0.384130, which was not significant. Figure 7 had a p-value of 0.318183, and figure 8 with a p-value of 0.789562, both were not significant. Statistical results were not calculated for figures 2 and 6.

As we look closely at each graph of survey answers we see a lot of clustering in the data. Most subjects don't have a progressive incline or a progressive decline. We can see those only a few times like subject 1 in the energy survey round 2 results, subject 2 in shooting data round 2, and subject 4 in the long term recovery survey round 1. While there are these little pockets of data that seem to suggest significant data, overall the data is all on the same level and clustered in the same areas. Similar ranges, similar numbers, and a lot of outliers make the data seem very erratic and suggest there is no correlation. Also, there is not a lot of difference in the short and long term recovery surveys or between the recovery and energy surveys. Overall, the data is all clustered into a similar range of numbers.

Conclusions

As we see from my results I acquired only one significant p-value for the energy average survey answer between the sugar and GU supplements at 0.020262. However, with only one significant p-value out of 6 I calculated, I will have to accept my null hypothesis that there is no difference in the subjects' energy levels and how they recovered between each supplement. Through this assessment, we can make the conclusion that consuming either sugar, protein, a combination of both, or none at all doesn't play a huge factor in the energy levels or how my high school subjects recovered. However, I still think there needs to be a follow up experiment or more experiments in regard to the relationship between carbohydrates and amino acids. One sign was a significant p-value between the sugar and Gu survey answers which means there could be more behind those findings. Also, there were multiple variables and factors that could have been controlled better, but it is important to note they were out of my hands.

The most obvious factor which is always a struggle in conducting sports nutrition experiments was the difference in experience, skill, and body types in my small subject group. Each member worked out differently, the height range was about 5'8 to about 6'5, and weight varied from 140 to 180 pounds. The differences in height and weight could have also been a factor of age since every body develops differently, however the age difference at most was about one year so I don't know if that would cause such a dramatic change in the body types. The next challenge that would have to be solved is the fact I have no real way of getting a quantitative data point. While I was able to turn qualitative data into quantitative through number scales, a better way to measure energy and recovery in a more concrete numerical way could possibly lead to more conclusive results. The next variable was the intensity of practice every day. Sometimes the team would have three games a week and two practices in between the three games. That caused practices to be different from when we had more practices than games in a week. Another factor was the timing of the recovery survey. The main problem was that each subject

wouldn't fill out the surveys at the same time. They mostly waited until they got home from practice that night and the second day after the specific practice date. Also, there were multiple instances of subjects forgetting to fill out surveys and me having to track them down to remind them to fill them out. It was difficult to control all these multiple variables in an environment like high school. A more suitable environment would be an exercise lab where the consumption, exercises, and data collection could all happen in one place, and having professional athletes or very serious amateur athletes could decrease the disparity in skill.

For future steps I want to continue to look for the connection between carbohydrates and protein. Some things I might change would be to give the supplement at different times. For example, I could give the supplement halfway through the activity to look for a difference in the first and second half or give them a supplement before and after an activity to see if that changes recovery time. The biggest goal for the next experiment would be to figure out the best way to measure energy or recovery in a quantifiable way. I could possibly find a certain power or strength test, or a particular workout that can easily be quantified. More investigation is still needed to fully figure out the relationship between carbohydrates and protein.

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"We look with favor on all forms of learning, but with particular grace we encourage philosophical studies, especially those which by actual experiments attempt either to shape out a new philosophy or to perfect the old."

KING CHARLES

from the 1661
Charter for the formation
of the Royal Academy of Science;
the proceedings of which
are the oldest journal in existence