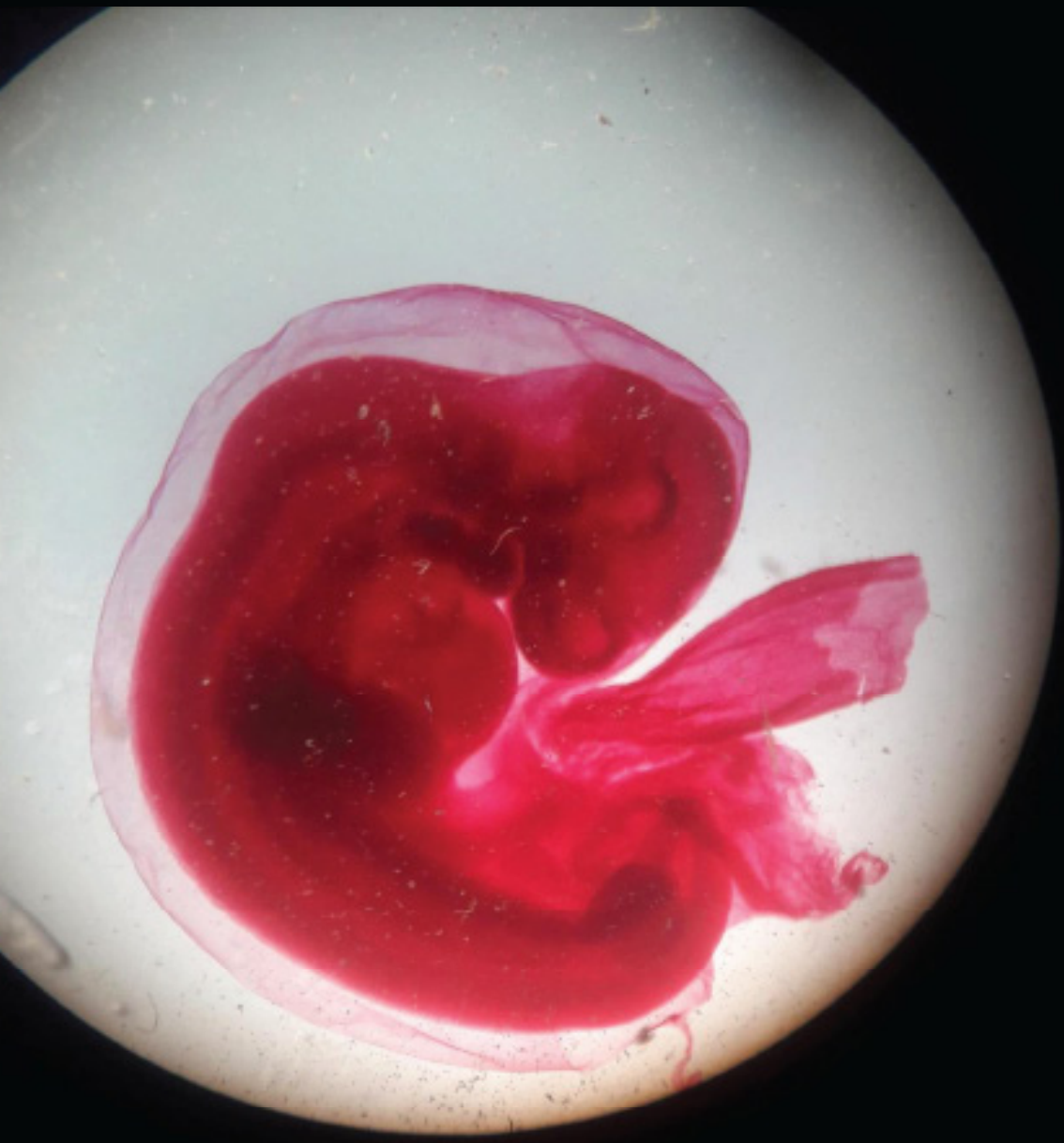
EUKARYON

Volume 18 March 2022



LAKE FOREST COLLEGE

LAKE FOREST BIOLOGY in ACTION

Engaged Scholars In & Out of the Classroom

About Eukaryon

Eukaryon is published by students at Lake Forest College, who are solely responsible for its content. The views expressed in Eukaryon do not necessarily reflect those of the College. Articles published within Eukaryon should not be cited in bibliographies. Material contained herein should be treated as personal communication and should be cited as such only with the consent of the author.

Eukaryon is an undergraduate research journal at Lake Forest College that publishes the very best of life science scholarship conducted by Lake Forest students. The journal's goal is to celebrate and highlight the academic accomplishments of students achieved within the research-rich classrooms and student-centered research labs of the Lake Forest College faculty. The word "Eukaryon" reflects the diversity of organisms with which the Biology Department faculty are involved through their scholarship.

The students and faculty of the Biology Department at Lake Forest College founded this peer-reviewed annual online journal in 2004. The inaugural issue was published in January 2005 and featured seventeen articles selected by the Biology faculty. In its inaugural issue, student work from a variety of biology courses (from First-Year Studies to advanced senior seminars and senior theses) was represented. Authors included freshmen, sophomores, juniors, seniors, and in one case, a graduate, who returned to audit a course! Diverse categories of articles were published, from research reviews and primary articles to Nature-styled News and Views and senior theses. These categories reflected the breadth and depth of scientific writing required of a life sciences student at the College.

An Editorial Board comprised solely of Lake Forest College students who selected articles through a peer-review process put forth the 2006 issue. In spring 2005, the biology faculty selected the student members of this Board. This Board not only reviewed articles, but it also authored all editorial policies of the journal. The 2007 issue demonstrated a continuing expansion of the journal with the co-publication of print and online issues, an increase in selectivity, diversity, and number of accepted manuscripts, as well as an increase in editorial board size. In 2009, we created the features board to encourage the writing of high-quality articles about student engagement beyond the classroom. In 2015, new board positions were created to aid in the editorial process. As Eukaryon continues to grow, maintaining high scientific journalistic standards and capturing the interdisciplinary, truly life-science experience of the journal remains a goal of this '21 – '22 editorial board. We hope to remain true to the founding principles of the first editorial board while still evolving the journal here at Lake Forest College.

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Front Cover: Photo by Katrina Topacio.

General contact : eukaryon2021-2022@mx.lakeforest.edu
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An UNDERGRADUATE JOURNAL of LIFE SCIENCE SCHOLARSHIP at LAKE FOREST COLLEGE

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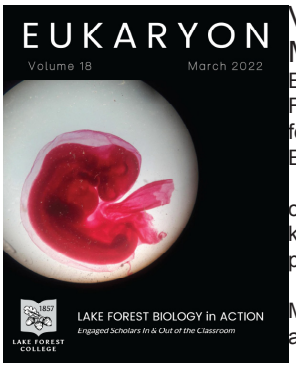

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Editor’s Corner

Katrina Topacio
Lake Forest College
Lake Forest, Illinois 60045

Dear Readers,
On behalf of the Eukaryon editorial board, I would like to extend my deepest gratitude for your continuous support as we publish our 18th year. The majority of the articles published in this edition were written during the peak of the pandemic when students and faculty spent the better half of the academic year learning remotely from all over the globe. Despite the difficulties we have endured these past few years and continue to endure, Eukaryon is prouder than ever to celebrate the academic achievements of the student body of Lake Forest College. The editorial board, which consists entirely of new members, has also demonstrated an admirable amount of dedication in order to make this publication possible. This year’s theme, “Evolution”, was inspired by our editorial board and their unwavering effort to see through Eukaryon’s revival following the tumultuous challenges brought forth by the pandemic.

Evolution is a process of gradual change that occurs within several generations. This simple yet monumental biological concept is the basis of all life on Earth. We see it throughout our history and how it has given shape to our society. From single-celled organisms to the first multicellular animals, from the adaptation of fins into limbs, evolution is apparent in every aspect of all living things. Similar to the generational change of physiology within a species, Eukaryon has gone through its own evolution. For the past eighteen years, Eukaryon has constantly been improving itself by building upon the successes of previous editorial boards. The success of this journal lies entirely within the collective work of individual members. This year, the editorial board truly excelled in this aspect, and I will be forever thankful for each and every one of them.

This year, we are honored to listen to Dr. Becky Delventhal, a recent addition to the college’s Biology Department. Dr. Delventhal received her undergraduate degree at Macalester College, another small liberal arts college, where she majored in Biology with minors in Chemistry and Psychology. She went on to complete her PhD at Yale University, before becoming a postdoctoral research scientist at Columbia University. At Lake Forest College, Dr. Delvental teaches Molecules, Genes, and Cells, Developmental Biology, and Molecular and Cellular Mechanisms of Neurodegenerative Disease. Her presentation for the 18th inauguration is entitled: “What Flies Can Teach Us About Human Biology and the World Around Us.” Despite her short time here, Dr. Delventhal has already proven herself to be a valued member of both the Science and Lake Forest College community and we are excited to have her celebrate this next milestone with us.

This publication would not be possible without the support of many individuals. First, I would like to thank Dr. Flavia Barbosa, who has graciously become the journal’s new advisor this year and has done a phenomenal job guiding us throughout the entire process. Her invaluable insight, encouragement, and genuine care about this journal inspired us to overcome any challenges and helped pave our way towards success. I would also like to acknowledge Dr. Shubhik DebBurman for his continued support and advocacy for Eukaryon. I would also like to thank my fellow Eukaryon members, all of whom are new to their positions and have demonstrated a remarkable effort in ensuring the successful revival of this journal. Lastly, thank you to all the faculty and students that submitted to Eukaryon. Your outstanding submissions are the core of this journal and we thank you for granting us the opportunity to publish your exceptional work. We hope you enjoy this year’s edition.

Sincerely,
Katrina Topacio
Editor-in-Chief, Eukaryon, 2021-2022

About the Editor

Katrina Topacio ’22 is from Grayslake, Illinois. She is double majoring in Biology and Neuroscience. She is a member of the Nu Rho Psi National Neuroscience Honor Society, the Tri-Beta Biology Honor Society, and a member of the Neuroscience Student Advisory Academic Committee 2021-22. She is also the current Editor-in-Chief for Eukaryon. She loved being a part of the Science community at LFC and enjoyed being a peer teacher and mentor for several introductory biology courses. She also enjoyed conducting research in Dr. Kim’s lab at Rosalind Franklin University of Medicine and Science. Her plans after graduation include taking a gap year before attending medical school.

Dr. Susan Long: Psychology, Statistics, and the Rest of Our Lives

Margaret Boersma
Lake Forest College
Lake Forest, Illinois 60045

Information doesn't live in a classroom, nor is research isolated in a laboratory. Scientific progress within modern society requires that people are always producing, absorbing, and sharing information through their actions, feelings, and words. In order to study the collective minds and behaviors of people around the world, the field of psychology has developed in leaps and bounds as modern technology bridges gaps between communities and issues. The variation of a sample (from which data is collected) can now be analyzed with complex statistical programs in order to better define the limits and applicability of a study's results. Dr. Susan Long of Lake Forest College leads the students in her classes to a better understanding of these ideas, focusing on psychological research, statistical analysis, and a variety of communities and cultures, all the while performing her own research outside of the classroom. Yet, her intention is not wholly to feed students information. She emphasizes the development of a student's critical lens and their ability to apply lessons to situations outside of the classroom even as she covers the subject matter.

Dr. Long's journey in academia is key to understanding her goals as a professor and researcher. In a recent interview, she explained that despite her current career, she did not always know that she wanted to be a professor. She took her first course on the psychology of women as a sophomore at Macalester College and found the subject powerful, realizing that research itself can be used to demonstrate how long-held beliefs and perspectives on gender were untrue. At that point, Dr. Long unofficially began her research career.

She went on to conduct undergraduate research on the perceptions of stay-at-home fathers compared to perceptions of stay-at-home mothers. When the results were finalized, Dr. Long was moved by the vast differences in those views and her own ability to collect information that might start to change them. Getting involved with undergraduate research also introduced Dr. Long to many faculty members. She discovered a love of connecting with those academics outside of regular classes. These developments also allowed her to recognize the strengths of her individual skill set, as she realized she was best suited for research and sharing knowledge.

However, her time in college was not entirely void of struggles. As only a second-generation college student, Dr. Long noticed a disconnect between her family's expectations and her own desired course of study. Life as a researcher requires years of college education for a relatively low monetary payoff, so she faced opposition and questioning from family throughout her academic career. Nevertheless, Dr. Long persevered, earning her B.A. in Psychology and Women's and Gender Studies and graduating with honors cum laude from Macalester College in 2001. She went on to obtain her M.A. and Ph.D. in Psychology in 2006 and 2009 respectively, both from the University of Illinois at Chicago. So far, Dr. Susan Long has contributed to 12 publications in various journals, with another under review and yet another paper being prepared.

Within Lake Forest College, Dr. Long is involved in many important roles. She is presently an Associate Professor of Psychology and has taught an impressive array of courses that include Community Psychology, Cross Cultural Psychology, Gender-Based Violence, Research Methods and Statistics, and Social Labels and Identities. Her students learn to analyze information beyond face-value while developing skills, ideas, and processes that will help them in the future. Through their assignments and projects, her students recognize topics and evaluate research in order to understand what is actually known about the world and what still must be studied and improved. These abilities carry over into many other fields, with students being encouraged to use those analytical skills to evaluate entirely separate studies and situations. Whether a study is based in psychology, biology, or even literature, the ability to critique and evaluate will always be a vital skill. In addition to her work as a professor, Dr. Long was appointed as Chair of the Psychology department in the summer of 2021. She works with faculty, administration, and students alike to develop course schedules and content for students, hire faculty members, and generally run the department.

Outside of the classroom, Dr. Long has been utilizing a grant awarded to Lake Forest College that allows her to join forces and perform research with community organizations in Chicago. She usually works with several undergraduate students, introducing them to research in the same way that she was introduced as a student herself. Currently, Dr. Long is conducting research alongside the *Freedom from Trafficking* program in Chicago. Run through the *Heartland Alliance*, the program serves survivors of forced labor as connected to exploitive labor practices. She works closely with the Housing Justice project, where housing and social services for those survivors are provided for 2 years. She evaluates current and past program clients on how they find (or previously found) housing after the 2-year expiration and how the pandemic affected that process. Dr. Long was quick to point out how going into lockdown actually mirrored some of the victims' past experiences, which was incredibly retraumatizing for those individuals. When I expressed surprise over the obscurity of the Chicago-based program, Dr. Long admitted that to many, "it seems like it's happening somewhere else, but it is happening here. It's almost impossible to not be a part of global exploitive labor, and sometimes also trafficking." In this pursuit of knowledge, Dr. Long hopes to improve the survivors' situations by directly working to spread knowledge and a more complete awareness of their struggles.

So, what is left for the future? The COVID-19 pandemic changed the paths of many Americans, including Dr. Long. Prior to the pandemic, her research focused on verbal sexual coercion, which is a relatively under-researched form of sexual assault. So theoretically, she could return to studying the importance of masculinity and shame, and their roles in the perpetuation of verbal sexual coercion. On the other hand, the opportunities for research are endless, and she could end up finding another subject altogether. For now, Dr. Long encourages students who are passionate about something, *anything*, to try out her courses. They will walk away from these classes and experiences developing skills, knowledge, and ideas that can and will apply to future passions and professions.

The new villain in SCA8

Tracey Nassuna
Lake Forest College
Lake Forest, Illinois 60045

Heading
The pathogenic consequences of RAN polyserine in SCA8 are poorly understood. Findings in mice and human autopsy show that the protein accumulates in white brain matter, causing demyelination, axonal degeneration, oligodendrocyte loss, and astrogliosis, and can be modulated by translation factor eIF3F.

Imagine being unable to control your movements when walking, almost resembling a dizzy child who has just gotten off a rollercoaster ride. Unfortunately, this is the reality for patients with SCA8. Spinocerebellar ataxia type 8 (SCA8) is a dominantly inherited neurodegenerative disease (Ayhan et al. 2018a). Onset is typically in adulthood and is characterized by slurred or slow speech, repetitive and uncontrolled movement of the eyes, and difficulty in coordinating movements when walking known as gait ataxia (National Institute of Health, 2020). It is caused by a CTG-CAG nucleotide repeat called a microsatellite expansion, which causes disease when the nucleotide repeats expand beyond a threshold length (Koob et al., 1999). The SCA8 mutation gene is expressed in two directions, producing both CAG (ATXN8) and CUG (ATXN8OS) expansion transcripts (Ikeda et al., 2008). The CUG expansion transcripts contribute to disease by conferring a new function onto an already existing protein, making it toxic (Li et al., 2018; Daughters et al., 2009). Before Ayhan et al. (2018b), the CAG expansion was thought to contribute to disease via two aggregation-prone proteins, namely ATG-initiated polyglutamine and repeat-associated non-ATG (RAN) polyalanine expansions that accumulate and cause toxicity in affected areas (Moseley et al., 2006). However, the authors report a novel RAN polyserine protein that contributes to SCA8, shows which areas of the brain it affects, and demonstrates a translation factor that modulates the protein's accumulation. Given that the CAG is a microsatellite expansion, there are three possible nucleotide base combinations that result in different amino acids. Glutamine is translated from CAG combination, alanine from GCA, and serine from AGC. Ayhan et al. (2018a) predicted that if repeat-associated non-ATG (RAN) translation of ATXN8 in AGC frame is possible, then a polyserine protein that contributes to toxicity in SCA8 will be formed. They confirmed its presence by the use of immunofluorescence staining of antibodies that bind specifically to polyserine to show the areas of the brain in which it accumulates. In both SCA8 mice and human autopsy tissue, they discovered polyserine aggregates in the cerebellum, brainstem, and cortex, which are the main areas affected by the disease. Polyglutamine expansion was the first confirmed protein to contribute to SCA8. Therefore, to understand polyserine pathology Ayhan et al. (2018a) compared the brain distribution of polyserine to that of polyglutamine in both mice and human autopsy tissue. They observed that polyglutamine aggregates accumulate in the Purkinje cell nuclei of the cerebellum, whereas polyserine aggregates accumulate in the cerebellar white matter regions and oligodendrocyte-rich subcortical regions, thus demonstrating strikingly different distribution patterns. Furthermore, polyserine aggregates increase with age and disease progression in mice. This applies to humans because over time, symptoms of the disease worsen, thus indicating an increase in the toxic aggregates. How does polyserine accumulation affect these regions? In both human autopsy and SCA8 mice tissue, Ayhan et al. (2018a) observed demyelination and axonal degeneration in the deep cerebellar white matter. Myelin is important to speed up the transportation of action potentials via saltatory conduct, therefore, its destruction coupled with axonal degeneration slows down the transmission of signals across the neurons resulting in a decline of motor function in patients. Consistent with demyelination, the authors also observed a decrease in the number of mature oligodendrocytes, which are the cells are responsible for producing myelin sheath within the central nervous system. When cell death occurs as a result of axonal degeneration and demyelination, it prompts the abnormal increase of astrocytes in a defense mechanism known as astrogliosis. Astrocytes are glial cells whose function is to repair damaged neurons. Taken together, the phenotypic effects of polyserine aggregate accumulation in white matter are demyelination, axonal degeneration, oligodendrocyte loss and astrogliosis (Fig 1) (Sharma et al., 2015).

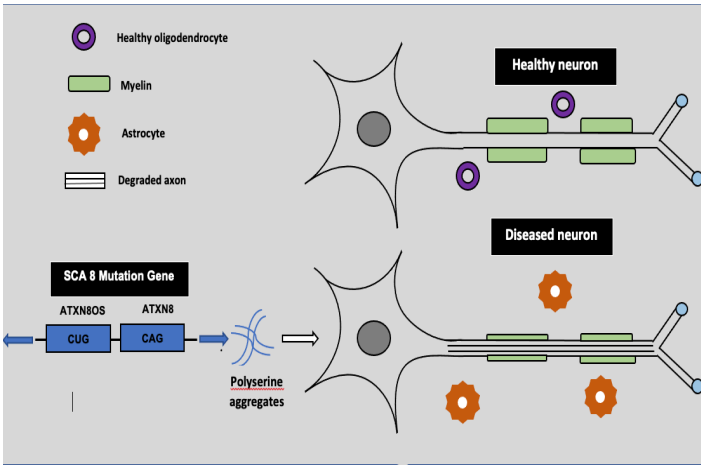


Figure 1. Polyserine contributes to SCA8 pathogenesis. SCA8 mutation gene is bidirectionally expressed. Ayhan et al demonstate that the CAG (ATXN8) expansion can be translated into polyserine whose aggregates accumulate in white matter causing demyelination, axonal degeneration, oligodendrocyte loss and increase in astrocytes

Probing further, Ayhan et al. (2018a) observed that the eukaryotic translation factor eIF3F was elevated in white matter brain regions compared to gray matter, hence the possibility that eIF3F increases RAN translation leading to accumulation of RAN polyser protein in white matter. This hypothesis was tested by knocking down eIF3F using small interfering RNA. The results showed a decrease in steady-set levels of RAN polyserine proteins expressed from the CAG frame that does not contain an AUG-start codon. This implies that RAN translation, which does not require the AUG start codon, is sensitive to eIF3F levels thus explaining the difference in distribution between the AUG-initiated polyglutamine and RAN polyserine. RAN translation has been shown to occur in other repeat expansion diseases such as Huntington's disease, therefore, understanding the common themes in RAN translation in one disorder provides insight into the molecular mechanisms of the others. This is why it important to note that although Ayhan et al. (2018a) demonstrated a correlation between eIF3F translation factor levels and RAN proteins, they did not experimentally show that reducing eIF3F levels caused the reduction in RAN proteins. Nonetheless, it provides a starting point for further research even in other disorders. The ultimate goal of understanding the pathogenesis of any disease is to find possible therapeutic measures. Even though Ayhan et al. (2018a) demonstrate that eIf3F plays a role in RAN translation and maybe a therapeutic target for microsatellite expansion disorders, it is important to consider the potential risks of reduced eIF3F activity on other brain functions. eIF3F is the largest eukaryotic translation factor and is highly conserved among species for a reason. It plays a critical role in cellular homeostasis by contributing to translation inhibition and programmed cell death known as apoptosis (Jiaqi S. et al., 2009). Also, its loss has been reported to contribute to the growth of cancerous tumors (Ayhan et al., 2018a). Future research should look into a way of countering the side effects of decreased eIF3F so that its modulation can serve as a therapeutic measure for RAN protein-associated diseases.

A key component of revenge: The "love" hormone

Fede Bertolotti
Lake Forest College
Lake Forest, Illinois 60045

Association of brain networks mediates propensity for revenge. A study of intergroup conflict in humans reveals stronger activation of the medial prefrontal cortex and higher levels of oxytocin increase the tendency to retaliate against out-group members.

Emotional contagion highly mediates humans' emotional experiences, this is to say that people are affected by the transmission of someone else's emotions (Thornton and Tamir 2017). Although this mechanism of contagion helps to socially interact, it can be detrimental to society when it involves the transmission of anger (Qu et al. 2016). The concept of people belonging to an ingroup (identified by the same characteristics) than to an outgroup (differing in identity) intensifies these emotions. Moreover, along with enhancing emotions, the tendency of revenge towards the other group as a response to their offenses is consequently strengthened (Pereira and van Prooijen 2018). The neurobiological responses to intergroup conflict still haven't been completely understood, nor the neural mechanism that drives revenge desire. Due to its worldwide relevance, of social dynamics and conflict, revenge propensity's neurobiological mechanism needs to be understood. In their paper published in *eLife*, "A neurobiological association of revenge propensity during intergroup conflict", Han et al. (2020) demonstrated that humans had a higher propensity to harm everyone outside their ingroup when their levels of endogenous oxytocin (OT) were elevated and mediated by the medial prefrontal cortex (mPFC) as a response of an outgroup member inflicting pain on an ingroup person. This association of the mPFC, OT, and revenge could potentially explain the neural underpinning that promotes the process of conflict contagion. Oxytocin is a hormone that gets the name "love hormone" from its influence on social bonding, trust, and conformism (Hertz et al. 2016). In the investigation of Han et al. (2020), they tested for endogenous OT levels—synthesized within the organism—in humans' saliva. Alternatively, in chimpanzees, OT was related to intergroup conflict and hostility towards outgroup members (Samuni et al. 2017). Furthermore, when OT is administered to humans, it seems to significantly activate the region of the mPFC (Skvortsova et al. 2020). This brain region promotes favoritism to an ingroup member over an outgroup member (Lin et al. 2018). This preference has prevented scientists from finding the real cause of revenge, whether it is negative outgroup behavior towards an ingroup member or a result of ingroup favoritism even in the absence of conflict. To control the bias created by favoritism, researchers classified the participants—healthy adults—into two groups (revenge and control) that matched in emotions, attitudes, and behaviors; however, they differ in the reason to punish the outgroup member. They also created the neural-behavior paradigm: the revenge group had to watch a competitive game, while two ingroup and outgroup members—both confederates—gave each other electric shocks. In addition, the control group had a computer that gave the shocks. Han et al. used these groups to test their hypothesis that the revenge group's OT levels would be higher than those in the control group—due to experiencing intergroup conflict— and thus, OT would mediate the tendency to mistreat the outgroup. Researchers mixed this paradigm with the functional magnetic resonance imaging (fMRI) neuroimaging technique. This method records the specific brain structures activated in a person during a certain task or experience (Roy et al. 2018). Simultaneously, Han et al. (2020) collected saliva samples to test their OT levels at three different points of the experiment: when the conflict started, when it ended, and after 15 minutes of conflict. They found that in the revenge group, after experiencing the intergroup conflict, their OT levels were significantly higher than those for the control group. Furthermore, their levels seemed to continuously increase throughout the other stages, thus suggesting a connection between intergroup conflict and high OT levels during intergroup conflict. Later on, they aimed to investigate the neural activation produced by OT. They analyzed fMRI scans taken after the second time - while also showing pictures of ingroup members with painful and non-painful facial expressions - and concluded that there was a greater activation, for both, in the mPFC. Researchers examined the association between the mPFC activity and the propensity of both groups to undertake vengeful behavior. They obtained that those in the revenge group had a higher potential to punish the outgroup members without caring whether they were directly involved or not in the conflict (punishing or just watching). Moreover, these results were followed by associating the levels of OT with the activation of mPFC in the experiment for both groups (revenge and control) towards the perception of ingroup suffering. Han et al. (2020) demonstrated that there was a significantly stronger association of mPFC activity with higher OT levels in the revenge group (vs. control group). This association suggested that the intergroup conflict made the OT levels increase and enhance its connection to the mPFC activity, which was a response to perceived ingroup pain in a conflict with an outgroup member. Researchers further these conclusions and aimed to investigate whether this association was linked to revenge propensity after experiencing the conflict. The researchers found that the increase in OT levels after experiencing the conflict and the tendency for punishment towards the outgroup was mediated by the activity of the mPFC. All in all, they showed that having ingroup identity alters OT function. Instead of promoting a bonding environment, it made them negatively respond to an outgroup during intergroup conflict. Similarly, the activation of mPFC was also related to higher OT levels and revenge propensity. Thus, it was concluded that the association between these is opening the understanding of the neurobiological explanation of the desire for revenge (Fig. 1).

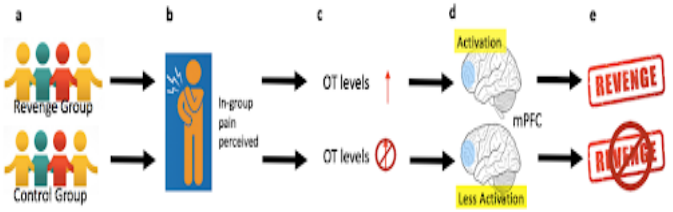


Figure 1. Humans' neurobiological association of mPFC and endogenous oxytocin during intergroup conflict. **(a)** Two groups of participants played a competitive game, ingroup vs. outgroup. Painful and non-painful electric shocks were given by a computer (control) or each other (revenge). **(b)** Each group watched ingroup getting painful shocks, and then pictures of them with a painful or non-painful facial expression. **(c)** The levels of OT increased significantly for the revenge group (vs. control group) that watched their ingroup get punished by an outgroup member. **(d)** fMRI scans showed higher activation of mPFC for the revenge group (vs. control). **(e)** The revenge group was most likely to undertake vengeful behavior (vs. control group). Han et al.'s (2020) research model helped them conclude their hypothesis. They aimed to investigate the neurobiological responses to intergroup conflict. Notably, they were able to establish an association between mPFC and endogenous OT levels both mediated by intergroup conflict and subsequently leading to revenge propensity. However, their results for punishment tendency rely greatly on participants' self-reports, and therefore it is difficult to determine real vengeful behavior. For a long time, the presence of intergroup conflict in the world has been a predominant factor of conflict contagion across communities. Even though this study investigated only one brain association, further studies need to delve deeper into other neural networks underlying revenge behavior. For example, pursuing research on serotonin, which is linked to the amygdala and mediating moral judgment and behavior (Crockett et al. 2010). By furthering knowledge about the neurobiology of intergroup conflict, people will gain insight into the importance of social identity, group dynamics, and how it connects to the brain— all leading to developing strategies to overcome group conflicts.

Sleep Deprivation: What is the Connection to Alzheimer's Disease?

Raneem Samman
Lake Forest College
Lake Forest, Illinois 60045

Sleep deprivation is so commonly experienced and observed around the world. It influences and impacts the human brain in multiple distinct ways; some were researched more than others. It also messes up the rhythm of the circadian clock, which controls humans' sleep-wakefulness cycles. A study showed the impact of one night of sleep deprivation on the risk factor of Alzheimer's Disease. Specifically, by studying the accumulation of amyloid beta plaques in the cerebellum.

Beta-amyloid plaques are waste products of the metabolic mechanisms and reactions that occur in the brain (Cheng, 2020). Thus, their accumulation indicates a malfunction in the intracellular proteasomal degradation and autophagy systems in the brain (Nedergaard, 2013), which are the systems responsible for breaking down and getting rid of the toxic molecules in the brain, such as beta-amyloid plaques. The degradation systems are influenced by multiple factors: some are inherited while others are environmental and can be controlled by individual decisions day-to-day (Liu, 2019). The work of Shokri-Kojori et al. (2018) explores the impact of sleep deprivation on human brains and its connection to Alzheimer's Disease. The researchers argue that the scientific literature lacks knowledge and evidence about the impact of sleep deprivation on the accumulation of amyloid-beta in the human brain. In order to explore this argument, the researchers used PET scans to show the impact of acute sleep deprivation on amyloid-beta burden. PET technology allows them to measure amyloid-beta burden (ABB) in the living human brain. Positron emission tomography (PET) has multiple radiotracers which are types of radioactive biochemical substances used for diagnostic purposes. Each PET radiotracer serves a specific purpose. Thus, for this study, the researchers used F florbetaben (FBB) because FBB binds to soluble and insoluble amyloid-beta plaques. Thus, the use of FBB with PET scans allow the scientists to detect amyloid-beta burden (ABB) in the human brain to a great extent and generate the most accurate results. The study consisted of two main goals. Firstly, the researchers aimed to evaluate one-night sleep deprivation's impact on the brain ABB in healthy participants, which were used as the controls for the study, using PET-FBB technology. They would then take the data collected and compare it to the measurements recorded from the same participants after a night of well-rested sleep, which was labeled rested-wakefulness (RW). The researchers referred to the data collected after a well-rested sleep night as the baseline brain ABB. Secondly, the scientists aimed to be able to generate and generalize their findings by looking at sleep history and brain amyloid-beta burden. The scientists focused on looking at the hippocampus, precuneus, and the medial prefrontal cortex because they are the regions of the brain that are mostly affected and altered by Alzheimer's disease. As a starter, in order to compare the data collected through FBB (a night of sleep deprivation vs a night of well-rested sleep), the scientists needed to quantify the FBB measurements. Thus, FBB was quantified as the relative standard uptake value (SUVr) and was used as a marker or indication of amyloid-beta burden. As a result, they were able to conduct a statistical t-test to analyze the data, which showed a significant increase in the amyloid-beta burden in the hippocampal region after one night of sleep deprivation. These observations were not seen after one night of well-rested sleep. In other words, more FBB binding occurred after one night of sleep deprivation in comparison to a night of well-rested sleep. In order to confirm the results and to ensure that there were no confounding variables causing this significant change, the scientists quantified FBB SUVr in a priori hippocampal ROI. ROI, also known as region of interest, is an analysis method that refers to selecting a cluster of voxels or brain region posteriori. Using that method, the results were consistently showing a significant increase in FBB SUVr (thus, amyloid-beta burden) after one night of sleep deprivation. Furthermore, the scientists looked at the number of sleep hours and the total score for sleep quality, which was self-reported using Pittsburgh Sleep Questionnaire Inventory. They found that those factors, sleep hours and the total score for sleep quality, were not associated with the increase of amyloid-beta burden after sleep deprivation. Thus, after supporting and testing their first finding, the researchers were able to explore their second aim. The scientists were looking at the longer-term effects of sleep deprivation and whether their first findings would be consistent when looking at the sleep history of participants and correlating it with the corresponding FBB SUVr measurements. To do so, the researchers tested the association between reported sleep hours, sleep quality, and amyloid-beta burden measured during well-rested sleep and sleep deprivation. They found that there was an inverse correlation between sleep hours and FBB SUVr at RW and a positive correlation under sleep deprivation. In other words, the results supported the hypothesis that there would be an increase in ABB with less SH, especially in the subcortical region of the brain. Furthermore, the scientists looked at different regions of the brain that showed an association between FBB SUVr at well-rested sleep and sleep hours as well as the APOE-based genetic risk for Alzheimer's Disease. APOE stands for apolipoprotein E, which is a gene that plays a major risk factor for Alzheimer's Disease (Kim, 2009). They found that the different observations of low and high FBB SUVr binding at different regions of the brain suggest that there are various brain factors for Alzheimer's Disease influencing each region semi-independently. However, most importantly for the goal of this research, they were able to show that the disruption of deep sleep increases amyloid-beta in the human brain, especially in the thalamus, which is one of the main regions for observing the development of early-onset Alzheimer's. Moreover, another significance to their results is that they reflect the decreased clearance of amyloid-beta in those regions, indicating a role for sleep in the glymphatic system, which is a macroscopic network of vessels for waste clearance in the brain and the overall processing of amyloid-beta clearance in human brains (Jessen, 2015).

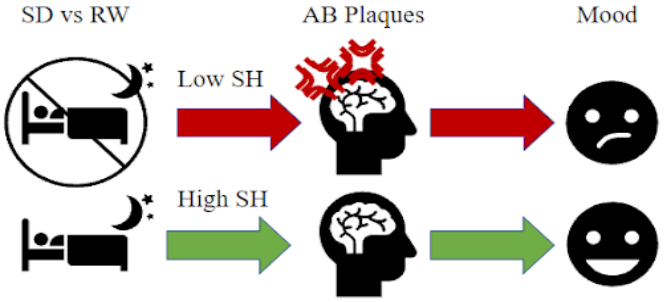


Figure 1: The effects of well-rested sleep and sleep deprivation on the human brain.
Sleep plays a crucial role in the degradation machinery of the brain. Shokri-Kojori et al. (2018) provides evidence that amyloid-beta burden is highly influenced by the sleep a person has. Statistical and screening tests that resulted in consistent PET FBB scans activities across the participants on the rested wakefulness and sleep deprivation tests showed higher FBB SUVr binding on the brain of participants after sleep deprivation in comparison to the scans after a night of well-rested sleep. This indicates a higher accumulation of amyloid-beta in the brain. Moreover, the figure shows that the scientists were able to test for the influence of sleep hours on amyloid-beta burden and found consistent results: the more sleep hours participants got, the less amyloid-beta accumulation detected. Similarly, mood was also negatively influenced by sleep deprivation, as participants were more likely to record negative moods after a night of sleep deprivation in comparison to a night of well-rested sleep (RW).
The research and work of Shokri-Kojori et al. (2018) provided supporting evidence for the role of sleep deprivation on increasing the accumulation of amyloid-beta in the human brain. This work is revolutionary as it is usually done on rodents and not human brains. Thus, we are now able to access data and recordings of the influence of sleep deprivation, sleep hours, and sleep quality on the different regions of the human brain. This is significant because the scientists were not only able to observe and record an increase in ABB after sleep deprivation in the hippocampus and the thalamus, which play a vital role in the development of Alzheimer's Disease, but also because no study was able to directly measure the effect of sleep and the glymphatic function in the human brain's amyloid-beta clearance.
Nonetheless, it is still plausible to argue that the accumulation of

amyloid-beta in the brain is reflecting an increase in the synthesis of amyloid-beta rather than a decrease in the amyloid-beta clearance or the glymphatic system's activity. This is where future studies can dive in and uncover more of the sleep-brain-Alzheimer's dilemma. Besides, the research in this field is of high importance as it is clinically relevant for the diagnosis of Alzheimer's Disease and enhancing both the detection of amyloid-beta accumulation and their degradation.

Vicious Cycles: Dysfunctional GCase and Parkinson's Disease

Ryan Osselborn
Lake Forest College
Lake Forest, Illinois 60045

Mutations in the GBA gene are one of the most common genetic factors associated with Parkinson's disease, yet it is still unknown how the enzyme coded by the gene, glucocerebrosidase (GCase), directly influences pathogenesis. However, recent systematic investigation offers answers regarding the important role GCase may play in the risk factor of developing the disease.

Imagine a disease that could strike at any time, where prevention is futile and nearly all cases have an unknown origin or cause. Parkinson's disease (PD) has a nearly identical epidemiology to this hypothetical disease, and though many breakthroughs in its pathology have occurred over the last 200 years, many of the risk factors and contributors to the development of Parkinson's disease are still shrouded in mystery. Using a combination of methods, Henderson et al. (2020) explores the complex interactions between the activity of a lysosomal enzyme, GCase, and the accumulation of dangerous forms of α-synuclein, the hallmark indicator of Parkinson's disease. This proverbial unveiling of the role of GCase activity, and by extension mutations in the GBA1 gene which codes for it, profoundly improve both our understanding of the pathology of parkinsonian diseases and opens the door to future therapeutic targets for treatment. Though many cases of PD are idiopathic, the prevalence of a relationship between GCase dysfunction and PD is apparent, with around 8-12% of sporadic cases involving a single mutation in the GBA gene (Avenali et al., 2020, p. 1). Although the majority with this mutation may not go on to develop Parkinson's disease, the likelihood of developing the disease increases five-fold for those who have it (Michel et al., 2016, p. 678). Individuals with mutations have also been shown to develop non-motor symptoms such as dysfunctional smell and psychiatric symptoms more than controls before developing any parkinsonian symptoms (Migdalska-Richards & Schapira, 2016, p. 80). Additionally, patients with GBA mutations often have much more aggressive disease development, slightly earlier onset, and more often develop dementia compared to non-GBA sporadic PD patients (Del Rey et al. 2018, p. 4), illustrating the importance of decoding these cellular interactions.

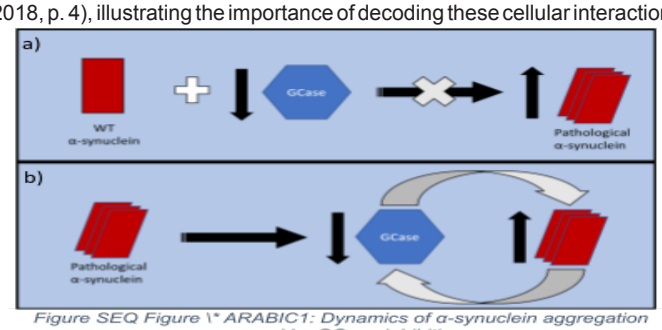


*Figure SEQ Figure * ARABIC1: Dynamics of α-synuclein aggregation caused by GCase inhibition*

- Wildtype (WT), properly folded α-synuclein found within the cell, when combined with decreased GCase activity, was not shown to cause new pathological α-synuclein aggregation. Rather,
- When pathological α-synuclein was already present within the cell, a positive feedback loop was shown to occur, where decreased or dysfunctional GCase activity instigated an increase in aggregation, which in turn further inhibits GCase activity, leading to a reinforcement to aggregation.

It is both unknown and debated whether the mechanism for PD pathogenesis due to GCase dysfunction is a loss-of-function mechanism, where reduction of GCase activity results in pathology. Likewise, it is similarly debated whether a gain-of-function mechanism is at play, where the mutated form is the cause of pathology itself (Migdalska-Richards et al., 2016, p. 83-84). Despite these two conflicting hypotheses, the former is more widely accepted (Blumenreich et al., 2020). Additionally, research suggests there may be a positive feedback loop where accumulation of α-synuclein may act as an inhibitor of GCase activity, which in turn allows for more accumulation of α-synuclein (Michel et al., 2016, p. 678). This leads to a vicious cycle that only amplifies itself and shows that the interaction may not be a simple linear relationship. However, the actual process of how mutations of GBA1 modulate and elevate the risk of developing dangerous forms of α-synuclein still remains a mystery for neuroscientists. Using a variety of in-vitro cell cultures and in-vivo mouse models, Henderson et al. (2020) systematically set out to answer this question once and for all. The researchers hypothesized that GCase inhibition acted as a modulator, rather than a direct cause, performing more like a dimmer knob on a lamp rather than a full-blown switch itself. To test this, they first set out to define the dynamics of the interaction between α-synuclein and GCase. First, Henderson et al. (2020) investigated the role GCase inhibition played in the formation of new pathological α-synuclein aggregates. Using primary hippocampal neurons (PHN), the brain cells found within the part of the brain responsible for memory, they inhibited GCase activity by injecting conduritol-β-epoxide (CBE), which is a small molecule that acts as an inhibitor. CBE effectively acts as a stop sign, halting all GCase activity without actually reducing the amount of GCase in the cells (Henderson et al., 2020, p. 824). Additionally, the researchers injected small clumps of pre-aggregated α-synuclein protein called pre-formed fibrils (PFFs) into some of the cells to act as an additional control. It was found that aggregation of α-synuclein, both wildtype and pathological forms, were not present with only GCase inhibition. However, with the addition of PFFs, aggregation significantly increased when paired with GCase inhibition. This shows that inhibition of GCase alone is not enough to cause new aggregation in healthy cells (Figure 1a) but supports the hypothesis that the enzyme modulates pre-existing pathology. Additionally, Henderson et al. (2020) found that with the addition of PFFs, healthy hippocampal cells with normal GCase function experienced a time-dependent reduction in GCase activity prior to addition, further supporting the idea of a positive feedback loop between the two proteins (Figure 1b). Additionally, Henderson et al. (2020) investigated whether different neuronal cells were susceptible to developing synucleinopathy more than others when plagued with GCase dysfunction. Using similar methods to the ones described previously, neurons from three different regions of the brain were shown to respond to GCase inhibition differently. In addition to hippocampal neurons, cortical neurons, which constitute the outermost part of our cerebral cortex, and midbrain neurons, most often associated with the connections responsible for motor functioning, were tested. Whereas hippocampal neurons showed very little elevation in pathological α-synuclein with the addition of CBE, when pathology was initially low as seen with the cortical and midbrain neurons, pathological α-synuclein aggregation increased by 50% and 4-fold, respectively (Henderson et al., 2020, p. 825-827)! These results show a significant susceptibility of midbrain neurons to aggregation related to GCase dysfunction, which is an important confirmation as midbrain neurons are both primarily and initially affected in PD. By providing a much deeper, more concrete understanding related to one of the most common genetic factors associated with Parkinson's disease, these findings not only shed light on intracellular aggregation of α-synuclein, but may also provide additional hints for how pathology spreads throughout the brain. Lysosomal dysfunction may lead to increased exocytosis, or the dumping of intracellular molecules out of the cell. This has been hypothesized as a possible way that pathological α-synuclein spreads from one region of the brain to another, as is the case in later stages of the disease (Abeliovich and Gitler, 2016, p. 212). In addition to simply illuminating this neurobiological interaction, these findings also confirm the possibility of a brighter future as this understanding can open the door to future therapeutic targets for treatment. One such treatment could arise from the use of small-molecule chaperons, which act like big brothers, guiding misfolded GCase proteins back into alignment to allow for increased activity, which may in turn affect the degradation of pathological α-synuclein (Aflaki et al., 2017, p. 743). One such small molecule chaperone that is currently in a Phase 2 clinical trial, Ambroxol, has shown promising results through increased GCase activity and subsequent α-synuclein (Blandini et al, 2019, p. 16). Though a cure for Parkinson's disease is still quite far from the grasp of neuroscientists, as our understanding of the disease expands and the weaknesses with which we can formulate treatments are illuminated, we are brought closer to a moment where the sporadic nature of the disease no longer lurks in the shadows. With their research, Henderson and colleagues have laid a strong foundation for future investigation into the mechanisms that underlay this relationship. As our knowledge grows, understanding of how GCase inhibition may play a part in purely sporadic cases may grow as well.

The Ability of SARS-CoV-2 to Transmit to New Hosts Evolved in Bats

Barry Khim
Lake Forest College
Lake Forest, Illinois 60045

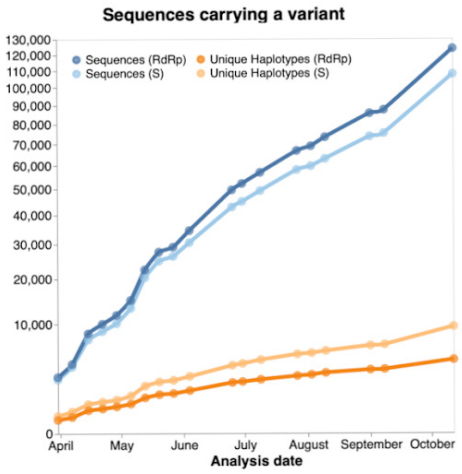
In December of 2019, a novel coronavirus was first reported in Wuhan, China. Evolutionary analysis identified the virus as a severe acute respiratory syndrome-related virus (SARS) with many similarities to the first SARS-CoV (Wu et al. 2020). It was subsequently named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Since then, SARS-CoV-2 has spread around the world, greatly disrupting social and economic interactions. The direct origin of the pandemic is unknown. While it is known that these viruses are found in horseshoe bats, the sudden transmission to human hosts represents decades of evolution (Boni et al. 2020). How exactly did this occur? What extent of evolution is required for a bat virus to transmit to humans? Researchers at MRC-University of Glasgow Centre for Virus Research set out to answer these questions. The ability to replicate efficiently and spread successfully is something most RNA viruses acquire after switching to the new host species. However, SARS-CoV-2 comes from the family of *Sarbecoviruses* which already transmit frequently between bat species because of their generalist properties. These viruses have evolved spike proteins to latch onto cells. They then bind to angiotensin-converting enzyme receptors for cell entry. Coincidentally, this allows them to successfully infect non-bat species, including humans, by binding to the human angiotensin-converting enzyme receptors. The researchers hypothesized that because of these prior adaptations that occurred in bats, SARS-CoV-2 did not need additional adaptations to transmit to humans.

To determine how SARS-CoV-2 came to be, the researchers investigated the evolutionary history of bat *Sarbecoviruses*. Historical evidence of virus adaptation in bats was contrasted with any evidence of virus adaptation that occurred in humans since the outbreak began in 2019. To do this, the researchers used an array of selection detection methods on two groups. One group consisted of 133,741 SARS-CoV-2 genomes sampled during the first year of the outbreak. The other group was 69 *Sarbecovirus* genomes that were separated and examined phylogenetically (MacLean et al. 2021). The researchers also analyzed the *Sarbecovirus* tree for changes in the patterns of CpG (site of a cytosine followed by a guanine in the 5′ to 3′ direction) because shifts in the CpG sites are an indicator of evolutionary changes.

The 133,741 SARS-CoV-2 samples, taken during the first 11 months of the pandemic, were examined for evidence of virus adaptation (MacLean et al. 2021). Each sample's genome was sequenced. The occurrence of unique sequences was plotted over time. The researchers noted that the rate of increase for unique sequences compared to the total number of sequenced genomes was quite slow [1] (MacLean et al. 2021). This indicated that the virus was evolving relatively slowly within the human population. Furthermore, statistical analysis revealed that selection was negative. The few mutations that were occurring were deleterious and failed to persist. Only after increasing levels of host immunity, due to vaccines, and SARS-CoV-2 circulation was it expected to see evidence of adaptation. However, they noted that any subsequent evolution in humans was not relevant for determining the origin of the virus' ability to efficiently spread between human hosts.

The researchers then turned to analyzing the 69 *Sarbecovirus* genomes found in bats. Viruses were separated and organized photogenically. The clade containing SARS-CoV-2 is referred to as new coronavirus (nCoV). To separate and organize the virus, they used an array of selection detection methods: BUSTED, hidden Markov model, aBSREL, mixed effects model of evolution, etc. Examination of the differences between virus phylogeny revealed evidence of historical positive selection (MacLean et al. 2021). In the nCoV clade, Orf1ab, a gene associated with SARS-CoV-2, had many sites that were subjected to positive and diversifying selection. Furthermore, analysis of CpG patterns showed that there was an adaptive shift in viruses of the nCoV clade (MacLean et al. 2021). These findings support the idea that the generalist properties of SARS-CoV-2 evolved in bats and not humans. This is further supported by the fact that SARS-CoV-2 can also transmit to other mammals (pangolins, mink, cats, etc.).

The implication of this research presents a concerning reality. The diversity and generalist nature of *Sarbecoviruses* allows it to transmit to new hosts frequently. This suggests that there are species of wild mammals infected with yet to be identified nCoV-like viruses. The threat of a new SARS-CoV that is genetically divergent enough to evade current, acquired immunity could possibly emerge in the future. For this reason, the researchers stress the importance of ramping up surveillance to better prepare ourselves against future outbreaks of SARS-CoV.



[1] The researchers looked at two traits of interest: spike proteins (S) and RNA-dependent RNA polymerase (RdRp). Total number of S and RdRp gene sequences (blue) and total number of unique S and RdRp variations (orange) were plotted on a graph.

An appetite for sleep?

Becca Ray
Lake Forest College
Lake Forest, Illinois 60045

Rapid eye movement (REM) sleep is one of the least understood aspects of neuroscience. Much of the mechanisms and purposes behind this extraordinary brain state remain unknown, but it has been associated with sensorimotor development (Rio-Bermudez et al. 2017), consolidation of memories (Li et al. 2017), and dreaming (Perogamvros et al. 2013). As opposed to the dominating stage of sleep, non-REM, REM sleep is characterized by its signature behavior of rapid eye movement during sleep, which is remarkably similar to eye movement during wake states (Figure 1).

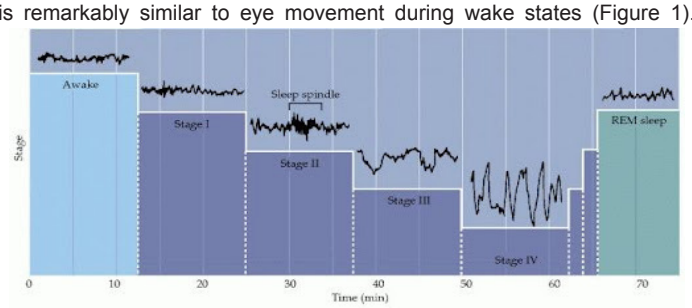


Figure 1. EEG recordings during first hour of sleep, showing wakefulness, REM, and the four stages of nREM sleep. (Purves 1970)

Among the areas in the brain that are most active during this sleep stage is the hypothalamus (Siegel 2005). It has been found that the hypothalamus plays an important role in sleep-wake cycles, and recent studies show that certain neurons in the hypothalamus are maximally active during REM sleep specifically (Hassani et al. 2010). The hypothalamus is also known to regulate a range of different functions, but it is most important for maintaining homeostasis and regulating behavior and metabolism in the brain and body (Zhou et al. 2020). One of these homeostatic behaviors is feeding habits and food consumption (Jennings et al. 2015). However, it is still unknown if REM sleep contributes to the hypothalamic control of food consumption. Answering this central question is important for understanding the role of REM sleep in maintaining certain biological functions and for the development of new treatments for disorders linked with sleep. A study by Oesch et al. (2020) reported in *PNAS* indicates there is a relationship between REM sleep and feeding in which REM sleep regulates and stabilizes the hypothalamic control of short- and long-term food intake. The authors first looked at the neuronal activity in the lateral hypothalamus (LH) of mice by assessing the calcium transients during feeding, food approach, and non-feeding. A neuron's activity is based off the concentrations of calcium inside and outside of the cell and can be calculated by measuring the influx and efflux of calcium. The more calcium flowing into the cell, the greater the activity. The mice began the trial deprived of food so they would have a high motivation to eat and then were given a free-feeding period, during which their LH neuronal activity was measured. The authors found that the LH neurons were most active during feeding and less active during food approach and non-feeding (Figure 2).

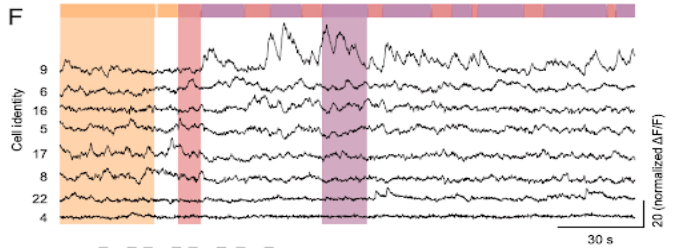


Figure 2. Calcium transients in eight LH neurons during non-feeding (orange), food approach (red), and feeding (purple). (Oesch et al. 2020)

Next, Oesch et al. (2020) turned to measuring LH activity during REM sleep. During the same trial, neuronal activity was also measured while the mice were sleeping. By comparing the calcium transients during both feeding and REM sleep, any evidence of a pattern in LH activity could be identified. The results showed that the LH activity pattern during REM sleep was similar to the activity during feeding but not any other stage (Figure 3). Not only did this finding suggest a connection between REM sleep and feeding, but it also showed that the relationship is unique to only those two stages.

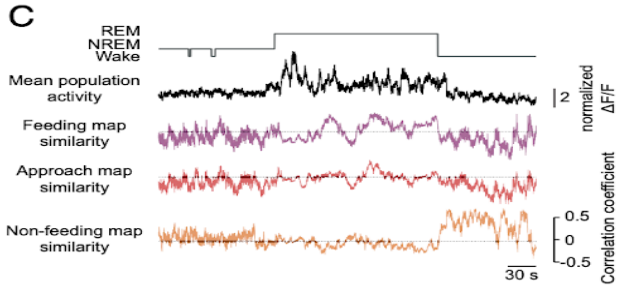


Figure 3. Feeding-associated activity compared to sleep-wake cycles. Colored graphs represent activity during different feeding patterns with black graph (lower) showing mean activity across all patterns. Corresponding sleep-wake cycles represented by black line (upper) (Oesch et al. 2020).

To test the significance of this relationship, Oesch et al. (2020) used optogenetics to ablate the LH neurons (Figure 4). Optogenetics is used to control neurons in a way similar to how a light switch controls a light bulb. While this method originated in neuroscience, it is used across many fields to study biological functions. Optogenetics is modeled off the ability of certain microorganisms to regulate ion flow across a membrane by harnessing visible light energy, and in 1971, researchers found that certain protein channels that transport these ions can be swiftly activated by light photons (Deisseroth 2011). Using this idea, scientists were able to rapidly activate or inactivate individual neurons using light. In the authors' experiment, a special light-sensitive gene was injected into the LH of mice that then allowed those select LH neurons to be activated or inactivated by light.

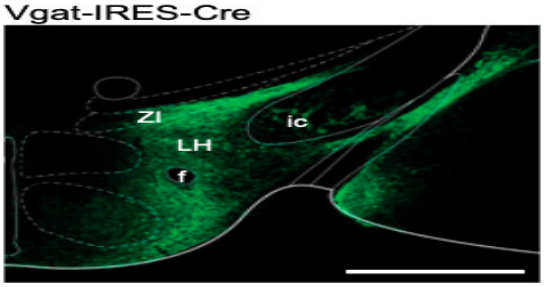


Figure 4. LH neurons infected with light-sensitive virus for optogenetic manipulation of neuronal activity (Oesch et al. 2020).

While the mice were in REM sleep, stagnant light was given in 30-second intervals during the duration of the REM sleep stage. This meant that while they were in REM sleep, those select neurons in the mice's LH were ablated and activity was inhibited for 30 seconds at a time. Following the optogenetic inhibition, Oesch et al. (2020) then observed the amount of food intake that occurred during a free-feeding period. They found that after the time of LH neuronal inhibition during REM sleep, the mice ate less food, spent less time feeding, and still had decreased levels of food intake four days after LH inhibition (Figure 5). This strongly suggests that neuronal activity in the LH during REM sleep is important for regulating short- and long-term food intake.

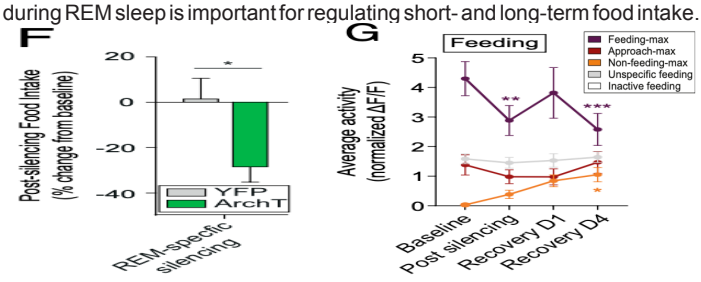


Figure 5. Average food intake post-silencing of LH neurons during

REM sleep (left). Feeding behavior across 4 days post-silencing (right) (Oesch et al. 2020).

Oesch et al. (2020)'s finding provides previously unknown functions of REM sleep and its association with LH neurons and feeding. Their discovery supports hypotheses surrounding how intuitive behaviors are modified during REM sleep as it shows its role in maintaining a necessary biological function. The continuation of the effects of the LH inhibition suggests that the impacts of LH synapses are preserved over multiple days and multiple changes in homeostasis. However, the study does not go into other possible behaviors controlled by REM sleep. The next step after considering Oesch et al. (2020)'s study is to follow up this question and investigate if REM sleep is responsible for regulation and stabilization of other homeostatic behaviors besides feeding, such as thirst or mating cycles. Additionally, the support of the idea that REM sleep plays a role in behavior modification and homeostasis is important for understanding possible implications for individuals who do not regularly reach REM sleep. Individuals suffering from sleep disorders may be at a greater risk of developing eating disorders associated with low intake of food, which could lead to other severe health implications such as loss of weight, hypothyroidism, and slow metabolism (NCEED 2018). The new connection between REM sleep and stabilized feeding behaviors could offer new therapies and treatments for those affected by sleep and eating disorders. Yet, there is still so little known about the functions of REM sleep, who knows what other treatments can be linked with this mysterious sleep stage. Understanding the relationships between REM sleep, behavior, and homeostasis is vital to fully understanding how the human brain and body are interconnected. Further research on REM sleep should help shed light on whether we are what we sleep.

Antibody Binds to Movement

María Jose Orozco Fuentes
Lake Forest College
Lake Forest, Illinois 60045

Memory B-cell antibody targets misfolded SOD1. Treatment with α-miSOD1 ameliorates ALS motor symptoms.

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease that affects motor neurons by biological mechanisms such as misfolded protein aggregation, which causes neuronal death. Lower and upper motor neurons allow the signaling of voluntary movement from the nervous system to the muscles (Taylor et al. 2016). There is not a clear cause for ALS as 90% of all patients are considered sporadic (SALS), which means there is no inherited gene mutation. Nevertheless, research on familial ALS (FALS) has allowed the identification of over 50 genes related to ALS: C9ORF72, FUS (fused in sarcoma), TDP-42 (TAR-DNA binding protein) and SOD1 (superoxide dismutase 1), among others (Mejzini et al. 2019). SOD1 is an enzyme that has a key role in the detoxification of superoxide anion radicals in the cell and when this gene is mutated, misfolding creates toxic inclusions and aggregation (Mejzini et al. 2019). Writing in *Science Translational Medicine*, Maier et al. (2018), explore the possibility of an immunological response triggered by misfolded SOD1 that generates neo-epitopes and B cell memory alongside with the possible use of it for immunotherapy. Memory B cells and antibodies are produced by plasma as an immunological response from the body (they are the soldiers of the body). These memory B cells identify the pathogen bound to and through interacting with T-helper cells (Akkaya et al. 2019). The production of antibodies is started by cell differentiation and proliferation of antibody-specific B cells (Akkaya et al. 2019). This mechanism is seen in human bodies to create immunity and faster immunological responses and has been used in vaccination as a preventive measure. Nevertheless, memory B cells have not been previously investigated in their efficacy to target and treat neurodegenerative diseases caused by misfolded protein aggregation. Maier et al. (2018) explores an advancement in understanding further mechanisms of ALS and possible treatments. The lack of a cure or an effective treatment of ALS drives the necessity of innovation in this field. One of the main obstacles for drug efficacy in neurodegenerative diseases is the crossing of the blood brain barrier (BBB). The BBB is a protective lining of endothelial cells that maintains the internal environment under regulated conditions. This barrier poses an obstacle for any foreign substances to enter the central nervous system (CNS) as it is a very selective membrane. In Maier et al. (2018), they will propose a treatment that will potentially improve motor function in ALS patients by crossing the BBB (Maier et al. 2018; Villabona-Rueda et al., 2019).

Maier et al. (2018) hypothesizes that the use of a human B memory cell can present an antibody with a high binding affinity to misfolded SOD1 without affecting the natural unmutated dimers. This recombinant antibody will improve the motor performance of ALS patients. In order to test this, the researchers performed an ELISA (direct enzyme-linked immunosorbent assay) to test the affinity of the antibody with misfolded SOD1 and native dimers. This ELISA assay is known as a *primary detection antibody* which is an immunoassay that tests the binding of a specific antibody to a protein of interest (Alhajj and Farhana 2020). This assay showed an exclusive affinity to misfolded SOD1 in denatured and oxidized conditions which indicates possible use for treatment of these toxic inclusions. Further, to confirm the possible detection of SOD1 in human post-mortem spinal cord sections, samples of SALS and FALS patients were injected with α-miSOD1. Microscopic images revealed that α-miSOD1 detected misfolded SOD1 in a majority of SALS patients and FALS patients with and without SOD1 mutation but with a hexanucleotide expansion mutation on C9ORF72.Moreover, there was no detection of misfolded SOD1 in non-neurological controls (NCC). Further, these results were congruent with transgenic SOD1 transgenic mice–α-miSOD1 detected aggregations from a pre-symptomatic stage (30-days old) to an end stage. To evaluate the effect of the chimeric derivative of α-miSOD1 on mice, researchers administered intracerebroventricularly a stable dosage of ^{ch}α-miSOD1 over regulated intervals of time using osmotic pumps after 60-days of age until end stage (Keraliya et al. 2012). A chimeric derivative is an antibody or glycoprotein molecule with various domains from different species, in this case, of rodents. By combining different domains and replacing as much of the non-binding antigen as possible with the species affinity, the chronic anti-human response to the antibody will be reduced (Chimeric Antibody 2020). This reduction of chronic response improves the reliability of the effects shown in the mice models by exposure to α-miSOD1. Some mice were presenting mitochondrial dysfunction and denervation as soon as 7-days old. Upon treatment with chα-miSOD1, mice showed an improved upright gait and wider angles between the iliac crest and the hind limbs which means that their motor function was better overall. Additionally, the survival rate of ^{ch}α-miSOD1-treated mice was significantly higher compared to the vehicle-treated littermate controls. This could partially be explained by the 51% decrease of aggregate load in the ventral horn of the lumbar spinal cord sections collected by the ^{ch}α-miSOD1-treated mice compared to the vehicle controls and a decrease in microglial activation measured in a Iba1 assessment. Next, they treated the mice from a peripheral administration by weekly injecting intraperitoneally ^{ch}α-miSOD1 into transgenic mice that showed slow progressing ALS motor symptoms. A comparing group was injected with an epitope isotype-antibody-matched within the amino acids 110 to 120 of SOD1. Decrement in motor symptoms and coordination was decreased in those treated with ^{ch}α-miSOD1 as well as motor impairment and survival rate. Additionally, a two-fold increase in motor neuron quantification indicated an overall improvement in the ventral horn health in ^{ch}α-miSOD1-treated mice compared to vehicle controls and isotype groups. This showed the efficacy of ^{ch}α-miSOD1 to cross the blood brain barrier from being injected intraperitoneally. Finally, Maier et al (2018) experimented on a fast-progressive ALS mice combined B6SJL background, which is a recombinant ALS-type of transgenic mice, to investigate the effect of intraperitoneal administration on neuroinflammation. They found that after three weeks of treatment with ^{ch}α-miSOD1, there was a 37 and 43% reduction in microgliosis and astrogliosis, respectively. Furthermore, there was a reduction of 25% of SOD1 aggregates and 66% of misfolded SOD1. This established an association between neuroinflammation, aggregates, and disease progression. The final issue arising when this information is integrated is whether α-miSOD1 is an effective treatment for ALS patients. The model mice that were used were highly over-expressive of SOD1 which might deviate the results of the treatment if it was applied on humans. Additionally, the human spinal cord samples were post-mortem which affects the evaluation of α-miSOD1 in on-set disease progression in humans. Nevertheless, this research sets off groundbreaking observations about how antibodies react to misfolded proteins, especially SOD1. This can lead to further studies on how immunotherapy can impact neurodegenerative diseases caused by aggregated and misfolded protein.

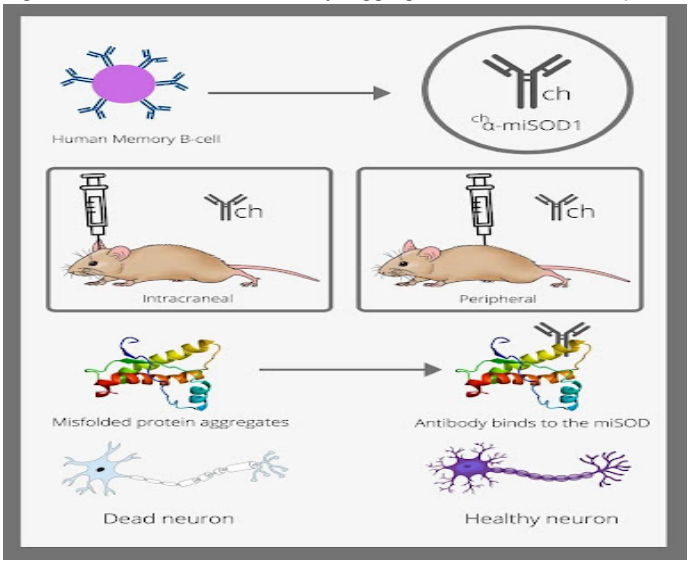


Figure 1. Memory B cells develop immune responses in humans. The use of the human memory B cells library indicated a potential antibody for the targeting of misfolded superoxide dismutase 1 (SOD1). SOD1 is

a common enzyme that helps with oxidative radicals regulation in the cells of the nervous system, but when misfolded, there is neuronal death due to mitochondrial dysfunction and superoxide accumulation. The development of a chimeric antibody (^{ch}α-miSOD1) was administered intracerebroventricularly and intraperitoneally to SOD1-Tg mice. This administration in low and high doses indicated correct targeting for misfolded SOD1 accumulation and improvement in motor function and survival rate.

Religion, Spirituality, and the Black Death

Brooke Carlock
Lake Forest College
Lake Forest, Illinois 60045

In Daniel Defoe's A *Journal of the Plague Year*, he writes, "… the People, from what Principle I cannot imagine, were more addicted to Prophesies, and Astrological Conjurations, Dreams, and old Wives Tales, than ever they were before or since…" (Defoe 2003). When examining documents during the fourteenth and seventeenth centuries about the plague, one can see that there was a variety of explanations regarding who or what was to blame for disease. Although the idea of contagion was just beginning to emerge and circulate in scientific communities, a prominent idea among many, as seen in Defoe's quote, is the idea that humanity itself is to blame for the Black Death. In this paper, I will argue that many believed humanity was so sinful that God either was the cause of the plague or was simply letting it happen, and therefore humanity needs to do penance for their sins to end the spread of disease. I will explore Defoe's book *A Journal of the Plague Year* as well as fourteenth-century documents from John Aberth's *The Black Death: The Great Mortality of 1348-1350* that demonstrate how people were determined to find "signs" in the sky or in the world around them that showed that the end was near, as well as how they decided to commit penance once they decided that humanity was being punished by God. To begin, I will analyze *A Journal of the Plague Year*, which goes into extensive detail about the signs from God that people claimed they saw. It is important to note that a potential bias within this novel stem from Defoe being five years old when this plague epidemic in London in 1665 was occurring, and it is theorized that he is writing from his uncle's perspective. Defoe was also a famed novelist who wrote *Robinson Crusoe*, and it is possible that some of the details that are described by Defoe were made up or exaggerated. Despite the problems with this source from a historian's perspective, it is still an important source for our modern-day understanding of what happened during the Great Plague of London. Defoe goes in depth into how the people of London tried to explain the oncoming plague through signs from God. He narrows his focus on a description of a scene that occurred before the beginning of the plague in which a woman claimed that she saw an angel in the sky. Defoe writes, "She described every Part of the Figure to the Life; shew'd them the Motion, and the Form; and the poor People came into it so eagerly, and with so much Readiness…" (Defoe 2003). The narrator (as we know that this is not Defoe's experience but someone else's) explains how he did not see the shape of the angel in the sky, and the woman told him "…that it was a time of God's Anger, and dreadful Judgments were approaching; and that Despisers, such as I, should wonder and perish" (Defoe 2003). The narrator has to leave the scene because he is so terrified that he is going to be "mobb'd by them" since he does not see the same image in the sky that they did. In addition to the clouds in the sky, Defoe describes how comets appeared over London and many people, including him, saw them as "the Forerunners and Warnings of Gods Judgements" (Defoe 2003). They even potentially had different colors and speeds of movements based on the type of judgement that God was giving. These descriptions from Defoe's book are crucial to understanding who the people considered responsible for the onslaught of disease. Clearly, the people of London are not using scientific reasoning such as the popular miasma theory for the cause of the plague but take a more religious direction. The intensity in which the people looking at the cloud in the sky defend their viewpoint against the narrator shows how desperate people were for an explanation to the plague, but it also shows how people were insistent that humanity as a whole was responsible for the plague because God was angry with them. The woman in this scene proclaims to the narrator that God was angry with humanity for their sins and thus was bringing His punishments, or judgements, as she says, to the people through the spread of the plague. The comets in the sky over London were another sign from God, letting humanity know beforehand that He was angry with them, and, therefore, He was going to allow or cause the plague and the Great Fire of London as punishment for their combined sins. Clergy begin to enforce this idea that humanity is responsible for the plague as well. Defoe writes that "…Ministers, that in their Sermons, rather sunk, than lifted up the Hearts of their Hearers; many of them no doubt did it for the strengthening the Resolution of the People; and especially for quickening them to Repentance…" (Defoe 2003). He describes people who frightened others to the point of tears, who "prophesying nothing but evil Tidings; terrifying the People with the Apprehensions of being utterly destroy'd" (Defoe 2003). The government becomes involved in this as well, with Defoe writing that "…The Government encouraged their Devotion, and appointed publick Prayers, and Days of fasting and Humiliation, to make publick Confession of Sin, and implore the Mercy of God, to avert the dreadful Judgement…" (Defoe 2003). London was not the only place in the Mediterranean world in which mass public penance and prayer events were conducted. The government of Damascus also takes action in the document "The Beginning and End: On History" in Aberth's collection of Black Death documents. The author of this document, Ibn Kathir, was a teacher of the Hadith, so it was easy for Ibn Kathir to witness how the city of Damascus and its people responded to the major threat of the plague. Throughout this document, Ibn Kathir describes the intensity in which the citizens of Damascus believed that they needed to pray penance to God to rid the city of plague. Ibn Kathir writes that "the people poured out their supplications that the city be spared the plague" (Kathir 2017). Supplications conducted by the people include the story of the flood of Noah being recited over three thousand times, fasting for three days, and were told to "humbly beseech God to take away this plague" (Kathir 2017). One of the most extraordinary scenes described by Ibn Kathir is when on the fourth day of the supplication of the city of Damascus, during the ceremonies that were being conducted, "…one saw in this multitude Jews, Christians, Samaritans, old men, old women, young children, poor men, emirs, notables, magistrates, who processed after the morning prayer, not ceasing to chant their prayers until daybreak" (Kathir 2017). After analyzing both Defoe and Ibn Kathir's descriptions of people from all different kinds of backgrounds participating in this mass repentance, it is clear what the common belief in the Mediterranean world of who was responsible for the plague: humanity. The cities of London and Damascus have common themes of fasting, public prayer and recitation, and supplication being conducted by the public; many believed that God was punishing humanity for their sins, and the correct way to abolish the plague was for the people to do everything in their power. This included doing penance repeatedly in fervent ceremonies, and constantly reciting relevant scriptures. As seen in the haunting description of the peoples of Damascus coming together to chant prayers, the belief that God was punishing humanity was not uncommon. Whether you were Jewish, Christian, or Muslim, Ibn Kathir's passage demonstrates how desperate the people were to appease God and to end the suffering that was the Black Death. Alongside the similar acts of penance that the people of the cities Damascus and London shared, Damascus also experienced events that were interpreted as signs from God about the judgements that are coming to humanity. Ibn Kathir describes a sandstorm that occurs over Damascus. He writes, "The population was in a state of anguish for about a quarter of an hour, imploring God, asking His pardon and lamenting all the more that it was afflicted by this cruel mortality " (Kathir 2017). In multiple cities, people were claiming that various natural events were signs from God; this demonstrates that this sudden onslaught of religious devotion was not exclusive to London. In addition, it also shows that the psychological effects of the plague were so strong that people were desperate to find any solution to or explanation for why so many people were suffering and dying from the plague. Surely, if humanity were innocent and free of sin, then the plague would not be happening in the first place. Therefore, the people of the Mediterranean world, as seen in both Defoe and Ibn Kathir's sources, decided to dedicate themselves to religious rituals and repetitive penance to try their best to cleanse their sins. To end this paper, I will look at the most prominent example of those who believed that humanity's evils were responsible for the plague: the flagellants. The flagellants, out of all the documents that I have examined, are probably the most extreme example of penance done for God's anger against humanity. In Fritsche Closener's "Chronicle," he describes the flagellants that would come through the town of Strasbourg and display their gruesome self-mutilation. Closener writes that twice a day, "they laid down in many ways, according to the sins which everyone had committed. After they had all lain down, the master began where he saw fit and stepped over one of them and hit his back with the scourge…" (Closener 2017).

The scourges in which Closener describes had “knots at the end into which thorns had been placed, and they whipped themselves across their backs so that many of them were bleeding a lot…”(Closener 2017). In fact, this self-harm was one of the most important parts of the flagellants’ penance to God. According to Closener, the flagellants went on pilgrimages for a little over thirty-three days (equilvalent to the amount of time that God/Christ lived on Earth) and that they should “never have a good day nor night and spill your blood.” Through this pilgrimage and self-imposed torture, God would supposedly forgive humanity and “forget His anger” (Closener 2017). Along with the self-flagellation that the flagellants were named for, they followed some of the same trends of public prayer and chanting in Defoe and Ibn Kathir. They would chant “Now we lift up our hands and pray/O God take the great death away!” for three hours (similarly to the repetition of the flood of Noah in Damascus), and they would read the letter they received from the king of Sicily informing them of how they should commit penance to God, where “there rose great lamentation from the people, because they all believed it was true” (Closener 2017). I think the last quote is particularly important because, again, it demonstrates how eager the people were to believe any religious explanation for the plague that was given. These people were publicly whipping themselves, which may be disturbing for many; however, they were also offering a potential explanation and solution to God’s anger against humanity, which may have been relieving to hear. Had the belief that the plague was God’s judgement not been as widespread or popular, then perhaps the flagellants would not have been as accepted and welcomed as they were. Although the popularity of the flagellants changed, they are still a prominent example of the lengths people went to appease God and to cleanse themselves of their sins that were the cause of the plague. On one end of the spectrum, we see simple acts like public prayer in Damascus and London, and on the other, we see extremist groups like the flagellants. People engaged in acts of penance in various ways during the Black Death. To sum up, during the time of the Black Death, there existed a variety of explanations for the immense suffering and mortality that was the plague. These reasons ranged from the scapegoating of the Jewish community to the miasma and earthquake theory. People were desperate to find some sort of solution and answer as to why there was so much agony in the world. The most common explanation to the plague and the group considered responsible for disease in the Mediterranean world was humanity itself. We explored documents from London to Damascus that demonstrated how there was a widespread belief that the reason why the plague was spreading was because humanity had accumulated so much sin that God was sending (or allowing) the plague as punishment for their collective sins. The only solution and way to end the plague was to fervently repent and commit acts of penance towards God. Many believed this would appease God, and He would forgive His anger against humanity. “Signs” from God included comets, clouds in the shapes of angels, and massive sandstorms, and there was mass recitation of scriptures, public prayer, ceremonies, and self-flagellation; ultimately, the Mediterranean world engaged in a variety of forms of repentance that reflected how desperately the people were clinging to religion and spirituality during a time of mass death and misery

Environmental Racism in Chicago

Sophie Rasof
Lake Forest College
Lake Forest, Illinois 60045

In the United States, there is a systematic disparity of exposure to environmental pollution in which low-income minority groups are forced to bear the burden of associated health problems and risks. This essay will highlight the systematic ways in which marginalized groups are targeted and exploited to work and live in unsafe conditions, which ultimately impacts their overall quality of life and health. The environmental justice movement and community organized groups' goals are to target the systematic structures that allows for the continuation of inequality. Throughout Chicago's history, there have been many cases of environmental injustice and inequality in the South and West neighborhoods consisting of predominately Black and Latinx members. The treadmill of production theory and history of exclusionary zoning practices provide a framework for the two case studies of environmental justice that will be presented in Little Village and the surrounding area. Ultimately, the work done by environmental justice organizations benefit the community by reducing exposure and risk, but even the process of "greening" the neighborhood can put its members at risk for environmental gentrification and displacement. Environmental racism is a term used to describe the disproportionate exposure to toxic and hazardous waste in low-income minority communities due to the inequality of environmental policymaking and laws (Pellow 2000 and Brulle and Pellow 2006). Environmental racism is an extension of the systematic racism that minority groups have faced in the United States throughout history. Specifically, Latinx and African Americans are at a systematic disadvantage, unable to access appropriate resources, and are put at a higher risk for health and economic disparity. In Chicago, factories and industrial manufacturing production plants are placed in predominately Hispanic and Black communities. Air, water, and ground pollution from these production facilities impede on the health of the overall community both directly or indirectly. The collective exposure to these pollutions is at much higher rate than the surrounding white-affluent neighborhoods. Improper storage of hazardous waste, illegal dumping, and lack of education and protection for the workers all contribute to unjust and unequal environmental protection. This is a human health crisis causing higher rates of asthma, cancer, respiratory illness, lead poisoning, and cardiovascular disease seen at exponentially higher rates in these communities (Brulle and Pellow 2006). The accessibility to healthcare is limited in low-income minority and immigrant communities. This, in combination with unsafe living conditions, leads to suffering of specific populations. Chicago's segregated neighborhoods allow for specific minority communities to be targeted and ultimately exploited. Chicago is currently one of the most segregated cities in America. Discriminatory housing practices in the 1920s segregated immigrant and minority communities into sectors of Chicago that lacked job opportunities, access to education, healthcare, and inadequate housing. This put these populations at a significant disadvantage, while offering high risk jobs working with hazardous materials without proper protocol and protection in place. Exclusionary zoning practices throughout the districts persist today even after the Fair Housing Act of 1968. Figure 1 depicts the racial distribution throughout Chicago's neighborhoods (Demographics Research Group 2010). We can see that Hispanic and Black communities are located in the South and West side of Chicago, while white populations are in affluent areas of the city and the Northern suburbs. This is consistent with historical redlining of racial and housing discrimination that began in 1934 with the National Housing Act. Figure 2 is a map identifying the most at-risk populations for exposure to pollution (Lam 2018). An overlay of Figure 1 and Figure 2 depicts a clear correlation between Black and Hispanic populations and high rates of environmental pollution. The foundational history of Chicago contributes to the overall inequality seen in these communities of marginalized groups today. The environmental movement beginning in the 1960s consisted of predominately white voices, excluding those who are most directly impacted from the negative effects (Brulle and Pellow 2006). In the 1970s, middle and upper-middle class community members used the phrase "Not in My Backyard" in opposition of industrial and manufacturing sites to be placed in their predominately white neighborhoods. Ultimately, the privilege in these affluent communities further pushed locally unwanted land use into low-income minority communities, externalizing the cost to already disadvantaged areas. The environmental justice movement emergence in the 1980s focused on the right that "all people and communities are entitled to equal protection of environmental and public health laws and regulations" (Brulle and Pellow 2006). The fight for environmental justice is just as prevalent today in 2021, especially in the city of Chicago. The main driver of environmental injustice is capitalism and the demand for capital accumulation, ultimately at the expense of poor and minority groups. Treadmill of production theory provides insight of why this occurs and how it benefits those who hold a position of power. The treadmill of production is sociological theory that highlights how economic growth and the drive for capital accumulation force businesses to seek out innovative ways to increase capital while decreasing the cost of production. This demand is reliant on the extraction of natural resources and production of pollution harming the environment. David Pellow, Director of the Global Environmental Justice Project, has done extensive research on the sociological drivers of environmental injustice in Chicago. He developed an intersectional approach to environmental justice while highlighting the role of stakeholders' position of power (Pellow 2000). Pellow explains the power dynamic between multiple stakeholders and uneven distribution of resources which keep poor and minority groups at a disadvantage. Those in power of the industry or state drive the demand for goods while finding ways to cut cost for production or targeting specific groups that they know they can take advantage of and exploit. This forces marginalized groups to be stuck on the treadmill that allows the capital accumulation for those in power. Meanwhile, laborers are underpaid and working in unsafe hazardous conditions. This theory will provide a framework throughout my essay in the ways in which the cost of hazardous production is externalized onto minority communities. Like I mentioned previously, the systematic structure of exclusionary zoning practices segregated Black and Hispanic communities into the South and West areas of the city. This is where the preponderance of factories, coal plants, steel production plants, and other industrialized facilities are located. These systematic structures will be applied to two case studies presented over the last 40 years. In 1995, after push from the state to increase the recycling rate in Chicago, Waste Management Inc. created a recycling program known as the "Blue Bag" (Pellow 2000). Chicago Recycling Coalition and Citizens for a Better Environment were two environmental organizations that pushed for improvement in recycling in the city of Chicago, which ultimately led to the state law that required an increase of recycling to 15% in 1994, and 25% in 1996 (Pellow 2000). Additionally, during this time the U.S. Supreme Court was investigating to see if Chicago's incinerator ash was compliant with the Resource Conservation and Recovery Act, in which they found Chicago was violating this law and was not properly disposing the hazardous waste (Pellow 2000). The ash from the incinerator in Northwest Chicago was being buried in the surrounding landfills which directly violated these laws since toxic compounds could leach into the soil and water (Pellow 2000). Eventually, the incinerator closed and WMI, the company known for putting hazardous waste sites, landfills, and incinerators into predominantly minority communities, took the opportunity to start a large-scale recycling program (Pellow 2000). The "Blue Bag" program allowed households to put their recyclable material in a blue bag along with their trash that would be picked up and brought to material recycling and recovery facilities (MRRFs) (Pellow 2000). The recyclable material was then hand sorted alongside household waste. The labor force consisted of predominately African American workers that were underpaid, overworked up to 20 hours a day, and deprived of proper protection for handling hazardous materials (Pellow 2000). Frequently, workers were exposed to hypodermic needles and syringes, unregulated medical waste, and toxic and hazardous substances (Pellow 2000). This is not only a human health concern but also a concern for racial injustice in the community. The initial implementation of the Blue Bag program was backed by state officials, environmentalist groups, and neighborhood communities because WMI claimed it would benefit the environment all while providing jobs in low-income communities in Chicago (Pellow 2000). This case study of environmental racism in Chicago in the 1990s exemplifies the treadmill of production in society. WMI took the opportunity to expand in the market after the closing of their incinerator. By constructing a recycling facility, they

were allowed to continue to gain capital, despite the harms to the individuals running the production. Strategically placing the recycling plant in a predominately low-income African American community permitted WMI to underpay and overwork the employees without much initial backlash. This was a tactic frequently used by this company. The introduction of the recycling facility coincided with a period in which unemployment was at an all-time high due to job decline from large scale "deindustrialization" (Pellow 2000). Therefore, community organizations backed up the introduction of the recycling plant because it would offer jobs to a community that was suffering immensely. Additionally, the environmental organization stakeholders supported the project because of the positive contribution to overall waste reduction and increase in recycling (Pellow 2000). The innovative initiative that WMI took used methods of "greenwashing" to present a project that seemed like a win-win to the other stakeholders. Ultimately, the "Blue Bag" project was unsustainable and put already disadvantaged groups at risk, leading to the work force to riot and halt production (Pellow 2000). The workers and community presented their resistance to the unequal and unjust exploitation of workers and exposures to toxins within the neighborhood. The protests, riots, and letters written to the press expressed their concern for these unsafe conditions reached the Occupational Safety and Health Administration (OSHA), which fined WMI for several labor violations (Pellow 2000). This case study provides one example of the environmental racism that marginalized groups in Chicago face. Unfortunately, this incident is not isolated but does provide a basis for understanding the mechanisms which continue the perpetuation of environmental inequality. Many of the tactics used by the industry as it pertains to the presentation of large-scale industrial operations are still used today. Crawford Generating Station, built in 1924, was a coal-fired power plant in the neighborhood South Lawndale in the Southwest side of Chicago. Eventually, South Lawndale became Little Village, a predominately Latinx community. Before the formation of Little Village, South Lawndale consisted of predominately Czech and Eastern European immigrants. In the 1970s white immigrants moved from the city into the surrounding wealthier neighborhoods leaving non-white minority groups to concentrate in areas of high demand for toxic industrial development (Isaacs 2020). Mexican immigrants moved into the South Lawndale area because of reclassification zoning from the University of Illinois campus, which ultimately displaced this population into Pilsen (Issacs 2020). The coal plants located in predominately Lantinx communities were known for extensive environmental pollution affecting the overall health of the surrounding community. The Crawford Coal plant in Little Village and Fisk coal plant in Pilsen were known for their excessive smokestacks which billowed smoke clouds into the air, covering the community in soot and ash. In 2001, the NAACP scored Crawford coal plant as the worst offender of environmental justice (Isaacs 2020). The surrounding communities' concerns of exposure from the coal plant were justifiable since emissions from this site included, "3 million tons of carbon dioxide, 9,000 tons of sulfur dioxide, 2,500 tons of nitrogen oxides, and 145 pounds of mercury [a year]" (Isaacs 2020). As a result of toxic exposure to the surrounding community, a study in 2001 from Harvard School of Public Health states that Crawford was responsible for "41 deaths, 550 emergency room visits, and 2,800 asthma attacks per year" (Isaacs 2020). In 2012, the Crawford coal plant, owned by Midwest Generation, closed because they did not want to upgrade their equipment to the up-to-date standards proposed by the Clean Air Act. Prior to their closure they were using outdated equipment from pre-industrial regulations (Isaacs 2020). After the coal plant closed, a vacant lot was available for purchase, so one environmentally hazardous site was replaced for another thus exacerbating the already high air pollution. In 2017, NRG hired Hilco Redevelopment Partners to purchase the land that the Crawford plant was on in order to start a development known as Exchange 55. Exchange 55 is an e-commerce shipping warehouse for Target, home to 176 diesel trucks (Isaacs 2020). Hilco's Exchange 55 website claims to be "eco-friendly" by planting trees on site, adding solar panels on the rooftops, all while benefitting the community by providing 1,000+ local jobs to support economic growth in Little Village (Exchange 55 2020). Again, companies are "greenwashing" their mission and goals by focusing on ecological benefits to a community without addressing underlying air pollution concerns from their facility. Placing emphasis on job creation and eco-friendly missions allows industries to manipulate stakeholders, investors, and the state to back projects that are ultimately not environmentally conscious. By emphasizing social and ecological contributions, companies can bypass the disproportionate risk they are exposing the community to when presenting their projects. On April 11, 2020, Hilco began the demolition of the Crawford Coal plant, and gave last minute notice to the surrounding community. Improper protocol was used during the demolition project resulting in a large plume of dust covering Little Village. The dust was likely to contain hazardous waste from the plant including lead and asbestos. This toxic dust plume coincided with the beginning of the COVID-19 Pandemic, a virus that targets the respiratory and immune system. Chicago's Department of Public Health report of City of Chicago's Air Quality and Health Report already listed the Southwest area of Chicago as highly vulnerable to the effects of air pollution, listing them as one of the most polluted areas of the city (CDPH 2020). Health associated risk for these areas include but are not limited to asthma, COPD, lead poisoning, coronary heart disease, low birth weight, and cancer risk (CDPH 2020). Within hours after the demolition project, Fernando Cantú, a 78-year-old man who lived in Little Village blocks away from the implosion site, died. Cantú had asthma and COPD, putting him at higher risk of the negative health implications because of the toxic dust that covered his neighborhood (Camarillo 2021). Environmental justice advocates fear that the policies, laws, and regulation in place to protect minority groups from pollution are not enough (Cherone 2021). Mayor Lori Lightfoot's response to the botched demolition requires more extensive permits and regulation for future demolition projects using implosion, including a city environmental inspector coming to certify that hazardous residue is not present prior to the demolition (Cherone 2020). Hilco was fined $68,000 for 16 citations and is paying $370,000 to settle a lawsuit for their failure to protect the community members in Little Village (Cherone 2020). The e-commerce facility is not currently operating, and concern for environmental justice is at the forefront of the Little Village community as production will shortly begin. The Little Village Environmental Justice Organization (LVEJO) is working with the community to address the environmental issues that their neighborhood faces, while advocating for legislative policies that protect their community. The closure of high polluter coal plants throughout Black and Hispanic communities improves and minimizes the exposure to toxic and hazardous wastes and pollution. With the loss of industrialized manufacturing sites, vacant land is often bought by other industries to produce facilities like the Crawford and Fisk sites. Deciding vacant land use also has the potential to put the community at risk for gentrification and displacement. Low-income communities are less likely to have private developer's purchase of land and use for the community due to lack of public or governmental investment (Maantay and Maroko 2018). Rather, larger companies come in and will buy the vacant land and convert it into to higher-end commercial or residential property. Gentrification through improving housing, bringing in new business, and renovating in low-income communities can displace minority communities as affluent white individuals begin to move into the previously vacant areas (Maantay and Maroko 2018). Additionally, a new problem emerges as environmental organizations push for increase in urban green spaces in low-income communities and begin cleaning up the communities. Urban green spaces have significant positive impact on the community's health and overall well-being (Kern and Kovesi 2018). A possible risk hindering the development of these neighborhoods is environmental gentrification. Environmental gentrification refers to the process of improving the environment of a community and "greening" of a neighborhood, resulting in the increase of local property values and displacing of the local community (Maantay and Maroko 2018). The process of improvement of the environment could include urban green spaces, community gardens, incorporating innovative green infrastructure, and cleaning up local pollution, all of which tend to attract affluent residents to neighborhoods that were previously undesired. It is necessary to provide the community with resources that many surrounding neighborhoods have already has access to, but this also can contribute to risk of displacement.

Low-income communities face challenges with food insecurity, pollution from transportation, inability to access nature, and lack of communal spaces (Kern and Kovesi 2018). Equality in the community means that accessibility issues must be addressed. Yet, there is a fine line between addressing the environmental hazardous risk to the community and risking displacement into overcrowded neighborhoods with worsened environmental conditions (Maantay et al. 2018). Often environmental groups, predominately wealthy and white, will come into low-income minority communities and contribute to the "greening" of communities. Community engagement is essential to avoid environmental gentrification while also addressing environmental equality. Improving access to jobs and the conditions of the workplace in the manufacturing facilities located in the

community can help mitigate these issues. Additionally, city investment in green transportation reduces air pollution for those in the community using public transportation to and from their employment. There is sufficient evidence of environmental injustice targeting low-income and minority communities that should be addressed in legislative policy to protect these vulnerable populations and diminish the effects of zoning and gentrification. Environmental racism is still pervasive in low-income and minority communities throughout Chicago. As local environmental justice groups fight for change in legislative policy, the continuation of industrial manufacturers fill the gaps in the economic sector in South and West neighborhoods of Chicago. Black and Latinx communities disproportionately bear the burden of environment risk and hazards associated with air, water, and land pollution. Health concerns associated with environmental pollution in an area with limitations in access to appropriate resource provides an exemplary struggle for environmental justice. The mechanisms for this cycle have been presented by the historical roots of segregated neighborhoods and exclusionary zoning while large industrial companies drive the treadmill of production. Workers are exploited because of their immigrant status and relatively low position of power and are subsequently forced to work in unsafe conditions with hazardous materials. Stakeholders that support the manufacturing and industrial facilities are being coerced through methods to create an idealized vision of what the company's goal and missions are by using eco-friendly language and persuading others with the potential to provide jobs to support the community. Meanwhile, the efforts of environmental protection within the community and improvement of pollution and quality of life can put the communities at risk for displacement due to environmental gentrification. Environmental racism is multifaceted and complex in the systematic mechanisms that contribute to inequality and the difficulties of implementing improvements to end the cycle.

Figures

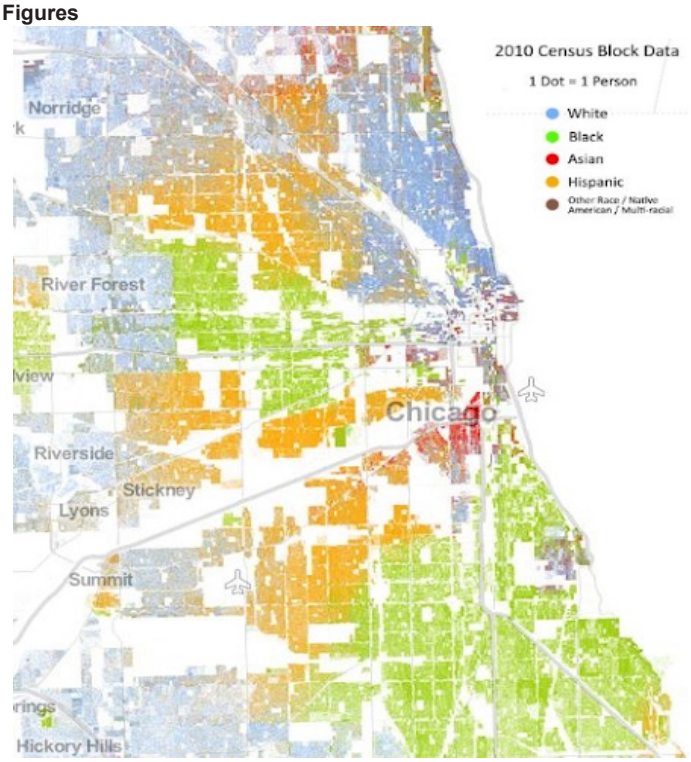


Figure 1. Map Of Racial Distribution in Chicago from 2010 Census Block Data (Demographics Research Group 2010)

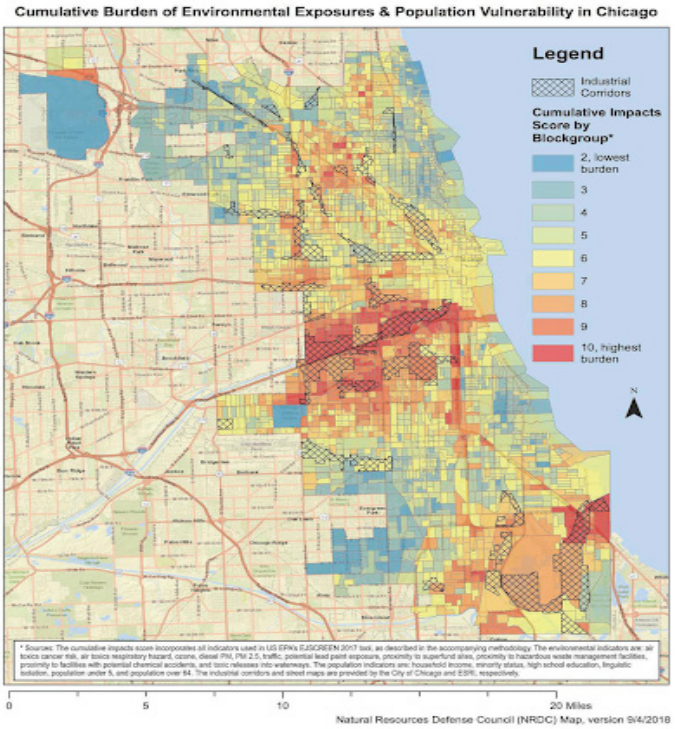


Figure 2. Map of Exposure to Environmental Risks and Hazards in Chicago by Neighborhood. (Lam 2018)

Matcha Saves the Day

Jasmin Montes
Lake Forest College
Lake Forest, Illinois 60045

Although sugary cookies are usually deemed unhealthy, Keiko Unno and colleagues conducted a study in which the effect of matcha cookies on university students and mice revealed that they can play a role in lessening stress.

Imagine taking a sip of ice-cold lemonade on a hot day. The refreshing feeling would make one feel good on a summer day. Similarly, imagine being able to destress by doing something as easy as eating delicious cookies. Keiko Unno and colleagues explored the possibilities of matcha's properties being able to destress individuals. They gave matcha to the subjects and recorded their effects on stressful participants.

The researchers used mice models as well as university students as participants to compare different dosages of matcha. The methods for measuring matcha were high-performance liquid chromatography (HPLC), mice model, and double-blind randomized controlled trial for the participants. Mice were kept under similar conditions and given different amounts of matcha; their behaviors were observed. Similarly, 19 university students were given matcha cookies, and 17 were given placebo-matcha. All participants answered questionnaires after each day's practice for 15 days. While studying the university students, the researchers measured salivary α- amylase (sAA) as a stress marker to assess the stress response.

Matcha is similar to green tea but with greater quality and made into a powder from the tea leaves (Ikegaya et al. 1984). A specific amount of matcha can contain Theanine which shows great stress-reducing properties (Kakuda et al. 2000). It is important to acquire an adequate amount of matcha for stress relief because differences in quantities affect the efficiency (Anan 1974). Also, caffeine is one of the factors that antagonize the function of theanine, so there must be a precise ratio (CE/TA) between the two in order to achieve the stress-relieving quantities.

Past studies have identified that ingestion of theanine suppresses psychosocial stress in mice (Unno et al. 2013). They investigated mice under stressful conditions and measured the stress response with adrenal gland observations. The researchers found that daily doses of theanine worked to block the effects of caffeine. Therefore, the idea that theanine prevents and relieves stress through hypothalamic-pituitary-adrenal activity was supported (Unno et al. 2013). By this same notion, researchers can reasonably assume that food with great amounts of theanine, such as matcha, can be beneficial to people that don't drink green tea often due to theanine's stress relieving qualities.

Moreover, with the introduction of caffeine in goods, there must be a balance with adequate amounts of theanine (Unno et al. 2016). Since green tea has similar properties to matcha, the researchers investigated the effects of drinking green tea with lowered caffeine and enriched theanine. Unno et al. (2016) found that drinking green tea exhibits anti-stress effects, and theanine abolishes the counter-effect of caffeine. Thus, the overexpression of theanine over caffeine can promote stress relief, and this can be an effective stress reliever for individuals that don't intake green tea often.

The researchers chose the mice and university students to observe the effect of green tea and matcha on their stress levels (Unno et al. 2018). Mice models are easier to manipulate because they are more accessible and easier to handle. They tried to connect this study to humans, and they did this by studying students as well. They used a self-report system by having them fill out a questionnaire describing their stress levels. This study was helpful because it allowed for the results to generalize to a broader population.

Keiko Unno and colleagues find that giving matcha with a CE/TA ratio exceeding 10 could not rid of physiological and psychological stress in the mice model. However, a CE/TA ratio of around 2 displayed a remarkable reduction in stress in the mice model. Participants in the study consumed 4.5g of a test or placebo matcha daily; the CE/TA ratio was 1.79 in the test matcha. They found that daily ingestion of the provided match lowered their stress levels.

The findings show that CE/TA ratios of the tea are essential for the subduing of stress. The study with the university students demonstrated that a ratio of 1.79 was sufficient for the relief of stress in the morning. Theanine inhibits the excitatory influence of caffeine, and other factors that work with theanine also regulate the nervous system.

Theanine reduces the ability for glutamate, an excitatory neurotransmitter, to be formed. When studying the mice after the intake of matcha, they found that GABA, an inhibitory neurotransmitter, had increased, and glutamate was reduced. This means that the presence of theanine may play a role in the balance between GABA and glutamate.

The work of these researchers aimed to discover a connection between matcha intake and stress levels. They found that a precise amount of matcha intake can relieve stress in university students. This revelation raises questions about future potential studies, such as examining the effect of matcha under more stressful conditions. Additionally, it would be helpful to study whether lower amounts of matcha can reduce stress and have an older range of participants partake in a similar study.

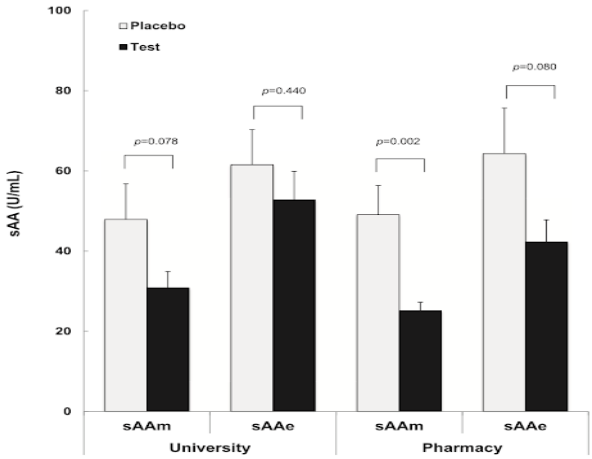


Figure 1: The graph above shows the sAA (salivary α amylase), which was used to measure stress levels, as a function of the two study groups, university and pharmacy students. The white bars are the placebo group and the black bar shows the experimental (matcha test) group. The sAAm and sAAe aim to express the salivary α amylase in the morning and evening, respectively.

College and COVID-19: An unprecedented learning experience

Nila Dilosa
Lake Forest College
Lake Forest, Illinois 60045

In the middle of the second semester of my first year of college, the unthinkable happened. It was a situation I could've never dreamt of in my wildest dreams, one that would shift the trajectory of my college career – and my life – as I knew it: a worldwide pandemic. College had dealt me a handful of surprises up until that earth-shaking March of 2020. I had made wonderful friends and gained experiences beyond what I'd ever imagined for myself. While college courses were hard and a major adjustment from high school, I felt that I was doing just fine overall. My outlook on the rest of my college journey was bursting with optimism. And then, COVID-19. Optimism was quickly replaced by fear, which was followed soon after by the transition to Zoom classes. With the start of online lectures came intense burn-out and trouble with focusing. It felt as if a rug I didn't know I was standing on had been swiftly yanked out from beneath me, sending me flying, only to inevitably crash to the ground. For me, learning during the pandemic became increasingly more difficult. But what made the whole experience bearable at its beginning was knowing that I wasn't alone in my struggle. Research has indicated that COVID-19 has not only impacted physical health but has also led to major damage to the mental health of many individuals, regardless of age. One particular study examined college students' early experiences in the face of the pandemic. In this article, researchers Madrigal and Blevins (2021) showed that the challenges most reported by students were struggles with mental health and negative emotions. In this study, negative emotions and mental health were operationally defined as severe or general feelings of anxiety, fear, sadness, depression, anger, and stress. These researchers suggested that a lack of social interaction between peers and a subsequent lack of emotional support might explain why the mental health of students has deteriorated since the start of virtual learning (Madrigal & Blevins 2021). Like the students who participated in this study, my mental well-being took a damaging blow. Being cooped up inside of my house all day while unable to spend time with my friends was difficult and only worsened the sense of isolation that I felt during the early stages of quarantine. The days began to blur, each one watered down with the same lack of sunlight and social interaction. While I have always considered myself an introvert and did sometimes enjoy getting to do college from the comfort of my bedroom, I struggled with being by myself all day more than I ever anticipated. Being alone with my thoughts day in and day out left room for worries over the health of my loved ones, especially as the holidays, usually spent in their company, passed by. These worries sometimes became overwhelming and only further distracted me from schoolwork. When this was coupled with burnout, both from school and witnessing repeated violence against my community, completing assignments began to feel like a burden. I went from someone who cared greatly about getting good grades to someone who carelessly slept the day away once classes ended. Reaching the end of the semester and the last of my final exams felt like climbing Mount Everest. Still, it ultimately felt good knowing I'd made it to the top and accomplished what had seemed impossible to me. While the majority of university students have braved and conquered the era of online courses, we are still very much in the thick of the COVID-19 pandemic. The definition of normal has shifted in meaning as we now wear masks in the classroom despite being less than 6 feet away from our classmates. To me, it feels as if we are collectively living on eggshells – a breakout of COVID-19 cases could occur at any moment, once again shifting the trajectory of our college careers. The most we can do is keep ourselves safe while trying our best to keep those around us safe as well. However, this isn't always enough, especially since not everyone is willing to abide by CDC guidelines. Overall, I find it incredible how college students have managed to survive the common stresses of academia while simultaneously dealing with immense burnout and less-than-desirable methods of learning that had to be adopted during the pandemic. To me, it indicates that we are stronger and more capable than we ever knew. Although returning to Zoom classes is the last thing most people (including myself) want, there's a spark of hope within that leads me to believe things would turn out just fine if we ever did have to return to remote learning. Though this may not be how I imagined my college experience would go, it's possessed beauty and meaningful moments in its unique way. For this, at least, I can say I'm eternally grateful.

Mind of the Damned: A Glimpse into a Psychotic Brain Portrayed in Film

Ilona Sergeeva
Lake Forest College
Lake Forest, Illinois 60045

Humanity has always been fascinated by the unknown. Such phenomena include the human psyche and all of its deviations. Despite centuries of research, our brain is one of our most mysterious and incomprehensible organs. In terms of mental illnesses, the earlier schools of thought differ in treatments with violent methods, such as lobotomy, and a more empathetic view, aimed at understanding the flawed mechanism. This placement of things provides an excellent plot for filmmakers and producers. In recent decades, films about mental illnesses have attracted more and more audiences with their thrilling mysteries and spectacular cinematography. One such piece is *Shutter Island* (2010), directed by Martin Scorsese and written by Laeta Kalogridis. The mystery thriller, based on Dennis Lehane's novel *Shutter Island* (2003), depicts an investigation led by Teddy Daniels, played by Leonardo DiCaprio, which takes place in a mental institution for the criminally insane, Ashecliffe (IMDB.com). The disappearance of one of the patients is a mystery for the U.S. Marshal and his partner, Chuck Aule, due to the fact that the institution is located on an inescapable island. As their investigation goes on, Teddy discovers the pieces to many conspiracies regarding the true nature of the institution and the patient's disappearance. The ending contains one of the most unexpected plot twists in cinematic history when it is uncovered that Teddy is a patient of Ashecliffe himself, and the investigation was all a well-thought-out role play to help him separate his delusions from a painful reality. Teddy Daniels, a hero of his fantasy, tries to uncover the mystery of the disappearance of Rachel Solando, a patient who drowned her three children, which caused her to end up in Ashecliffe. Teddy unravels the tangle of mysteries as he questions the nurses, patients, and the lead psychiatrist, Dr. Cawley. The strange events begin when he starts to hallucinate the tragedy of his past. Dolores, his wife who died in an apartment fire, facilitates his conspiracies against Ashecliffe as he sees her in his dreams. The more steps he seems to take, the more sinister secrets pile up. The audience is watching the events unfold through Teddy's eyes, and are led to believe what Teddy believes. Siding with his gut, the protagonist is certain that he was manipulated into investigating this case and was about to be used for the psychiatrists' alleged "bizarre brain experiments" along with Chuck. The climax of the movie shifts the past meaning of the plot in a completely different direction. It is revealed that Teddy Daniels is a former U.S. Marshal whose real name is Andrew Laeddis. He is a highly delusional and violent patient of Ashecliffe who created this alternate reality and persona as a method of psychological protection. His inability to accept his tragic crime causes him to dive into the world of delusions where he is a hero. After World War II, he began heavily drinking and did not notice that his wife, Dolores, was going insane. After Dolores drowned their three kids, he was responsible for shooting her to set her free from her internal suffering. The aspects of his trauma were carefully scattered throughout his fantasy. Dr. Cawley planned out the extreme roleplay where Andrew can live in his fantasy as Teddy Daniels and, over time, realize how unrealistic it really is. Chuck Aule turned out to be Andrew's primary psychiatrist, Dr. Lester Sheehan. Due to the nature of Andrew's aggression, his last resort is getting lobotomized, and Dr. Cawley did everything to prevent that. A traumatic event can have different influences on a variety of aspects of a person's life. A study of the effects of trauma on proneness to psychosis done by Spauwen *et al.* (2006) found that 17.5% of the individuals in a large adolescent sample reported having experienced at least one psychotic symptom (i.e. hallucinations), concluding that exposure to a traumatic event has a positive correlation with psychosis proneness. Andrew carried the blame for the death of his wife and kids on his shoulders to the point when it became unbearable. Knowing he could have saved his children if only he noticed his wife's bizarre and suicidal behavior makes it impossible for him to continue living as Andrew Laeddis, thus leading to his psychotic delusion and loss of touch with reality. The cinematography contains big, yet easy-to-miss hints throughout the movie that insinuates the possibility of things being not exactly what they seem. For instance, there is important symbolism behind the use of fire and water. The very first scene of the film is the main character Teddy, or Andrew, feeling seasick on a boat as he and Chuck are on their way to the island (*Shutter Island*, 0:01:11). His dislike of water is not just a simple proneness to seasickness, since similar moments arise later in the movie. In a later scene where Teddy and Chuck are interviewing the patients about Rachel Solando, one of them asks Chuck to bring her a glass of water. She uses this moment to grab Teddy's notebook and write the word "Run" on one of the pages (*Shutter Island*, 0:37:18). However, the more interesting part of this scene is when the patient actually grabs the glass of water and brings it to her lips. There is a split second where it is evident that there is nothing in her hand (*Shutter Island*, 0:37:37). This hint is very obvious, yet fairly unnoticeable. A plausible explanation for this motif of water is Andrew associating it with Dolores drowning their children. He does everything not to be Andrew and blocks out any memory of this reality. In contrast, every scene that contains his dreams or delusions always includes fire in the form of matches, fireplaces, etc. It is not a question that Andrew is intelligent and these defense mechanisms are helping him move on. Nonetheless, is he able to accept his reality? The shocking plot twist is followed by a final scene of Andrew and Dr. Sheehan sitting on the steps of the entrance to one of the wards. A storm that was present throughout the majority of the film has died down, correlating with the peaceful end to Andrew's delusions. Nevertheless, the audience receives one last jaw-dropping twist as Andrew refers to Dr. Sheehan as Chuck and regresses to questioning their next moves of getting off the island. Dr. Sheehan signals to Dr. Cawley, who is standing in the distance, implying that their hard work with attempting to save Andrew has failed after all. As the camera shifts to a nurse walking towards Andrew with an ice pick for the lobotomy, Andrew leans over to Dr. Sheehan and asks "This place makes me wonder which would be worse… To live as a monster, or to die as a good man? (*Shutter Island*, 2:09:11)" With those final words, Andrew gets up and walks over to his inescapable finish line of getting lobotomized. At first glance, it may seem as though Andrew has regressed back to his made-up persona; however, this last line sends a message from Andrew to Dr. Sheehan and the audience, that this is an act. He is still aware of the reality, however, he cannot continue living as Andrew, the 'monster' who feels responsible for the deaths of his wife and children, and is choosing to 'die' as Teddy, the hero he so badly wanted to be. Overall, this movie does a spectacular job of enlightening the audience about trauma-induced delusional disorders and psychosis. Throughout most of the film, the viewers see Teddy as a sane man as they observe him work his investigation. If the audience went into the movie knowing that Andrew is rather insane, they most likely would not have seen him the same. His conspiracies would have been a part of his delusions and his high guard would be backed up by his inability to accept the truth. The audience uncovers the truth along with Andrew and gets a clear look into his past, shining a light on the question "Who can we blame?". This kind of screenplay gives us an empathetic view of the main character. His history and his struggles are very raw and tragic, but also explain his wounded psyche. Dr. Cawley's approach educated the public about the power of understanding the patient, instead of jumping to extreme methods as a first response. Although the story of Andrew Laeddis did not contain a happy ending, it is only proof that even damned minds reside in human beings that deserve to be remembered as they too have lived, loved, and laughed.

Cane toad invasion: documentary review

Iman Shepard
Lake Forest College
Lake Forest, Illinois 60045

You see an ominous figure lurking in the shadows of a bush outside your front yard; cognitive dissonance overcomes you as you curiously, but cautiously, approach the frightening entity. As epinephrine is released in your brain, fear builds in your body and your subconscious considers flight or fight; your hand lifts the bushes and… a wide, stumpy, forest green creature with large brown eyes stares back at you. You have found yet another cane toad in your yard. *Cane Toads: An Unnatural History* (1988) is an iconic documentary directed by Mark Lewis that explores the introduction and invasion of *Rhinella marina*, more commonly known as cane toads, along the coast of eastern Australia. Cane toads are native to Central America, where they are vital contributors to the ecosystem by controlling insect populations. Cane toads were purposefully released by humans with the hope of controlling *Dermolepida albohirtum* (commonly known as cane beetles) because cane beetles were decimating sugar cane populations and subsequently decreasing crop yield. This film explores the scientific aspects of the cane toad both anatomically and ecologically and addresses the cultural impacts of cane toads in Australia. Comedy is uncommonly used in the scientific documentary genre; the unique aspects of this film are its use of satire through audio, visuals, and jump cut scenes. The film dives into the toxins and unique features of the cane toad that allow it to protect itself, reproduce, and spread in Australia. Cane toads have parotoid glands located on each shoulder that release a potent, fast-acting toxin known as bufotoxin. Bufotoxin is made up of adrenaline, serotonin, and other compounds that are found in bodily tissue. When ingested, bufotoxin can cause tachycardia, increased salivation, convulsions, paralysis, and even death. The release of this toxin is caused when significant pressure is placed on the cane toad's glands, causing them to burst; a thick, white, opaque substance ruptures from the glands. This would typically occur if another organism were attempting to eat or grab the cane toad with their mouth, which illuminates why the toxin must be ingested to be effective. This protective mechanism is not a common occurrence recognized by native Australian animals and is part of the reason cane toads are extremely effective at surviving in Australia. Additionally, cane toads are r-selected species, which means they produce a large amount of offspring with limited parental investment. R-selected populations are controlled by a high death rate in the early stages of the species' life cycle—few individuals make it to adulthood. In Australia, the cane toads have no natural predators and are prolific breeders. Females deposit between 8,000 and 30,000 eggs one to two times a year. Rather than a large die-off early in life, many of the juvenile cane toads reach adulthood because of the lack of predators or environmental pressures placed on them. Cane toads are a successful invasive species because of their unique characteristics—they have mechanisms to protect themselves from predators and are r-selected species. Australia is an ideal environment for a cane toad invasion because the native species are not adept in dealing with cane toads. The impacts of cane toads stretch beyond the ecological and into the cultural setting of Australia as well. Along with being informative, *Cane Toads* considers the cultural impact of the toads on affected Australians. The Australians featured in the film had a wide variety of reactions to the invasion of the toads, ranging from anger to fondness. Many find the cane toads endearing; the film features an interview of Councilor Max Ackland, who recalls attempting to resurrect a statue of a cane toad in the middle of his town (Mulgrave) outside a government building. Additionally, the film included an interview with Elvie Grieg, a resident of Redcliffe, in which she fondly describes viewing and treating the cane toads akin to pets by feeding them cat food and providing them with shelter. Despite this positive response to the cane toad invasion, not every Australian shares in these sentiments. A vlog created by Brent Vincent, a resident of Cairns, shows him filming himself while purposefully running over cane toads on the road; he finds himself empowered by directly helping with the pest control of the cane toads. He expresses frustration with the government reaction and discusses his vexation with the toads' invasion. Along with contentment and anger, there is also an element of fear surrounding the cane toad invasion. The fear of cane toads especially concerns the safety of pets and young children. If either is left unaccompanied and happens to disturb a cane toad enough to trigger a release of bufotoxin, there would be serious (and potentially deadly) consequences. The film depicts a satirical scene of this occurrence in which a mother takes a phone call inside and leaves her child unattended; the camera angles, jump cuts, and suspenseful music make a cane toad appear to be stalking and trying to harm the child. This unrealistic scene adds an element of humor to the film while also addressing the real fear parents and pet owners face regarding the invasion. Ultimately, the comical aspect of the film that is a vital part of its charm and differentiates this documentary from others of similar subject matters. The satire is the main draw of the film for me. I would rate this film 5 out of 5 stars because of its flawless combination of humor and science; this is a difficult feat to overcome, but I learned about invasion ecology, Australian ecosystems, and cane toad anatomy in a fun and engaging way. I recommend this movie to any non-specialist looking for a fun documentary or for anyone interested in invasion ecology.

Drosophila Meeting Report

Mathieu Norrcross
Lake Forest College
Lake Forest, Illinois 60045

The 62nd *Drosophila* conference tied different research themes together using the model organism *Drosophila*. This talk was attended by professors and students from across the world, with my attendance following my research using *Drosophila* looking at iron supplementation effects on fertility and developmental timing. I attended talks centered around topics discussed in class and ones I found personally interesting. The conference consisted of five talks, four posters, an undergraduate platform, and interviews/questions with presenters. The topics/themes I focused on were diversity in STEM, factors affecting oogenesis & ovarian cell migration, stem cells in organogenesis, bacteria in the gut microbiome, dealing with physical/mental traumas, and new databases determining gene expression patterns. All of the talks throughout this conference demonstrated the depth of research that can be performed with *Drosophila*. Each talks presented potential uses for *Drosophila* in solving complex scientific research questions. Many of the presentations discussed novel findings and methodologies that could help inspire an informed scientific audience to pursue the research further or incorporate these novel methodologies into other experiments. The general format of the conference consisted of live presentations and poster halls. Talks lasted for 15-30 minutes, while posters were open for two weeks. The theme of diversity in STEM was demonstrated in the undergraduate platform session, where students showed the diversity in research using *Drosophila*. There were a total of four undergraduate researchers from across the country presenting research on everything from gene regulation of histones to basolateral polarity and Bisphenol A's role in neurodevelopment. All of these talks demonstrated the extreme breadth of research using *Drosophila* to study all sorts of mechanisms within biology and beyond. The second talk surrounding diversity was titled *Managerial Engagement to Promote DEI in STEM* by Mala Htun. This talk addressed the promotion of diversity in STEM, stating current procedures like harassment training are unproductive in promoting diversity and inclusion. Htun instead proposed three projects, including institutional change in faculty hiring processes, team projects focused around solving issues of DEI, and the creation of a curriculum designed to fight against microaggressions and sexual assault in STEM. Another theme focused on factors affecting oogenesis and ovarian cell migration in *Drosophila*. The first talk titled *Obesity and Drosophila Oogenesis* asked the question on how obesity affects oogenesis. Flies were fed a high sugar diet (HSD) causing them to become obese, which lead to increases in germline cyst and vitellogenic follicle death along with decreased egg production and quality. Obesity alone, however, was not sufficient to elicit these affects. Knockouts of lipase Brummer showed that no stage of oogenesis was affected by obesity alone, and only egg production was decreased, indicating that an HSD has other mechanisms that led to detrimental oogenetic outcomes. How oogenesis was affected by different levels of chronic temperature stress (18, 25, and 29 C) was discussed in a separate poster presentation. Both cold and hot stress saw a decrease in egg laying, with the heat effect being a reversible phenomenon. In cold temperatures, GSCs underwent more cell maintenance, while more GSCs died in heat. Heat also showed increased follicle death and decreased hatching rates. The effect of Ferritin on Ovarian cell migration was presented in a poster by Susan Afolabi. This talk was particularly interesting because Ferritin is an important player in iron sequestration, which is important to my previous research. Ferritin is an important downstream molecule in migration pathways and RNAi was used to determine if Ferritin is important to border cell migration. Results indicate Ferritin is important for border cell migration as misexpression delayed migration. I interviewed Susan and asked her about the potential of feeding flies a high/low iron diet, which I performed in my study. Susan discussed how oversupplementing iron would likely disable Ferritin, affecting cell migration negatively. I also asked her to clarify results she found, and she stated she wanted to perform further imaging studies to help clarify phenomenona demonstrated in her results.

The final developmental biology session I attended was a talk titled *Positioning a stem cell niche during organogenesis*, which analyzed how anterior niche assembly was regulated in gonads. Through lineage tracing and knockout experiments, the visceral mesoderm was determined to be important for niche placement as it used paracrine signals (Slit & FGF) to influence E-cadherin polarity and expression of islet transcription factors necessary for niche assembly in the embryo. All of this is dependent on the proximity of the niche to the visceral mesoderm surrounding the gut of the *Drosophila*. On the topic of the *Drosophila* gut, I attended a poster session discussing the gut bacteria defending against invasive microbes. The study looked at interactions between gut bacteria *Lactobacillus plantarum* (Lp) and *Escherichia coli Nissle*. Plating the bacteria together showed that Lp inhibits EcN due to Lp's antimicrobial secondary metabolites. Looking at the bacteria interactions in vivo in *Drosophila* showed similar inhibition of EcN, demonstrating gut bacteria possess a defense mechanism against invaders. Another theme surrounding talks I attended is centered around repairing physical/mental damages. The repairing of physical damages was discussed by the talk *Cell wound repair: Dealing with life's daily trauma*. The presentation wounded *Drosophila* in multiple areas and used microarrays and FlyTrap screens to isolate RNA 5 minutes and 30 minutes following the wound. No transcriptional response was found after 5 minutes, but 30 minutes showed 80 upregulated and 172 downregulated genes. The top 16 up and downregulated genes were shown to be important to wound healing processes such as wound expansion, and accumulation of internal actins through RNAi studies. The study was able to quantify the genes upregulated and downregulated during wound closure. I also look at a poster titled *The psychedelic drug psilocybin has long lasting antidepressant-like effects in male Drosophila*, which demonstrated the effectiveness of psilocybin as an antidepressant by analyzing flies using Forced Swim Test (FST) and Drosophila activity monitoring system (DAMS). Results suggest pulsed dosing (1x) of psilocybin leads to increased mobility function, while constant dosing leads to reduced function, demonstrating the drug's potential use for combatting depression. The final talk focused on a new methodology, particularly a database of gene expression surrounding the *Drosophila* embryo. There are many spatial/temporal images of gene expression in *Drosophila* embryos. The presenters have been developing an imaging database that is easily accessible to researchers. This would help approximate the dorsal gradient and WntD mutant phenotype in the developing *Drosophila* accurately through an easily accessible database built from JSON trees. This conference provides valuable insight for future scientific research. There were many talks focusing on current important scientific topics such as the effects of obesity and the importance of the gut microbiome, among other topics. There were other talks that focused on other issues within STEM, such as the lack of diversity, and how we should combat these issues. The talks presented future studies that should be investigated further in order to make progress on the many issues the scientific community faces. For this reason alone, undergraduates like myself should be required to attend this conference. The conference introduced me to what sorts of research is being done on the major biological issues of today while also introducing me to new issues I originally didn't know about. The conference also introduced me to new methodologies I could incorporate into future research designs while also allowing me to work on talking to professionals. Attending networking events and interviewing participants helped me think critically about the materials being presented, leading to the development of critical thinking questions. All of these skills are important for future interactions within the scientific community and for future success in academia, which is yet another reason more undergraduate students should attend events like these. Personally, the conference provided me with new knowledge about topics I'm personally passionate about, while also reinforcing topics discussed in class, which made the talk a scientifically educational event. The talks also presented research topics that I would be interested in joining potentially in the future, while helping me understand areas I need to work on when networking and asking critical thinking questions.

Fly Meeting Report

Hannah Kobienia
Lake Forest College
Lake Forest, Illinois 60045

Introduction

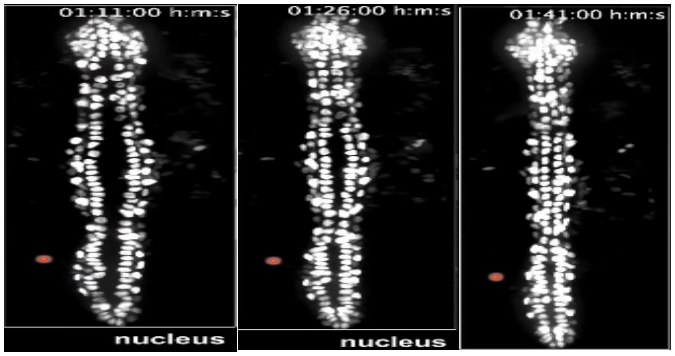
This virtual conference covered diverse biology topics ranging from developmental concepts, cellular and molecular mechanisms, neuroscience pathologies, and even biomedical aspects that could lead to new developments in prospective clinical treatments. Undergraduate and graduate students were given the opportunity to listen, observe, and learn from the researchers that had made biological discoveries/advancements, and a select few were even given the opportunity to speak and present research that they conducted themselves. Other attendees included postdocs, faculty, and principal investigators. The purpose of this conference was to update the science community on new discoveries from research conducted on the *Drosophila melanogaster*, as well as provide learning experiences about the research side of the STEM field.

One example of a learning experience offered through this conference was the Undergraduate Platform. This was one of my favorite sessions from the conference. The ability to look at other researchers around my age that are using techniques I have learned about, such as ChIP-seq and CRISPR, as well as perform research remotely, which I have experience with because of my Biochemistry course from last semester.

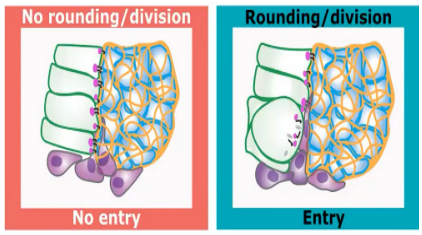
Another learning experience was the Tools and Techniques webinar, which went through investigative research techniques that study a wide range of biological mechanisms and structures. Some of the most interesting techniques would have been on high-speed 3D microscopy advances that were made by using a light sheet and apparatus with the galvo mirror. The SCAPE microscopy technique (Swept Confocally-Aligned Planar Excitation) was the real new advancement which allows for the observation of a single oblique plane of an object in real time. It is important because it is the only light microscopy system that allows for movement of only the plane without a hold on either the mirror or organism, and therefore a more comprehensive video can be observed. Another technique that piqued my interest and will play a largely important role in developmental biology in the future is going to be the connectome analysis tool: neuPrint. It will allow researchers to find neurons that are involved in certain pathways, or determine neurons involved in certain pathways that affect a certain place in the brain. This tool would be extremely helpful in determining where neurons are located within the embryo and their migration pathways during embryogenesis and embryo development.

Presentations

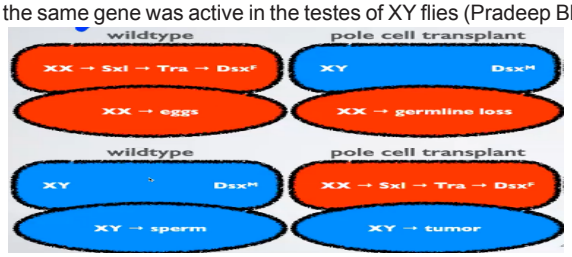
The first live talk I attended was given by Negar Balaghi. Her research investigated the mostly unknown underlying causes of congenital heart defects through cellular migration and alternating polarity of myosin in Drosophila. Their lab located the cardio blasts in the embryo as they migrated to the dorsal midline to form a tube. They used watershed algorithm imaging to track the migration of the myosin inside the nucleus of the cardio blasts as they oscillated forwards according to their dipoles. When they disrupted myosin motor activity, a higher variability in oscillation and contralateral communication between cardio blasts was observed.



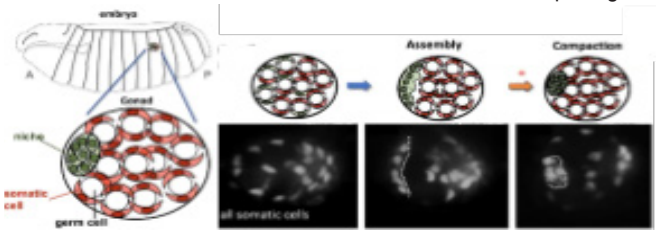
The second live talk I attended was done by Maria Akhmanova and covered the topic of macrophage invasion of embryonic tissues after epithelial cell division. They started by identifying the macrophages path as it penetrates and parts the ectoderm from the mesoderm, which companies miotic rounding and division of the surrounding epithelial cells. Division profiles determined that macrophage entry was always and only occurring at the same time as mitotic rounding and/or division. So, if the ectodermal rounding/division was required (necessary), which is typical of the "Lose It" type of developmental experiments. They ended up determining that focal adhesions disappear during cleavage events in the meso-ectoderm cell layers, further determined that mitotic rounding is indeed what enables macrophage tissue infiltration.



The third live talk I attended was "Zooming in on gonadogenesis." This talk was given by Brian Oliver of NIH and researched the sex transformations that occur when inserting male germ cells into a female, and vice versa. In addition to the transplanting of genes into the opposite sex, this experiment also investigated how important sex gene locations are. The results from this experiment found a gene that was active in the abdomen of an XX fly, while the same gene was active in the testes of XY flies (Pradeep Bhaskar).

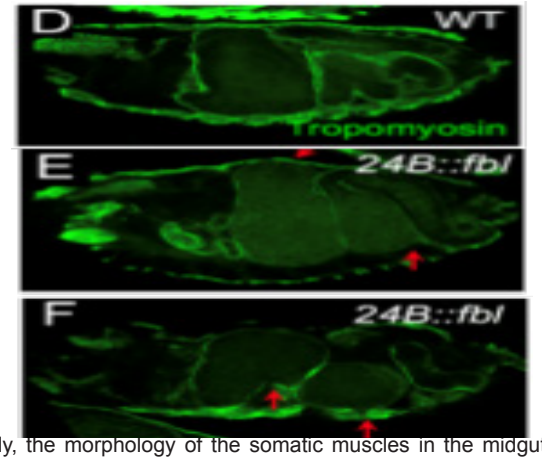


Their research from the germ cell insertion resulted in data that found a loss in the germline cells when a WT XX germ cell was inserted into a XY fly, and that the opposite resulted in a tumor. The fourth live talk I attended was given by Lauren Anllo. She spoke about the location of the stem cell niche in male flies which resides in the apex of the spiraled testes. They then looked at the testes during embryogenesis and found that niche cells seemed to be covered in visceral mesoderm (Vm). They then removed the Vm and observed irregular placement of the stem cells in the testes and determined that without the Vm, E-cadherin does not receive polarization and therefore cannot create the proper cell to cell connections in the stem cell niche in male Drosophila gonads.



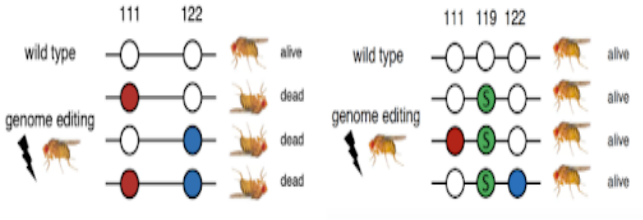
Posters

The first poster presentation I attended was given by one of the two presenters (the other had internet issues). Bronwyn Tollefson, of University of St. Thomas (UST) in Minnesota, investigated the ECM protein, Fibulin, and its role in structure development during embryogenesis. The first responsibility of this protein is to aid in corrected patterning of somatic muscles. When fibulin was knocked out through RNAi, abnormal fusions and spacings occurred between different ventral-longitudinal somatic muscle segments.



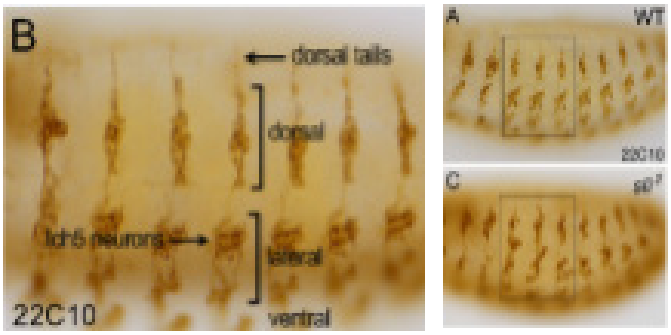
Additionally, the morphology of the somatic muscles in the midgut were defected when the embryos were altered to over-express fibulin (**D-F**).

The second poster presentation I attended was given by Arya Rao of Columbia University in New York. She had conducted research on the genetic basis behind the cardiac glycoside (CGs) resistance in WT Drosophila. She identified two amino acid sites with adaptive substitutions (mutations) that allowed these insects to inhibit the toxicity of CGs within their systems by inhibiting the Na+/K+ ATPase. These sites were 111, 122, and eventually 119. Genetic engineering of the first two amino acid sites in combinations, resulted in the death of all the WT flies.



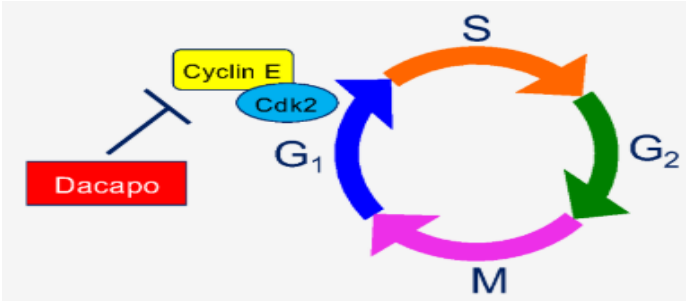
When the third site was then identified (119), it was determined to correlate with sites 111 and 122. When a substitution was inserted into this site, the WT flies were instead able to live through the mediation of the CGs toxicity.

The third poster presentation I attended was on the mechanism of slit in the PNS of *Drosophila*. This poster was presented by Maria A. Pizarro Salazar of UST. She researched the effect of the slit ligand in the PNS based off previous research on the function and importance of this ligand in the CNS. Through staining, it was found and over-expressed in glial cells, the ectoderm, and Ich5 neurons; specifically, their axons and muscle attachment sites of dorsal tails (**A, B**).



When over-expressed, slit in glial cells caused defects in abnormal axonal branching, loss of dorsal neurons, and mislocated neurons (**C**). In the ectoderm, the dorsal tail development was hindered, and resulted in a shortened dorsal tail. When over-expressed in PNS neurons, slit resulted in the incorrect localization of neurons in the embryo through 22C10 stains.

The fourth poster presentation I attended was given by another researcher fellow named Kaitlyn Solberg from UST. Her research attempted to identify the role of protein *dacapo* in the development of PNS neurons. They were found to reside in the abdominal segments of the flies. They then used its known function as a cyclin-dependent kinase (Cdk) inhibitor. This means it inhibits a subunit of Cdk's which normally activates the cyclin pathway. *Dacapo* was also found to play a role in determining whether a cell will continue to divide or if it will terminally differentiate.



Conclusion

I connected these posters to the lectures that involved the ECM and how influential the concentration of many cellular molecules and structures are to proper development, as well as the importance of embryonic patterning for proper physical development. I connected the live presentations to the themes from lecture that we have learned about, such as gonadogenesis, embryo cell migration and patterning, and the crucial proteins in the cell and the ECM that drive proper development. All these talks resulted in discoveries that further reiterated the importance of these themes in developmental biology. These discoveries will aid scientists in continuing to better understand *Drosophila* genes in the hopes that their research can help learn more about specific disease pathologies in humans. During these presentations I spoke and formed connections with researchers from UST, such as Bronwyn Tollefson, Maria Pizarro Salazar, and Kaitlyn Solberg. I also have emailed and connected with Arya Rao on LinkedIn to discuss her research more in-depth, and hopefully form a professional relationship with her. It is my belief that undergraduate students should attend conferences like this one, because it provided us with an avenue to further understand what research in the world after education consists of. It also allowed us to network with individuals in the STEM field, and make connections between material covered in lecture and how these themes are used in research today. After attending this conference, I feel that I have a better understanding of morphogenesis gradients, gonadogenesis, and the importance of embryo migration patterns.

Peroxisome-Mitochondria Interplay in Adrenoleukodystrophy: Effect of Diminished Acetyl-CoA β-Oxidation Product

Grace E. Dodis, Dana S. Midani, and Courtney P. Stickling
Lake Forest College
Lake Forest, Illinois 60045

Summary
X-Linked adrenoleukodystrophy (X-ALD) results from a mutation in the *ABCD1* gene. This gene codes for a protein that imports very-long-chain fatty acids (VLCFAs) into the peroxisome for degradation via β-oxidation (β-O). Previous research has labeled the loss of function of the ABCD1 protein, and thus, the build-up of VLCFAs as the primary cause of neurodegeneration. However, VLCFA levels do not correlate with disease severity. Therefore, we aim to investigate the downstream effects of a lack of β-O acetyl-CoA (a-CA) product and how this may impact functioning of specific molecules involved in peroxisome-mitochondria interaction. The goal of the present proposal is to investigate how the products of VLCFA β-O are shuttled to the mitochondria from the peroxisome, how lack of product affects the functioning of metabolic and transport proteins Cit2, Cat2, and Pex34, and whether the function of these proteins is altered in X-ALD. We will first examine the relationship between behavioral and cellular markers of disease severity and peroxisomal a-CA levels, both *in vivo* with an established *Drosophila* ALD model and *in vitro* with human fibroblast cell cultures. Peroxisomal a-CA is hypothesized to negatively correlate with behavioral and cellular markers of disease severity in *Drosophila* and human fibroblasts. Next, we will focus our attention on Cit2 and Cat2 functioning and their relationship with diminished a-CA by creating fibroblast groups with Cit2 and Cat2 genes knocked out. We will then measure fatty acid β-O in the peroxisome. Mitochondrial integrity (MI) will also be measured via CO2 levels and mitochondrial membrane potential (MMP). If lack of a-CA impairs Cit2 and Cat2, mitochondrial function will be impaired and further contribute to disease phenotype. Finally, we will look to establish a correlational relationship between Pex34 gene silencing and molecular function/severity in ALD fibroblasts by measuring peroxisomal a-CA levels in a cell culture of human fibroblasts. We aim to show that a-CA is diminished in ALD-Pex34 knockdowns (KD) and that this causes a chain reaction leading to mitochondrial dysfunction and overall neurodegeneration.

Background
X-linked adrenoleukodystrophy (X-ALD) is a progressive and often fatal neurodegenerative disease that affects the white matter of the brain and spinal cord (Gordon et al. 2018). The cause of X-ALD is an autosomal recessive mutation in the *ABCD1* gene which codes for the ABCD1 transporter protein residing in the peroxisomal membrane (Gordon et al. 2018). ABCD1 is responsible for the import of very-long-chain fatty acids (VLCFAs) into the peroxisome for degradation via β-O (Gordon et al. 2018; Kawaguchi et al. 2018). However, the mutations that result in X-ALD are loss of function, and as a result VLCFAs, are not imported into the peroxisome for degradation, resulting in several toxic effects (Kawaguchi et al. 2018). First, due to a lack of VLCFAs being degraded, there a lack of product that would normally be produced by β-O of VLCFAs, which include a-CA (a-CA) and medium-chain fatty acids (MCFAs) (Gordon et al. 2018). Additionally, the accumulation of VLCFAs in fibroblasts of X-ALD patients is a well-known biomarker for this disease and thought to be the first sign of a mutated ABCD1, since they cannot enter the peroxisome to be degraded and have been shown to be substrates for further elongation (Ofman et al. 2010). However, the levels of VLCFAs in cells and tissues do not correlate with phenotype nor with disease severity (Gordon et al. 2018; Stradomska and Tylki-Szymańska 2018; Schirinzi et al. 2019). Therefore, a novel hypothesis of the downstream effects of mutated ABCD1 is that the lack of product of VLCFA peroxisomal β-O is causative of disease rather than a buildup of the VLCFA/VLCFA-CoA substrate (Gordon et al. 2018; Stradomska and Tylki-Szymańska 2018; Schirinzi et al. 2019). Additionally, it has been shown that β-O of fatty acids in the mitochondria and function of the citric acid cycle (CAC) is coupled with β-O in the peroxisome (Violante et al. 2013; Violante et al. 2019; van Roermund et al. 1995; Shai et al. 2018). Indeed, a-CA, a product of peroxisomal β-O, has been shown to be shuttled from the peroxisome to the mitochondria. This is important given the fact that the peroxisomal membrane is impermeable to a-CA and NAD(H), another product of β-O (van Roermund et al. 1995). One of the pathways implicated in shuttling a-CA from the peroxisome to the mitochondria involves conversion of a-CA into citrate by citrate synthase (Cit2) (van Roermund et al. 1995; Visser et al. 2007) in the peroxisome; another pathway involves the conversion of a-CA to acetylcarnitine by carnitine transferase (Cat2) (van Roermund et al. 1995; Visser et al. 2007) in the peroxisome. Then, both citrate and acetylcarnitine seem to be shuttled to the mitochondria by citrate acetyl transferase protein (van Roermund et al. 1995; Visser et al. 2007) or the glyoxylate cycle where they can be converted back into a-CA (Visser et al. 2007). It remains an open question whether citrate and acetylcarnitine are lacking in X-ALD patients because there is a lack of a-CA product from β-O of VLCFAs not occurring. Additionally, Pex34 is a peroxisome-mitochondria tethering protein found on the peroxisomal membrane (Shai et al. 2018). Pex34 has been previously found to be involved in transporting a-CA from the peroxisome to the mitochondria (Shai et al. 2018). However, further investigation is needed to assess how these pathways and contact proteins interact as well as how a lack of product might affect their function. Lack of product from peroxisomal β-O of VLCFAs might cause downstream damage to the mitochondria, among other possibly impacted organelles and cellular functions. The goal of the present proposal is to investigate how the products of VLCFA β-O are shuttled to the mitochondria, the implication of a lack of product on the functioning of Cit2, Cat2, and Pex34, and how the functioning of these proteins are affected in X-ALD.

Significance
Identifying the features of the pathway of ABCD1 dysfunction and alterations in peroxisomal β-O dysfunction in ALD sheds light on the key mechanistic properties that can be targets for future therapeutics and interventions. Furthermore, investigating the role that product loss plays in disease severity would indicate that therapeutics focused on restoring this product loss should be investigated.

Specific Aims
Aim 1: Is lack of a-CA product causative of ALD phenotypic severity?
The goal of this aim is to directly connect lack of peroxisomal product with ALD phenotypes, as a lack of β-O product a-CA has been implied in previous studies to be causative of disease (Gordon et al. 2018); however, a dose-dependent relationship between levels of a-CA product and disease severity has yet to be characterized. Firstly, a dose-dependent relationship between the levels of a-CA product and behavioral and cellular measures of disease severity will be assessed. Disease severity and phenotype will be measured by locomotor activity and retinal degeneration in the *bgm dbb Drosophila* model of ALD (Gordon et al. 2018) as well as peroxisomal a-CA levels and MI in both *bgm dbb Drosophila* and human fibroblasts derived from ALD patients and healthy controls (Morita et al. 2016; Ofman et al. 2010). It is hypothesized that levels of peroxisomal a-CA will correlate with disease severity such that the lower the levels of peroxisomal a-CA, the more severe the disease as measured behaviorally and cellularly. Then, the rescue effects of introduction of MCFA will be assessed both in *Drosophila* and human fibroblasts. This will be done by measuring if introduction of MCFA reduces severity of neurodegeneration, returns a-CA levels to normal, and prevents loss of mitochondrial integrity. Additionally, the level of rescue (assessed by measuring mitochondrial integrity) will be correlated to the level of peroxisomal a-CA restoration to see if there is a correlation between the two. This might suggest that MCFA diet supplementation has rescue effects at least partly due to providing β-O product. It is hypothesized that the magnitude of rescue by treatment with MCFA, measured by mitochondrial integrity, will correlate restoration of peroxisomal a-CA levels, such that the greater the restoration of peroxisomal a-CA, the greater the rescue.
Aim 2: Does lack of a-CA product result in impaired Cit2 and Cat2 functioning or mitochondrial integrity?
The objective of this aim is to explore whether a lack of a-CA product impairs Cit2 and Cat2 functioning, and how this impairment may impact MI. Cit2 functions by converting a-CA into citrate before being shuttled from the peroxisome to the mitochondria (van Roermund et al. 1995). Cat2 is involved in converting a-CA to acetylcarnitine before being transferred to the mitochondria via citrate acetyl transferase protein (van Roermund et al. 1995; Visser et al. 2007). Because VLCFA build-up does not seem to be causative of disease severity (Gordon et al. 2018; Stradomska et al. 2009; Schirinzi

et al. 2019), we will be investigating the possible correlation between lack of a-CA product and Cit2/ Cat2 functioning. There will be five groups of fibroblasts: healthy controls, ALD, KD of Cit2, KD of Cat2, and double KD of Cit2 and Cat2. A-CA levels will be measured in each of the cells after giving pyrene□C12:0 since pyrene□C12:0 is only broken down in the peroxisome. This allows us to measure the peroxisomal activity for fatty acid β-O. Mitochondrial functioning will also be measured by assessing levels of CO2 as well as MMP. Cit2 and Cat2 convert a-CA into citrate and acetylcarnitine, respectively (van Roermund et al. 1995). If there is a lack of a-CA product in ALD, then Cit2 and Cat2 may be impaired in their conversion functioning, further impairing mitochondrial function (van Roermund et al. 1995).

Aim 3: Does Pex34 silencing correlate with a lack of a-CA product, mitochondrial dysfunction, and phenotypic severity?

The objective of this aim is to investigate whether silencing Pex34, a protein involved in transporting a-CA from the peroxisome to the mitochondria (Shai et al. 2018), results in a lack of a-CA product from VLCFA β-O. There will be four groups of fibroblasts: healthy control cells, ALD cells, ALD + Pex34 silenced cells, and healthy + Pex34 silenced cells. We will determine what silencing of Pex34 in the presence or absence of a functional ABCD1 transporter does to levels of a-CA as well as assess MI by measuring levels of CO2 and MMP. We will then compare the groups of cells to see if ALD fibroblasts have similar a-CA levels and MI as healthy cells with Pex34 KD. If the a-CA levels and MI are found to be comparable, this could indicate that Pex34 may be impaired in ALD fibroblasts due to a lack of a-CA product from β-O of VLCFAs in the peroxisome.

Design and Methods

Aim 1: Is lack of a-CA product directly related ALD phenotypic severity?

Rationale. If a lack of product is causative of disease (Gordon et al. 2018), then a direct link between the level of a-CA in the peroxisome and ALD disease severity should exist. Despite previous studies indicating that rescue effects of introduction to MCFA was due to restoration of a-CA product, this was not directly measured (Gordon et al. 2018). Therefore, characterizing the levels of peroxisomal a-CA might establish that a lack of product correlates with disease severity and phenotype; utilizing both *in vivo* and *in vitro* methods will strengthen these findings (Gordon et al. 2018; Morita et al. 2016). Additionally, comparing behavioral and cellular measures of disease severity before and after treatment with MCFA will isolate the effects of MCFA treatment alone on rescue of disease severity measures. This might provide evidence for a causal relationship between restoration of peroxisomal β-O product a-CA and reduction in severity of disease, further supporting that cellular damage in ALD is due to lack of the β-O product.

Methods. To see if the level of a-CA in the peroxisome correlates with disease phenotype *in vivo*, *bgm dbb* double mutant *Drosophila* (a previously established model of ALD (Gordon et al. 2018)) and WT Drosophila would be assessed for behavioral and cellular markers of disease severity as follows. First, levels of locomotion would be recorded for a behavioral marker (Gordon et al. 2018). Then, animals will then be sacrificed, and the level of retinal degeneration will be recorded a cellular marker of disease severity (Gordon et al. 2018). The rest of the bodies of the *Drosophila* will be used to make homogenized tissue samples. The samples will be assessed for levels of peroxisomal a-CA by first isolating the peroxisome by differential and density gradient diffusion (Graham 2001). Then, after isolation, the amount of a-CA will be determined using a commonly-used HPLC and ultraviolet detection procedure (Various Species Acetylcarnitine ELISA Kit). The cell cultures will also be assessed for CO2 levels following protocols outlined by Shai et al. (2018) and MMP following protocols outlined by Baarine et al. (2015). A one-way ANOVA with the one independent variable of disease type with four dependent variables that measure disease severity will be conducted to assess the differences in these measures of pathology in the flies. Specifically, the independent variable will have two levels (WT vs *bgm dbb*) and the four dependent measures of disease severity will be locomotor activity, peroxisomal acetyl Co-A, mitochondrial CO2, and MMP. Within-group comparisons might elucidate if severity of neurodegeneration is related to a-CA and mitochondrial function. Between-group comparisons would elucidate how levels of peroxisomal a-CA differ between WT *Drosophila* and the *bgm dbb* ALD model *Drosophila*. To see if peroxisomal a-CA correlates with severity of disease in human cells, human fibroblasts from ALD patients and healthy controls will be assessed for peroxisomal a-CA, mitochondrial CO2, and MMP, with the same protocols as was used with the *Drosophila* samples. A one-way ANOVA will be conducted with disease type (control vs ALD) as the independent variable, and the following three dependent variables: peroxisomal acetyl Co-A, mitochondrial CO2, and MMP. Within-group comparisons will compare levels of peroxisomal a-CA with MI to see if there is a direct interaction between the two. Between-group comparisons will compare levels of peroxisomal a-CA and MI between control and ALD fibroblasts. To expand on previous findings implicating a relief of ALD symptoms using MCFA supplementation in the *bgm dbb Drosophila* ALD model (Gordon et al. 2018), the effects of MCFA diet supplementation on levels of peroxisomal a-CA and MI will be measured. Then, rescue of disease phenotype with MCFA diet supplementation will be compared to possible restored levels of a-CA, to see if magnitude of rescue correlates with restoration of β-O product. This will be investigated *in vivo* in *Drosophila* fly models and *in vitro* in human fibroblast cell cultures, as follows. *In vivo* assessment of MCFA diet supplementation will include WT and *bgm dbb Drosophila* with three groups within each: standard diet, MCFA diet, and long-chain fatty acid (LCFA) diet. The rescue effect of diet supplementation will i assessed by measuring locomotor activity in live flies, then, after sacrifice, severity of retinal degeneration, levels of peroxisomal a-CA as previously described (Graham 2001, Various Species Acetylcarnitine ELISA Kit), and MI via levels of CO2 (Shai et al. 2018) and MMP (Baarine et al. 2015). Data analysis will include a multivariate analysis of variance (MANOVA) with two independent variables: disease type (WT or *bgm dbb Drosophila*) and diet type (standard, MCFA, LCFA), with four dependent variables: locomotor activity, peroxisomal acetyl Co-A, mitochondrial CO2, and MMP). *In vitro* assessment will involve human fibroblasts from ALD patients and healthy controls. The fibroblasts will be exposed to medium with fatty acids of differing lengths (MCFA, LCFA, standard diet), where the standard diet will consist of an array of fatty acids that represent a well-balanced human diet. The three dependent measures will be peroxisomal a-CA, measured as previously described (Graham 2001, Various Species Acetylcarnitine ELISA Kit), and MI via levels of CO2 (Shai et al. 2018) and MMP (Baarine et al. 2015). To analyze these data, a MANOVA will be run with two independent variables of disease type (ALD vs control) and diet (MCFA, LCFA, standard diet), and three dependent measures of disease severity (peroxisomal a-CA, mitochondrial CO2 levels, and MMP).

Predicted outcomes. Previous research found that diet supplementation with MCFA reduced phenotypic disease severity in *bgm dbb Drosophila*, and the authors speculated if this could be due to a restoration in β-O product that might otherwise be lacking (Gordon et al. 2018). However, an experiment relating ALD to lower levels of β-O products has yet to be done. Additionally, establishing a dose-dependent relationship between peroxisomal a-CA and level of cellular damage can extend previous findings (Gordon et al. 2018) by establishing that a direct relationship between lack of peroxisomal β-O product and disease severity exists in ALD. Severity of disease measured in vivo (locomotor activity and retinal damage of *Drosophila*) and *in vivo* (molecular functioning in cellular assays from both the *Drosophila* and human fibroblasts) in the same study will establish these findings are present both *in vivo* and *in vitro*, as they have been found in separate studies until now (Gordon et al. 2018; Ofman et al. 2010; Various Species Acetylcarnitine ELISA Kit). Then, these findings could be extended by directly relating the behavioral and cellular neurodegeneration measures with abundance of β-O product and mitochondrial integrity. Previous findings assumed that providing MCFA provides an alternate pathway for production of β-O products (Gordon et al. 2018), but directly measuring these products after introduction of MCFA could further establish this causality. Assessing rescue via changes in β-O product and MI upon MCFA diet supplementation will further point to a lack of β-O product as causative of disease while elucidating components of the β-O pathway impacted by ALD product loss. Additionally, assessing if restoration of peroxisomal a-CA levels through MCFA diet supplementation directly relates to MI would further establish that lack of peroxisomal a-CA contributes to cellular damage and neurodegeneration. Furthermore, it may provide insight into possible targets for therapeutic intervention, such as restoring peroxisomal β-O products to slow disease progression.

Aim 2: Does lack of a-CA product result in impaired Cit2 and Cat2 functioning or mitochondrial integrity?

Rationale. Cit2 and Cat2 work by converting β-O product, a-CA, into citrate and acetylcarnitine, respectively (van Roermund et al. 1995). If there is a lack of a-CA product established in aim 1, then Cit2 and Cat2 will have no product to transfer into citrate and acetylcarnitine, two molecules involved in many cellular processes (Violante et al. 2013; van

Roermund et al. 1995; Shai et al. 2018). Thus, a lack of a-CA product may contribute to a lack of citrate and acetylcarnitine, diminishing the effects of Cit2 and Cat2 and resulting in neurodegeneration.
Methods. To elucidate the functional response of the Cit2 and Cat2 signaling pathways, five groups of fibroblasts will be used: healthy controls, ALD, Cit2 knock-down, Cat2 knock-down, and Cit2 Cat2 double knock-down fibroblasts. KD of Cit2 and Cat2 will be achieved by utilizing short hairpin RNAs (shRNAs) to target and silence the Cit2 and Cat2 genes via RNA interference (RNAi), similarly to how genes have been knocked down in previous research (Alsayegh et al. 2015). Then, levels of citrate and acetylcarnitine will be assessed in each of the cells and correlated to measures of mitochondrial CO2 and MMP. Then, the cells will be introduced to MCFAs, and after this, the change in levels of citrate and acetylcarnitine will be assessed to see if the lack of product was restored and could therefore be seen by an increase in production of citrate and acetylcarnitine. This will also be correlated with changes in mitochondrial integrity. To measure levels of citrate, Citrate Assay Kit ab83396 will be used (Citrate Assay Kit). A one-way ANOVA will be conducted to compare levels of citrate between cell groups. In order to assess levels of acetylcarnitine, an ELISA acetylcarnitine detection kit will be utilized in conjunction with methods from Stejskal et al. (2008) to assess acetylcarnitine levels in each group of fibroblasts (Various Species Acetylcarnitine ELISA Kit). A one-way ANOVA will be conducted to compare levels of acetylcarnitine between cell groups. MCFAs will be introduced by being added to the fibroblast medium (Jones et al. 2006). Once MCFAs are introduced, citrate and acetylcarnitine levels will be reassessed using the methods above. A repeated measures ANOVA will be conducted to compare citrate and acetylcarnitine levels in each group before and after the introduction of MCFAs.To assess CO2 levels, protocols previously outlined by Shai et al. in 2018 will be followed. CO2 levels will then be measured as a percentage relative to the rate of oxidation in control groups (Shai et al. 2018). A one-way ANOVA will be conducted to compare the mean levels of CO2 in each group of fibroblasts. MMP will be examined as a measure of mitochondrial dysfunction. Protocols previously described by Baarine et al. (2015) will be followed. A one-way ANOVA will be conducted to compare the mean MMPs of each group of fibroblasts.

Aim 3: Does Pex34 silencing correlate with a lack of a-CA product, mitochondrial dysfunction, and phenotypic severity?

Rationale. Aim 3 attempts to establish a relationship between Pex34 silencing, mitochondrial dysfunction, and phenotypic severity in ALD fibroblasts. Despite a significant amount of research focusing on the impact of a build-up of VLCFAs on neurodegeneration, it has recently been established that phenotypic severity may be due to a lack of β-O product (Gordon et al. 2018). A-CA product is involved in many downstream processes in the mitochondria (Shai et al. 2018), so a lack of this product might negatively impact mitochondrial integrity. If it is found that a-CA levels are lower in ALD and ALD + Pex34 KD fibroblasts compared to control fibroblasts, it can be hypothesized that lack of a-CA product diminishes functionality of Pex34. The diminished function of Pex34 might then contribute to mitochondrial dysfunction due to a decreased ability to transfer a-CA from the peroxisome to the mitochondria. Thus, downstream effects of impaired Pex34 function may add to the neurodegeneration seen in X-ALD patients.
Methods. The current proposal will compare a-CA levels, CO2 levels, and MMP of four experimental groups. Fibroblasts from healthy controls (HC-F) and fibroblasts from ALD patients (ALD-F) will serve as control groups to compare to healthy control fibroblasts + Pex34 silencing (HC-F/Pex34-/-) and ALD fibroblasts + Pex34 silencing (ALD-F/Pex34-/-). Pex34 will be silenced in the latter two experimental groups by utilizing short hairpin RNAs (shRNAs) to target and silence the Pex34 gene via RNA interference (RNAi). Methodology for Pex34 silencing was modeled after a previously described protocol by Alsayegh et al. (2015), although researchers utilized RNAi for KD of another gene in human fibroblasts. First, a-CA levels will be measured in each of the four cell groups to determine whether ALD-F and ALD-F/Pex34-/- groups have lower levels of product compared to both the HC-F and HC-F/Pex34-/- groups. In order to measure a-CA levels, high performance liquid chromatography will be utilized as previously described by Shurubor et al. in 2017. This technique allows for measurement of a-CA in whole cells by analyzing the supernatant left after centrifugation of cells. If there is a lack of a-CA product being transferred to the mitochondria, this should be demonstrated by lower overall a-CA levels in the whole-cell samples. A one-way ANOVA will be conducted to compare the mean levels of a-CA in each group of fibroblasts. To assess CO2 levels in fibroblast samples, the current proposal will follow protocols previously outlined by Shai et al. in 2018. Protocols previously described in aim 2 will be followed. A one-way ANOVA will be conducted to compare the mean levels of CO2 in each group of fibroblasts. Finally, MMP will be examined as a measure of mitochondrial dysfunction. Protocols previously described by Baarine et al. (2015) and outlined in aim 2 will be followed. A one-way ANOVA will be conducted to compare the mean MMPs of each group of fibroblasts.
Predicted outcomes. Gordon et al. (2018) was the first to provide evidence that a lack of β-O product is causative of disease, rather than a build-up of VLCFAs. Thus, we predict lower levels of a-CA in ALD-F and ALD-F/Pex34-/- groups compared to control cells. If it is true that Pex34 functioning is impaired in ALD due to a lack of a-CA product, ALD-F and ALD-F/Pex34 -/- should have similar a-CA levels. Also, HC-F/Pex34 -/- might show decreased A-CA, but levels should not be as low as ALD-F and ALD-F/Pex34-/- because these cells still have an intact ABCD1 protein to bring VLCFAs into the peroxisome for degradation into a-CA. Furthermore, Shai et al. (2018) found that overexpression of Pex34 increased CO2 levels, so it is possible that KD of Pex34 may decrease CO2 levels. Because CO2 is a product of the Krebs cycle in mitochondria, decreased CO2 levels may indicate mitochondrial dysfunction. Correlating with decreased CO2, Pex34 silencing may also contribute to impaired MMP, further suggesting mitochondrial dysfunction. Mitochondrial dysfunction may be attributed to a lack of VLCFA β-O product. These predicted findings would suggest that phenotypic severity in X-ALD is due to a lack of β-O product ultimately impacting downstream mitochondrial processes involving Pex34.

A Battle of Intelligence: Review of Organismal Eavesdropping and Predation

Tate Rosenhagen
Lake Forest College
Lake Forest, Illinois 60045

ABSTRACT

In the complex natural world, organisms constantly seek to gain an advantage in any way possible to survive, including eavesdropping on signals from other organisms. Eavesdropping is the interception and reading of signals that are not intended for the recipient. Those signals can be both interspecific communication between similar trophic level organisms and predator-prey relationships. Both the predator and the prey can eavesdrop on each other's signals, whether they be auditory, vibrational, or pheromonal. In this review paper, I examine the different forms of eavesdropping that exist, beginning with intraspecific eavesdropping and interspecific eavesdropping of the same trophic level to examine the mutualistic and commensality of such eavesdropping and the multiple ways they exist. This will be followed by an examination of predator and prey eavesdropping and the multiple sensory modalities in which they eavesdrop, auditory, vibrational, and chemical, as well as a specific dive on the eavesdropping involving sexual pheromones. I also examine the unique relationship of a three-way parasitoid eavesdropping relationship, and a plant trapping prey pheromonal eavesdropping relationship. This paper aims to summarize multiple forms of eavesdropping and the need for further study on eavesdropping of sexual pheromones.

INTRODUCTION

Since the beginning of life, organisms have been in a constant search for nutrients in order to survive. As time progressed, some organisms became predators and others became prey. Both predators and prey have evolved ways to communicate in order to signal food availability, warn of threats, and find mates, such as winter moths releasing pheromones to find a mate and bees stingers releasing pheromones to encourage other bees to sting its target, which are all driven by survival.

Naturally, each organism, both predator and prey, evolved methods to locate and either hunt or avoid each other, respectively. This act, known as eavesdropping, is where an organism intercepts and responds to a signal not meant for the receiver, and locates the signaler or recipient via said signal. Eavesdropping can occur across a variety of modalities such as sounds, pheromones, and vibrations and can be intraspecific, prey eavesdropping predators, and predators eavesdropping prey.

This article aims to review the multiple forms in which eavesdropping occurs in nature. While this review paper is not exhaustive of all possibilities and relationships in which eavesdropping occurs, it serves to create a baseline of knowledge and inform the readers of the ways eavesdropping exists in nature, beginning with predators eavesdropping on their prey. Within this section, I review literature showing eavesdropping through vibrational stimuli and pheromone eavesdropping, two different media in which predators locate their prey, and where multiple signals are intended for mating but observed by predators. I also discuss the reverse relationship, prey eavesdropping on their predators to avoid predation, again through the media of vibrational stimuli and pheromone eavesdropping, with a further emphasis on the duality of this relationship between predators and prey. Finally, I move into intraspecific competition, within both predator species and prey species, discussing vocal stimuli and varying response to warning cries in prey animals. I then end by briefly discussing some of the complex real-world examples of eavesdropping, such as a fungus eavesdropping on its prey and an example of a vector placing hormones on a plant for the plants parasite to locate, then consuming the parasite from the plant, also known as an eavesdropping web.

This paper is designed to educate readers on the topic out of an abundance of interest from the writer. Understanding of the complex ways in which organisms eavesdrop on each other helps create a better understanding of the ecosystem, niches, and how organisms coexist with each other in such complex ecosystems. It is also vital to understand how invasive species may have an edge in eavesdropping in their new environment. Invasive species create mayhem throughout an ecosystem or even towards one organism, such as what happened to the Kiwi, a small, flightless bird in New Zealand that has no native predators but has since become endangered after the introduction of dogs and rodents to the island.

Predator eavesdropping prey

Organisms communicate across a variety of media when trying to signal for danger, food, mates, among others. One such media over which they communicate is through vibrations, something commonly only thought of after auditory and pheromonal signaling but is an efficient way to communicate and one way in which predators can eavesdrop on prey. For instance, the sand scorpion, a nocturnal, burrowing predator that can locate its prey by eavesdropping on vibrational stimuli conducted by the sand (Brownell & Van Leo Hemmen 2001). This serves as a huge benefit to the scorpion as, given the time and way in which it hunts, tapping into the vibrational network in the sand will aid a scorpion in knowing when prey is nearby. The desert scorpion, *Paruroctonus mesaensis*, evolved specialized receptors on the tips of its arms in order to eavesdrop on this vibrational network, known as the slit sensilla. With this receptor, *Paruroctonus mesaensis* are able to sense vibrations through the sand approximately 20 centimeters away from their receptor, as calculated by vibration source localization model designed by Brownell and Van Leo Hemmen (Brownell & Van Leo Hemmen 2001). This study attempted to mathematically model the sensory field of the scorpion, calculating the degrees at which the slit sensilla are angled when the scorpion in the hunting stance, and then making an 'informed guess' to account for the neuronal mechanism through which the stimulation reached and activated the scorpion's brain (Brownell & Van Leo Hemmen 2001). Their theory involves an educated guess along with their mathematical model and lined up very well with their anticipated results to the stimuli presented in their experiment. Thus, suggesting a slight similarity to the auditory pathway (Brownell & Van Leo Hemmen 2001).

Furthermore, one of the most common ways in which predators can eavesdrop on prey is through pheromones, which are hormones that are given off and sensed by another member of the same species, typically for mating purposes. However, pheromones are also used in social insects to communicate colony identity and coordinate group behavior. Predators are believed to cue into these pheromones to locate prey easier, such as the case of the great tit and blue tit picking up pheromones from the winter moth. The winter moth, *Operophtera brumata*, emits a pheromone to attract mates when landing on trees. To further examine its behavior, an experiment was set up involving placing artificial larvae on trees (Saavedra & Amo 2018). Half of the trees were sprayed with the winter moth's reproductive pheromone, and half were not, and it was found that more trees sprayed with the pheromones were attacked by their predators. In addition, regarding the trees sprayed with pheromones, a greater percentage of larvae were attacked on those trees than the trees without the pheromone (Saavedra & Amo 2018). This is similar to the eavesdropping performed by *Odontoponera transversa*, a termite raiding ant that uses pheromones to track its prey. In this example, *O. transversa* was able to perceive two different pheromones, DDE and DEO, given off by the various termites during their foraging activities (Wen et al. 2017). Between the two pheromones, the termites first studied the secretion of DOE on their initial foraging trail while they initially searched for food. At this stage, there are few termites on the trail or around the foraging termite. However, once food was found, DDE was released along the trail, which acts as a recruiting pheromone to other termites, leading to a larger number of termites moving along the newly marked trail. *O. transversa* were found to distinguish between these two hormones. Moreover, when both hormones were present, they hunt along the DDE trail as it leads to more abundant prey (Wen et al. 2017). By being able to perceive their prey, both *O. transversa* and pheromones, the blue and great tit, were able to successfully locate prey, giving them an advantage for finding food in their environment.

Furthermore, the ability of predators, such as O. transversa, to distinguish between pheromones and act upon the pheromone that gives them the largest amount of prey is clearly incredibly important to their predation habits.

Finally, it should be noted that predator-prey eavesdropping is not limited to insects and animals. An interesting example of this is the nematode-trapping fungus, which is capable of eavesdropping on pheromones given off by its nematode prey. While most commonly known eavesdropping relationships between predator and prey are between animals and insects, carnivorous fungi have been found to eavesdrop on ascarides, which are a conserved family of molecules secreted by soft soil nematodes (Hsueh et al. 2013). These fungi developed a relatively small number of traps in their neutral state. However, when certain concentrations of ascarides are present, the fungi observed, *Arthrobotrys* oligospora, not only perceives the pheromone but rapidly develops more traps to catch their nematode prey (Hsueh et al. 2013). This serves as a reminder that eavesdropping is occurring on various scales and involving organisms we may not see or think about daily. Besides, it provides evidence of the complexity of the ecosystem as well as the intensity of the fight for survival between a multitude of creatures living in it.

Prey eavesdropping predators

While predators can eavesdrop on their prey to discern their location and hunt them down, prey are not defenseless in this struggle. Studies have shown that just as predators are able to perceive prey's signals across multiple media, prey are also able to do the same thing. In the previous section, I described how predatory ants are able to track pheromones given off by termites, but termites are able to eavesdrop on the ants as well. Furthermore, in the relationship between the termite, *Coptotermes acinaciformis*, and their predatory ant, *Iridomyrmex purpureus*, *C. acinaciformis* eavesdrops on the footsteps of the ant (Oberst et al. 2017). Originally, they were unsure whether the method of eavesdropping was chemical or vibrational in nature, since termites communicate amongst themselves in both manners. They tested this by playing recordings of ants walking while termites were in containers with no ants present. They found that termites responded in the manner matching how they responded to ants in the wild, with termite soldiers banging their heads into the substrate or shaking their bodies to sound the alarm (Oberst et al. 2017). This information helps to paint the larger picture of the relationship between predators and prey; neither of them exist in a vacuum, rather, they both eavesdrop and react to each other.

Another example of prey eavesdropping on predators is seen through the Giant Asian honey bee, which detects pheromones from predatory ants to avoid predation during foraging. Often, as previously discussed, predators use pheromones, specifically sexual pheromones, to locate their prey. However, in such instances, the opposite occurs. In this relationship, line weaver ants, *Oceophylla smaragdina*, ambush the Giant Asian honey bee, *Apis dorsata*, by hiding underneath a flower and waiting for the bee to land before leaping up and attacking them. While this is a somewhat rare event for the honeybee, it still poses a threat. *A. dorsata* has developed olfactory senses to sense the ants and avoid them (Li et al. 2014). They tested this by placing wire near an ant colony and waiting until about thirty ants, the average number of ants which ambush the bees, have crawled along the wire (leaving their pheromones on the wire). Then, they extracted the pheromone from the wire and administered it to flowers in the nearby area. Other flowers were baited naturally with live ants. The researchers found that bees avoided both the live ants and the pheromone, which pointed to the eavesdropping on the pheromone (Li et al. 2014). Moreover, the fact that the bees avoided both the live ants and the pheromones, which will be circled back to later in the paper as an example of intraspecific eavesdropping, is the fact that *A. dorsata* releases alarm pheromones when harmed on a flower to alert other honeybees not to land on the same flower. To remove this error from their data, researchers removed the bees as soon as they landed on the flower, so no attacks could happen and no pheromone was released, which was important as honeybees avoided flowers with this alarm pheromone present (Li et al. 2014). This prey-predator eavesdropping assists in helping the Giant Asian honey bee avoid predation by both picking up on their predator's trail pheromones and being able to leave a warning pheromone to others in the event of an attack, highlighting the multiple warning signs and complexity of the eavesdropping relationship.

Further exemplifying the complex relationship is the fact that both predators and prey are able to eavesdrop on each other, which has already been briefly discussed. This complex scenario is highlighted in the relationship between brown rats and house mice, both of which can perceive the other's pheromones and hunt or avoid the other, respectively. In this study, the 'counterespionage' hypothesis was tested, which, as previously stated, claims that both predator and prey perceive the opponents' pheromones and react accordingly, to locate and hunt prey or locate and avoid predation (Varner et al. 2020). In this experiment, an experimental plot was set up containing food and testosterone on either side of the plot. One side contained the pheromone of the other species pheromone as well as testosterone, which was present to make sure the organisms were not simply avoiding any pheromone present. While their data did show that each organism was aware of the others presence, their results were rather confusing at first glance as both mice avoided the rat pheromones, as expected, but also the rats avoided the mice, which was the opposite of what should happen according to their hypothesis (Varner et al. 2020). Even though this data seems surprising, it makes sense when you put into context the set-up of the experiment, where the rats had an abundant and constant supply of food. The authors believe that the rats weighed the benefits of hunting the mice versus the risk of injury and, with a constant supply of food there, found no reason to take that risk. Furthermore, the brown rats are opportunistic predators of mice, not specifically mice hunters, which may have further weighed into them avoiding the mice. However, even with this data, the scientists were able to show that both the rats and mice can sense each other's pheromones and respond to them, setting the table for further tests on the specifics of the organisms' responses (Varner et al. 2020). With a better experimental set up, such as making the rats hungry before the test and with a different breed of rat or another test organism all together, like feral cats, the authors may find data that aligns more with their hypothesis and depicts an accurate reflection of predator-prey dynamics (Varner et al. 2020).

It should be noted that this method of prey-predator eavesdropping, or eavesdropping in general, is not limited to animals: plants perform this as well. While plants cannot move to avoid their predators, they are able to increase defenses to protect themselves from further predation. This eavesdropping can be explored through a variety of medias once again, but one astonishing way in which the plant *Arabidopsis thaliana* eavesdrops on its predators is by picking up on vibrations of their predatory caterpillars, *Pieris rapae* (Appel & Cocroft 2014). When exposed to vibrations simulating caterpillar feeding mechanisms, the authors found that chemical defenses, particularly glucosinolate and anthocyanin, give these plants a higher chemical defense to their predators than unstimulated plants. This is fascinating to see as it means the plants are able to distinguish between vibrations caused by caterpillar predation and those caused by wind, bumps, insect songs, and raindrops, only eliciting a response in the presence of predation (Appel & Cocroft 2014).

Prey-Prey and Predator-Predator eavesdropping

As I have already established, predator-prey eavesdropping results in an interesting dynamic between the two organisms, with each organism using the information provided by eavesdropping to locate and either hunt or avoid the other. However, eavesdropping is not purely limited to the predator-prey relationship and can happen both intra- and inter-specifically by both predator and prey organisms. For example, in the case of the Brazilian free-tailed bats, *Tadarida brasiliensis*, intraspecific eavesdropping is used often to help them locate prey. As is very well documented, bats use echolocation to help them locate and hunt prey at night. These echolocation calls emit a strange buzzing sound that can be audible if one is near bats while they are hunting. As *T. brasiliensis* approaches a prey target, it emits a specific 'feeding buzz' right when it attacks its prey, which other bats are able to recognize as a signal of nearby prey (Gillam 2007). F urthermore, the author tested if it was the sound alone that made the bats swarm to an area as a signal prey was nearby; he found that when he played the feeding buzz, the feeding buzz backwards, and a silence control, the feeding buzz created the largest response by the bats (Gillam 2007). The sensitivity of *T. brasiliensis* to the call shown by recognizing the forward playing feed buzz best shows how specific this eavesdropping mechanism is and the advantages the bats gained in intraspecific competition by eavesdropping on their fellow bats. This ensured the individual eavesdropping was aware of any prey found by the rest of the swarm and allowed them to quickly move to and consume prey before it was gone.

While predators compete interspecifically by eavesdropping as we just discussed, prey can eavesdrop on other prey species signals to find food or avoid predators. In the case of the African plains, numerous predators are present and able to attack at any moment, including lions, leopards, and cheetahs, making it both important and challenging for prey animals to keep an eye out for their predators. In an interesting paper written by Palmer and Gross, alarm calls by Impalas, Wildebeests, and Zebras were examined and played to each species to examine their response to said calls. Not only did they respond to the alarm calls of other species, but they also reacted differently to each call based on the predators of the species giving the call (Palmer & Gross 2018). The authors found that Zebra calls elicited the largest response by all of the study species as the Zebra's predator is the Lion, a common predator for all of prey mammals, while the Impala's warning cries elicited the smallest response, as Impalas are much smaller than the other prey and thus had a larger variety of predators that did not prey on the Zebra and Wildebeest (Palmer & Gross 2018). The responses were sorted into vigilance, grouping, alarming, and fleeing, though every animal with every response exhibited signs of vigilance. The Impala can be seen to flee at any call, including the control, further showing their understanding that any predator in the area is likely to play upon them, while the Wildebeests and Zebras were more likely to only be alarmed. On the other hand, Zebra calls were responded to by every species, although the response of fleeing, grouping, or alarming varied in response to common predators (Palmer & Gross 2018). This interpretation of each call serves to benefit each animal by allowing them to be more aware of their surroundings and potential predators while tailoring their response based on the organism the alarm came from.

Eavesdropping Webs

In the context of the three different eavesdropping relationships we have already discussed, there are a few unique eavesdropping examples that I believe warrant their own section. One such example is a nematode-trapping fungus, which is capable of eavesdropping on pheromones given off by its nematode prey. While most commonly known eavesdropping relationships between predator and prey are between animals and insects, carnivorous fungi have been found to eavesdrop on ascarides, a conserved family of molecules secreted by soft soil nematodes (Hsueh et al. 2013). These fungi developed relatively small number of traps in their neutral state, but when concentrations of ascarides are present, the fungi observed, *Arthrobotrys* oligospora, not only perceives the pheromone but rapidly developed more traps to catch their nematode prey (Hsueh et al. 2013). This serves as a reminder that eavesdropping is occurring on scales and involving organisms we may not see or think about daily, and that the ecosystem is an incredibly complex fight for survival between a multitude of creatures living in it.

The final unique example I plan to discuss in this section involves a unique, three-wayrelationship between an insect, plant, and parasitoid. In this relationship, *D. citri* is a specialist parasitoid that harms its host plant, citrus trees. *D. citri* is able to eavesdrop on a bacterial vector known as Las, *Candidatus Liberibacter asiaticus*, to find its host plant (Martini et al. 2014). In this relationship, Las is also incredibly harmful to the citrus trees and can cause huanglongbing, a deadly disease in the citrus plant and the release of MeSA, which is a plant-defense-hormone that attracts the parasitoid's predator, *Taxarixia radiata*, to act as a 'bodyguard' of the plant. This bodyguard then consumes the parasitoids after a slight amount of damage has been done to the citrus tree, and the combination of the damage by the parasitoids and the infection by Las makes the citrus plant no longer attractive to parasitoids; this allows the tree to continue to grow with the Las infection inside of it (Martini et al. 2014). The parasitoids not preyed upon will also leave the plant after consuming some of the citrus tree leaves due to the increased chemical defenses, which can spread the Las infection to nearby trees, wreaking havoc upon a tree farm. This serves as a fascinating example of an incredibly complex eavesdropping relationship between a bacteria, plant, parasitoid, and predatory wasp to continue to spread the infection to nearby plants while preserving the initial plants health.

Eavesdropping webs like this add a further layer of complexity to the ecosystem that cannot be noticed when looking closely at an individual organism but can be observed when looking at the bigger picture. If we look throughout other ecosystems, there may be other examples of complex eavesdropping webs that may give us a greater understanding of the ecosystem where the relationship resides.

Conclusion

In review, these findings of eavesdropping interactions through different medias, in different relationships and ecosystems, and between animals, plants, bacteria, and insects across a range of complexity is of great importance to the scientific world. To truly understand the complexity of an ecosystem, eavesdropping relationships must be considered throughout the various organisms present in this ecosystem. While the authors all found eavesdropping relationships in the organisms they studied, the common thread amongst all the papers is the need for further study, further relationships to be explored, and further research into the mechanism behind the eavesdropping relationships.

Furthermore, the possibilities of future studies are endless. In the case of the citrus trees I discussed earlier, study on the bacterial vectors can be done to help farmers recognize infected plants early to prevent spreading and save crops. On the other hand, the complexity of eavesdropping relationships needs to be considered when introducing species that may become invasive to non-native ecosystems. It would be an interesting study to observe invasive species relationships in their native environment versus their invasive environments to observe any eavesdropping relationships that give them an advantage in their new environment.

In short, eavesdropping is a simple idea with incredibly complex mechanisms, relationships, and implications for the natural world. Further understanding of these relationships may aid in our understanding of the natural world, habitat reconstruction, and identification of possible invasive species to prevent them from ever being introduced.

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The how and why in the origins of sexual reproduction

Zubair Mohammed
Lake Forest College
Lake Forest, Illinois 60045

Abstract
The origin of sexual reproduction, how it evolved, and its benefits have been topics of active research. Many different theories have been proposed and various models have been made to explain sexual reproduction in species. There is an inherent problem of which proponent of the evolution of sex is correct or whether it is a combination of theories that is correct. Although not exhaustive, the current review aims to synthesize and explore this issue and ideas based on some of its arguments including the generation of sex differences, the molecular basis for sex, and why it may have been selected for and kept in certain species. By providing a baseline for the discussion, further research may be executed in deriving the reasoning behind the evolution of sex and its pros and cons compared to asexual reproduction.

What is obligatory sex and who has it?

The definition of sex has generally been accepted as a means of reproduction that requires the genetic material of two parents combining (via gametes) to form a zygote (Bai 2015). However, this is not the only means of reproduction as organisms can also pass on their genetic material through asexual reproduction where the parent clones its genes for the offspring. It is important to note that about only 0.1% of animal species reproduce asexually (Phillips et al. 1990). An even more curious and interesting note is most asexual lineages have come from sexually reproducing ancestors which may indicate that asexual reproduction is probably a niche tool for evolution in certain evolutionary situations (Geodakyan 1991). Both sexual reproduction and asexual reproduction have a facultative side and an obligate side that can be observed in species. Obligate asexual reproduction has been thought to arise because of inbreeding or mutation in large populations (Scheuerl et al. 2011). Facultative sexual reproduction is relatively rare, being only observed in a few animal species. The reason for this seems to be a response from species for lack of viable mates in certain environments. On the other hand, obligate sexual reproduction is when species reproduce exclusively via sexual reproduction and is the reproductive method most observed in the animal kingdom. It may initially appear evident and obvious as to why and how sexual reproduction is advantageous and thus so prevalent. Unfortunately, this is not the case, as the origin and maintenance of sexual reproduction is still one of the most elusive and hotly debated topics in evolutionary biology. The reasons behind this are because sexual reproduction requires more resources and has more costs associated with it compared to asexual reproduction, and yet it is still prevalent. Therefore, there must be a greater payoff to the risks and costs that keeps sexual reproduction the main mode of propagation in species. In face of this logic, no concrete and agreed upon theory has been presented which explains how sexual reproduction came to be and how it is maintained. In the current review, some major theories and hypotheses will be discussed to provide a foundation for future research.

The sexual cons

One of the most famous costs of sex is known as the “two-fold” cost of sex. The idea is that a female can reproduce asexually and make many clones of herself which can then propagate further unhindered. Compare that to a sexual female who has a 50% chance of producing a male and cannot reproduce by themselves, decreasing the growth rate of the sexual population. Besides that, males and females have to first find each other to mate and produce one male and one female at the very least to keep the population at equilibrium (Gibson et al. 2017). This is significantly more difficult than cloning and duplicating which allows the asexual population to quickly double and outgrow the sexual population. Also, there is the loss of genetic information on both sides, known as the “cost of meiosis”, where each parent is only contributing half of their genetic material (Williams 1975). This is obviously a problem as the genetic traits that helped the parent survive in the environment are not fully transferred from parent to the offspring. There are other problems that contend with the evolution of sexual reproduction. For example, recombination can and does destroy successful gene combinations which reduces individual fitness (Lehtonen et al. 2012). Logically, natural selection should therefore act against recombination. Furthermore, the act of having sex can increase the transmission of sexually transmitted diseases (Otto 2009). These diseases can be crippling to populations of animals if the sexually reproducing members are dying from them. Genetic problems to consider include genetic linkages where certain areas of DNA are inherited together based on proximity and can carry disadvantageous alleles that may cause disease. There is also evidence showing that some genetic sequences can enhance their transmission and decrease other gene transmission regardless of whether they have no effect or are detrimental to organismal fitness (Hickey 1993). A simple way to avoid these problems would be to just not reproduce sexually, so what possible advantages could sex offer that overshadow its cons?

The sexual pros

Many experiments and explanations have been offered to demonstrate why sex may be advantageous. One explanation as to why obligatory sex can evolve over facultative sex is that sexual selection exists in sexually reproducing species (Hadany & Beker 2007). It is usually the case that the sex investing less in the offspring have varied fitness. Via sexual selection, natural selection is sped up in these species, and this presents the long-term benefits of quicker adaptation to the environment. The short-term benefits of sexual reproduction include allowing sexual organisms to compete in the “evolutionary arms race” better than asexual organisms. This is known as the Red Queen hypothesis (Lively 2010). By recombining the genotype, sexual organisms can better adapt to the fluctuating environments that are deterministic. In other words, the back and forth of the arms race may continue without sex, but sex enables the “race” to continue efficiently. Similarly, the haploid-diploid nature of sexual organisms is thought to exploit a very simple form of the Baldwin effect, where changed needs lead to learned behavior and natural selection takes care of the rest (Bull 2017). Simply put, the diploid stage of sexual organisms acts like the learned behavior stage and associated phenomenon, such as recombination or the varied lengths of haploid-diploid stages, can be evolution fine tuning the learning experienced by organisms. In this way, sex would be maintained as it allows for this learning stage to exist. Gandon and Otto (2007) looked at the specifics of the Red Queen hypothesis and argue that there are multiple factors that contribute to the phenomenon, including fluctuating epistasis, drift, and directional selection. They show that fluctuating epistasis is likely the most important factor in the development of sex. Natural selection often leads to certain allelic combinations that work well together, but recombination can break these links. In the short-term, this is reducing fitness for the organism; however, over the long-term, sufficiently high rates of recombination can work to help break those linkages which do not work well together (Otto & Feldman 1997). In this way, recombination and sex would be favored as they increase the ability to correct linkages if the incorrect ones are being broken, thus increasing the fluctuations in epistasis. Some phylogenetic evidence has also been provided showing that obligate sex organisms may have evolved from facultatively reproducing ancestors (Kleiman & Hadany 2015). It begs the question: why is obligate sex so common? One important realization to note is that most facultatively reproducing organisms are unicellular whereas multicellularity has been correlated with the development of sex (Kleiman & Hadany 2015). Unicellular organisms that reproduce asexually would accumulate DNA damage from the environment and pass it on until it becomes debilitating. This answers the question of why organisms may develop recombination, but that fails to account for the development of sex and sexual dimorphism, which allows sex to happen in the first place. It seems that there are many intertwining theories that are filling in different pieces of the puzzle, but there does not seem to be one apparent theory everyone can agree with. For this reason, the field has taken a “pluralist” approach in addressing the origin of sex. As stated before, so many different factors have contributed to the development and maintenance of sex, including the development of meiosis, anisogamy, sexual dimorphism, recombination, environmental constrains and pressures, and many more unlisted contributors, that this view is the best way to approach the problem. In this review, a few early theories will be discussed along with some modern theories. This is not an exhaustive list as many topics are outside the scope of this review, but a foundational understanding of the field will be posed.

Some early theories on why sex evolved

Fisher-Muller hypothesis

In a set number of asexual populations, the efficacy of natural selection is impeded by elements of genetic drift. The linkage of alleles talked about previously cannot happen by any efficient means. Recombination offers selection at individual loci; over time, the mean fitness should increase. It has been shown in the past that recombination in conjunction with sex increases the genetic variability in the genome of the species. Therefore, natural selection will then eliminate the unfavored combinations and have a better chance at bringing together successful allele pairs compared to asexual organisms. This was experimentally formalized by Fischer (1930) and Muller (1932) in a case applied to a set of 2 alleles, which are both beneficial but arise in two different loci that are linked. They both logically conclude that sexual organisms would have an easier time combining the two alleles into a new genome than asexual organisms. Asexual organisms would have to wait for the mutation to be made and to fix it into their sequence in the same generation. This would naturally take a much longer time period, comparatively.

Muller's hypothetical ratchet

Muller (1964) proposed another hypothesis in which recombination and sex could evolve in a set population. Muller argued that most mutations, due to random chance, are deleterious and restoration of the original wildtype allele, even in long periods of time, is rare. So, imagining a scenario where there is an initial population of asexual organisms of which some incur this mutation, there will be a few which do not. However, this initial subset of mutation-free organisms will be lost over time and cannot be recovered evolutionarily. This constitutes as one "crank" of Muller's Ratchet. Allowing for multiple generations to continue as such would result in the least loaded group being weeded out. Eventually, this would result in the irreversible degeneration of the genome to a point of no return as the entire species is driven to extinction. Recombination and sex evolved to combat these deleterious mutations as even a little bit of recombination can stop the cranking of the ratchet.

Geodakyan's evolutionary theory

Geodakyan (1991) suggests another interesting theory involving sexual reproduction and why it has become the most common form of reproduction over the three other ones discussed prior: asexual, hermaphrodite reproduction, and sexual reproduction. The theory is broken down into two components: the principle of conjugated systems and the theory of asynchronous evolution. The first hypothesis involves the variance produced in a sexual population in the males and females and compares the two. It has been shown in subsequent analysis that males have higher variability compared to females in humans and in other plants and animals. (Lehre et al. 2009) Geodakyan proposes then that sexual dimorphism is inherent to the development and maintenance of sex because the male acts as an experimental ground for recombination and variation whereas females function as a conservative subsystem. The second hypothesis takes these subsystems and separates them further by stating that they evolve slightly differently. The trait is first adapted to the males and then in future generations, the females. In this way, they evolve asynchronously. This theory was also described in subsequent studies (Andersson, & Wallander 2004).

Hickey's theory

Hickey (1993) helps establish an even more rudimentary aspect of sex, describing how recombination came to be. Hickey relates that conjugation, or the linking of two unicellular organisms for genetic transfer, had to be a precursor step to recombination. After conjugation, karyogamy would have to develop, and then right before recombination is meiosis. He also suggests that outbreeding is very much in line with what evolution wants and is therefore favored. By having multiple copies of a successful transposon spread within a population, it would be favored, and this ability would be kept in the genome. This somewhat relates back to the selfish gene theories mentioned prior. Sex may have evolved as a byproduct of ensuring rapid transmission of such elements, even if they cause a substantial reduction in the host's fitness (Hickey 1982). Although this explanation is sound for the initial emergence of sex, it cannot similarly explain how sex is maintained. Once selfish genes invade a population and reach a high frequency, asexual individuals should be able to propagate selfish elements just as quickly as sexual individuals. Therefore, multiple theories are needed in order to explain each facet of this paradox.

Current working theories

There is some recently emerging evidence that helps explain and expand on the early working theories. One such study helps us to understand how meiosis may have developed originally as a response to errors in spontaneous auto-ploidy or whole genome reproduction. This would combat problems like aneuploidy, gene-overexpression, or even other negative effects of polyploidy (Niklas et al. 2014). This study also asserts that the reason for the continued maintenance of sexual reproduction has to do with decreased competition between siblings via increasing variability. Although their research is incomplete in addressing everything, they do point out important ideas. A more focused hypothesis looks at the Red Queen hypothesis once again, but this time from a genetic standpoint and as a cancer prevention theory. Aubier (2020) and his colleagues point out that early multicellular organisms may have been afflicted by transmissible cancers. In this scenario, the cell line of the host will continue to generate cancer cells (also known as neoplasia). They propose that although it is difficult to be horizontally transmitted, it is easily transmitted vertically, and this is what promotes the evolution of sex through a mechanism known as similarity selection. To further elaborate, the study asserts that sexual reproduction evolved as a defensive strategy against vertically transmissible cancers, but only in certain circumstances where the host life history is slow, neoplasia is slow, and the transmission rate is high. Once that occurs though, sex can allow faster development of immunity to cancerous cell lines much like the arms race posed by the Red Queen hypothesis. Another recent study confirms the longstanding and previously discussed hypothesis that sex is beneficial and that recombination speeds up adaptation to the environment. McDonald et al. (2016) show the specific mechanisms that allow sex to speed up adaptation. Looking at sexual and asexual Saccharomyces cerevisiae populations, they arrive at the conclusion that sex alters the spectrum of mutations that fix compared to deleterious mutations can easily fix in asexual organisms. Recombination actually prevents this substantially deleterious mutation from fixing in the genome.

Summary

As is evident from the varied fields of research, there are many angles from which to tackle the evolution of sex. This review only covered a select few, including the Red Queen hypothesis, Cancer deletion theory, the Fisher-Muller hypothesis, and more. Although each question comes from and leaves on different roads, the central theme of each theory is that sexual reproduction evolved to reduce the amount of errors in the replicated genome being passed down and to increase variation in the genome to increase the chances of survival of the organism via natural selection.

Discussion

Even after many decades of research, it is still not concrete why obligate sex is so ubiquitous. However, it is clear from the findings how each facet and underlying mechanism works to a certain degree. Theories like the Red Queen hypothesis and selection interference only go so far in providing cases for the maintenance of sex in relation to its costs. They have not yet yielded models in which sex appears in a large range of biological scenarios. It is true that even in the models and papers posed in this review that constraints are put into the models which do not reflect all the possible situations. Also, we need evidence for diploid organisms that addresses both questions of origin and the maintenance of sex in order for them to be robustly answered. One of the most important tasks for evolutionary biologists is to determine all the different situations in which it would be the most conducive in making the maintenance of sex necessary for organisms. Some other direction could possibly be how multiple processes coexist and maybe even balance each other in producing both advantageous and deleterious mutations. An example could be if parasitic infections and cancerous cell lines were both generated and studied in a Red Queen model. Regardless of the direction, it is pertinent to the field that more experimental data must be generated. There are a great number of phenomena and theoretical predictions that need to be tested. The situation has seemingly gotten better over the past few years with many studies showing the advantageous sides of sex, dimorphism, parasitic co-evolution, and breaking selection interference. However, more empirical evidence is needed to showcase the main mechanisms that select for sex in nature. There were a few discussed in this review but there must be verification studies and replications to really establish these leads. It is certainly possible that a combination of theories may be the best bet in understanding the selective pressures for sexual reproduction to evolve, but most of the foundational data has been in the theoretical papers that introduced them. There should be some work done on the theoretical predictions, such as if the evolution of sex and recombination truly do lead to an increase in fitness variance. The outcomes of these studies will no doubt be providing key insights in determining what key factors are the main determinants of the evolution of sex and its maintenance.

What explains genital variation in snakes? A review of the hypotheses of genital co-evolution

Frances Rice
Lake Forest College
Lake Forest, Illinois 60045

Abstract
Genitalia can be defining traits for many species with internal fertilization. In general, genitalia of males and females typically coevolve, as copulation is one of the most mechanically direct interactions in biology. Snakes, in particular, have quite complex and elaborate genitalia. Male snakes possess paired copulatory organs, known as hemipenes. There is immense variation in these structures, including spines, scoops, size, and the presence of bilobes. Females accordingly have complementary variation in genital structures. Three hypotheses exist to explain this variation and coevolution: the lock-and-key hypothesis, sexual conflict, and cryptic female choice. In an attempt to identify the main driver of this variation and coevolution, this review will investigate each of these hypotheses to explain genitalia variance, and it will evaluate the primary evidence behind each of them. Present evidence majorly supports the sexual conflict hypothesis and the lock-and-key hypothesis. Ultimately, however, further research on female snake genitalia, and on snake genitalia in general, should be completed, as there are still many unanswered questions.

Introduction
In many animals with external genitalia and internal fertilization, the genitals are often elaborate and unique. Males typically have intromittent copulatory organs, and females have an internal genital tract which receives the male intromittent copulatory organ during copulation. Traditionally, male genitalia have been considered more diverse and variable, compared to female genitalia (Eberhard 1985). Female genitalia have not been considered as variable due to lack of research, primarily due to male researcher bias and the hidden nature of female genitalia (Ah-King et al. 2014; Brennan & Prum 2015). Furthermore, female genitalia can be complicated to study, as they are subject to other selective pressures. The female reproductive tract not only receives the male intromittent copulatory organ during copulation, but it also performs birthing functions, sperm storage, and ovipositing (Brennan & Prum 2015). Despite these multiple functions, female genitalia are still considered variable enough for coevolution to occur (Brennan 2016; Brennan & Prum 2015). When discussing genital coevolution, it is vital to have variation in both the male and the female genitalia.

Genital coevolution occurs when evolutionary changes in one sex's genitalia drives evolutionary change in the opposite sex's genitalia (Brennan & Prum 2015). As copulation is one of the most mechanically specific interactions between individuals, genitalia of the male and female must evolve closely together in order for copulation to be successful (Brennan 2016; Brennan & Prum 2015). Initially, Charles Darwin proposed that natural selection influences genital coevolution, as copulation is a function of gamete transfer and is necessary for successful reproduction (Darwin 1871). In addition to natural selection, genital coevolution can occur through other mechanisms, such as sexual selection. Experimental evolution has confirmed that genital coevolution occurs under sexual selection pressures and through mate choice (Simmons & Garcia-Gonzalez 2011). Nonetheless, there are three hypotheses that are frequently used to explain the roots of genital coevolution that incorporate both natural and sexual selection: lock-and-key, sexual conflict, and cryptic female choice.

The lock-and-key hypothesis is the most popularly known hypothesis regarding genital coevolution (Eberhard 2010). This hypothesis proposes that genital coevolution occurs through natural selection in order to prevent hybridization and to reinforce reproductive isolation (Dufour 1844; Eberhard 2010; Shapiro & Porter 1989). This is advantageous because hybridizations can be quite costly, especially for females, as they lose a large investment if they produce an inviable or unfit offspring from mating with a heterospecific male (Eberhard 2010). It can also be costly for males, although to a lesser extent, as they do not typically invest as much in the offspring as females do (Bateman 1948). Thus, the genitalia coevolve to only fit with the lock or key of their own species (Brennan & Prum 2015). Under this hypothesis, the genitalia are expected to have a close mechanical fit during copulation and specific coevolution of genital shape (Brennan & Prum 2015). It would also be expected that this would occur when closely related species are living in sympatry, as this is when the risk of hybridization would be the highest. The lock-and-key hypothesis tends to be refuted, however, as female genitalia are not viewed as variable enough to possess species-specific locks (Eberhard 2010). This claim is largely based on the fact that female genitalia are largely understudied, due to male researcher bias and the hidden nature of female genitalia (Ah-King et al. 2014). However, there is now evidence that demonstrates the falsity in this claim, and that female genitalia are in fact also variable (Simmons 2014). With variation in both male and female genitalia, there is a good foundation for supporting the lock-and-key hypothesis.

Another hypothesis that can explain genital coevolution is sexual conflict, which results in sexually antagonistic coevolution, initiating a coevolutionary arms race. Under this hypothesis, males and females compete for control over reproduction, but the genital adaptations that benefit each sex are detrimental to the opposite sex (Chapman et al. 2002; Eberhard 2010). Sexual conflict may occur through male-male competition for a successful fertilization or through natural selection on female behavior, physiology, or morphology, allowing the female to reduce physical harm or resist coercion (Brennan & Prum 2015). One of the best-known examples of sexually antagonistic coevolution is in waterfowl, where males are forceful in their mating attempts with females; this comes at a disadvantage to the female (Brennan et al. 2007). Males have a corkscrew shaped penis and females have a vagina shaped in a way to prevent coercive mating attempts from the males (Brennan et al. 2007). Here, the genitalia of the male and female waterfowl oppose each other, as they fight to gain control over copulation. It can be expected under this hypothesis that the female genitalia or body would be harmed, but that is not always the case when genitals coevolve through sexual selection.

The final hypothesis that can explain genital coevolution is cryptic female choice (CFC), which operates through sexual selection. Under this hypothesis, genital coevolution occurs because females prefer certain sensory stimuli from male genitalia to facilitate a mate choice or become pregnant (Brennan & Prum 2015). Some features of the male genitalia will be more stimulating, and thus will be selected for (Eberhard 2010). In domestic pigs (Sus domesticus), the females require stimulation from a filament on the male penis in order to become pregnant; if they do not receive this stimulation, such as in artificial insemination, pregnancy rates drop (Bonet et al. 2013). In this case, the female need for stimulation drives the evolution of penis shape. This hypothesis also requires female promiscuity, as females must be choosing from multiple males (Brennan & Prum 2015). Additionally, under this hypothesis, male genitalia should not harm the females, as it may under the sexual conflict hypothesis (Friesen et al. 2014). All these three hypotheses mentioned—lock-and-key, sexual conflict, and cryptic female choice—can be investigated further in snakes—a good model for studying genitalia.

In snakes (class Reptilia; order Squamata), the genitalia are elaborate and unique. Male snakes possess paired intromittent copulatory organs, termed hemipenes, and females accordingly have hemiclitores, as well as vaginal pouches (Gredler et al. 2014). Hemipenes extend from the lateral edges of the cloacal opening when everted (Leal & Cohn 2015). These paired copulatory organs are present in lizards as well, which are also part of the Reptilian order, Squamata. Interestingly, unpaired intromittent copulatory organs are present in two other Reptilian orders, Testudines and Crocodylia, but not the order which contains the tuatara, Rhynchocephalia (Gredler et al. 2014). During embryonic development, hemipenes arise from lateral swellings on each side of the cloaca, and as the embryo develops, these swellings grow to form the right and left hemipenes (Leal & Cohn 2015). These organs undergo further development and differentiation as they transform into their mature form.

Once sexually mature, both male and female snakes have immense genital variation, both in physical structure and appearance. Male snake hemipenes have been photographically documented to have spines, scoops, and bilobes, or to have no complex structures at all (Andonov et al. 2017). Similarly, female genitalia, although still widely understudied in snakes, have some documented variation as well. Female pouch morphologies and vaginal shapes differ among species (Showalter et al. 2014; Siegel et al. 2012). The variation has been documented across many snake species and suggests a close genital coevolution. However, it is still unknown what explains this genital variation and coevolution,

and what the main force of evolution is behind it. In an attempt to locate the main driver of this genital variation and coevolution in snakes, this review will investigate each of the hypotheses that explain genital variance and coevolution, and it will evaluate the primary evidence behind each of them. This review will begin with the discussion of evidence behind the classic hypothesis of genital coevolution, the lock-and-key hypothesis. It will then transition into the two other hypotheses, sexual conflict and cryptic female choice, and highlight the evidence behind each of them.

Lock-and-key

The lock-and-key hypothesis states that genitalia of males and females evolved to prevent a costly hybridization event with another closely related species and also to reinforce reproductive isolation (Shapiro & Porter 1989). Under this hypothesis, a close mechanical fit between the genitalia is expected (Brennan & Prum 2015). In snakes, the evidence for the lock-and-key hypothesis of genitalia coevolution is limited, but still substantial.

Most broadly speaking, in male snakes, hemipenes are extremely variable, in regard to their shape, presence of spines, width, and length (Andonov et al. 2017). In simplest terms, if male hemipenes present such variation, it would make sense for the female genitalia to also be variable, in order to fit with these hemipenes. Accordingly, female snake genitalia have shown to be diverse. In female snakes, a "pouch" is present in the reproductive tract, and it is involved with the receiving of the male copulatory organ (Siegel et al. 2012). Siegel and colleagues (2012) found these pouches to be variable interspecifically in Colubridae snakes. This variation was substantial, as some pouches were bifurcated, separated, or classified as "simple." According to the lock-and-key hypothesis, variation should exist interspecifically, as to prevent costly hybridization events (Shapiro & Porter 1989). Because this variation was observed interspecifically, this evidence supports the lock-and-key hypothesis. This study, however, did not investigate if these species were living in allopatry or sympatry; only if they were living in sympatry would this be substantial evidence for lock-and-key, as closely related sympatric species have the highest rates of hybridization.

Further evidence supports genital variation in female snakes, which is suggestive of coevolution. Showalter and colleagues (2014) investigated interspecific genital variation in two closely related species of watersnakes (*Nerodia sipedon* and *Nerodia fasciata*) and discovered that the vagina is different in these two species living in sympatry. The vaginas were both bifurcated, but they differed enough in the degree of bifurcation. As these species are closely related and living in sympatry, this evidence greatly supports the lock-and-key hypothesis. Because these two species are already somewhat genetically similar and living in sympatry, further reinforcement of reproductive isolation is necessary to prevent a costly hybridization event (Brennan & Prum 2015). Genitalia specific to each species represents that reinforcement. Further supporting this hypothesis is evidence from King and colleagues (2009) which demonstrates that the male genitalia in these two species were morphologically similar; they were bilobed in accordance with the bifurcated female vagina (King et al. 2009). As expected under the lock-and-key hypothesis, the male and female genitalia in these two watersnake species demonstrate a mechanically close fit, also known as copulatory adjustment (Edgren et al. 1953), thus supporting that this hypothesis explains the variation present in these two species. Although there is substantial evidence supporting the lock-and-key hypothesis, there is also some strong evidence against it. Inger and Marx, two herpetologists from the Field Museum of Natural History in Chicago, Illinois, found that the genitalia of *Calamaria lumbricoidea* (family Colubridae) varied not interspecifically as expected under the lock-and-key hypothesis, but intraspecifically (Inger & Marx 1962). Interestingly, this variation was observed in populations not living in sympatry. This could indicate that a reproductive isolation event occurred, possibly leading to the beginnings of speciation. However, the female genitalia from one population fit well enough with the male genitalia of the other population; the same was true for the reverse. This refutes the lock-and-key hypothesis, as this example does not demonstrate a mechanically close fit, or copulatory adjustment. This instead supports that sexual selection via female choice is driving this variation, as females within the species may be selecting for certain male genitalia, driving intraspecific variance (Gilligan & Wensel 2008). All these stated examples denote that the lock-and-key hypothesis may explain variation in some species, but not in others, hinting that there must be other explanations for this variation and coevolution.

Sexual Conflict

Sexual conflict can result in sexually antagonistic coevolution, initiating a coevolutionary arms race in an effort to gain control over reproduction. In this instance, genitalia can coevolve to benefit the one sex, but harm the other. In the case of genital coevolution via sexual conflict, genitals can be expected to evolve very fast. In male Anolis lizards, their genitalia have been observed to evolve faster than any other trait they possess (Klaczko et al. 2015). Like snakes, lizards are in the order Squamata, and they possess hemipenes. This example, although not in snakes, may be surface level evidence of sexual conflict driving genital coevolution, as coevolutionary arms races are characterized by rapid genitalia evolution (Hosken & Stockley 2004). The sexual conflict hypothesis of genital coevolution can be further investigated by looking at male and female genital adaptations, as well as adaptive behaviors in each sex that respond to the reproductive conflict.

Male genital adaptations

Male hemipenes have a wide variation in traits that are characteristic of conflict, such as the presence of spines, scoops, and bilobes (Andonov et al. 2017; Friesen et al., 2014; King et al. 2009). Although these characters are not inherently linked to conflict—they have the possibility of stimulating the female in a way that signals male quality—there is substantial evidence claiming that they are working against the females, leading to conflict. There is also evidence that they are not simply a vestigial structure, as once thought, relating to the loss of limbs in snakes. Nunes and colleagues (2014) presented evidence that limb reduction is not related to the presence of hemipenile spines in lizards that have limb loss; it can be inferred that the same applies for snakes, as snakes and lizards are closely related. As this idea has not been supported, it seems even more probable that hemipenile spines are involved in sexual conflict.

In support of spines leading to sexual conflict, Friesen and colleagues (2014) observed copulatory behavior in red-sided garter snakes (*Thamnophis sirtalis*) and found that spines function to extend copulation by holding the female in place; spines allow the male to secure the copulatory organs together. When spines were removed, copulation was observed to be much shorter, as females could then end copulation when they wished (Friesen et al. 2014). Interestingly, females were also bleeding during or after copulation. Together, these results indicate that hemipenile spines are not only an adaptation to extend copulation past the point of the female's preference, but they are also physically damaging to the female—a characteristic of sexual conflict. Both these findings support that male snake genitalia possesses an adaptation that leads to conflict between the sexes. In other animals as well, penile spines have been studied in relation to the harm they cause females. Penile spines in male seed beetles damage females after just one copulation event (Ronn et al. 2007). Interestingly, female seed beetles can adapt to these spines; this study demonstrated that as penile spines increased, the thickness of the reproductive tract wall in females also increased. As both the males and the females are evolving adaptations in response to the genitalia of the opposite sex, this demonstrates that a sexually antagonistic arms race is occurring, indicative of sexual conflict. Although studies have not investigated the relationship between increased hemipenile spines and the thickness of the reproductive tract wall in female snakes, this relationship is highly probable, as hemipenile spines are clearly damaging to females (Friesen et al. 2014). Female snakes could be expected to adapt in a way that protects them from these spines, as the female seed beetles do.

Another variation in the shape of the male hemipenis in snakes is the scoop. This scoop shape is present in the Eastern Montpellier snake (*Malpolon insignitus*), and it is quite prominent (Andonov et al. 2017). To date, there is no explanation for this shape, however, evidence in other non-snake species may point to a possible function for this scoop shape. In damselflies, the scooped shaped tip of the penis functions in removing the sperm of other males from inside the female reproductive tract (Waage 1979). Many female snakes can store sperm (Friesen et al. 2013a) and mate with multiple males, so this may explain the scoop shape in the hemipenes of *Malpolon insignitus* (Andonov et al. 2017). If true, this male genital characteristic would support the sexual conflict hypothesis as an explanation of variation and coevolution in snake genitalia.

Further, another hemipenile characteristic that may explain the coevolution of genitalia via sexual conflict is the presence of bilobes. King and colleagues (2009) observed longer copulations in Plains garter snakes (*Thamnophis radix*) with bilobed hemipenes; this was paired with female body rolling behavior and resistance to the copulation (King et al. 2009). This demonstrates that a characteristic of the male genitalia is causing conflict, as the females did not want to engage in copulation that long. The presence of bilobes enables males to gain control over copulation in a way that directly benefits them. Moreover, as time goes

on, female snakes in species with males possessing bilobed hemipenes may evolve resistance to this trait through a physical genital adaptation. Aside from hemipenile characteristics, males have other genital adaptations, such as the ability to insert a mating plug into females, which is observed in garter snakes (*Thamnophis sirtalis*) (Friesen et al. 2013b). Sperm plugs enable the sperm to be inserted inside the female and gradually released, improving the mating success rate. It may also prevent sperm from another male from fertilizing the female (Mangels et al. 2016). This ability represents a male genital adaptation to override the female behavioral adaptation to end copulation early and prevent the male from depositing enough sperm; in this instance, the male and female adaptations are in conflict with each other, demonstrating sexual conflict. Additional studies have also demonstrated this behavior in female snakes, indicating that sperm plug deposition may be common (King et al. 2009; Shine et al. 2003). With these examples, it is important to note that behaviors that exist to prevent or sustain copulation can be included in the definition of genital coevolution, as they too can influence the evolution of genital structures (Brennan, 2016). Behaviors can be just as important as genital structure in driving genital coevolution.

Female genital adaptations

Female snakes also have genital adaptations that work against the male genitalia, leading to sexual conflict. Friesen and colleagues (2014) found that after anesthetizing the female pouch in red-sided garter snakes (*Thamnophis sirtalis*), copulation was much longer, ending when the male ended it. It is important to note that the male also possessed hemipenile spines to support a long copulation (Friesen et al. 2014). Through this experiment, they discovered that female pouches are muscularized, allowing the female to contract the pouch and expel the male hemipenis prematurely. This demonstrates that the female pouch, which is shown to have large interspecific variation (Siegel et al. 2012), is an adaptation to gain control over copulation and counter male genital adaptations, such as hemipenile spines or sperm plugs. Thus, the female pouch is a genital characteristic that opposes male genital adaptations, supporting the sexual conflict hypothesis for genital coevolution.

Female behavioral adaptations

Similarly, as briefly mentioned prior, female snakes also have behavioral adaptations that provide evidence of sexual conflict driving genital coevolution. In several different snake species, female behaviors to end copulation early have been observed, which supports the idea that male snake genitalia are causing sexual conflict. Females have been observed body rolling to terminate copulation (King et al. 2009), and they have also been observed displaying behavior to directly counter forcible insemination (Shine et al. 2003). Shine and colleagues (2003) demonstrated that male garter snakes (*Thamnophis sirtalis*) exploit the physiology of the female snakes, initiating hypoxic stress which causes them to raise their tail and gape their cloaca. Although these behaviors are not directly related to genitalia, they provide an opportunity for genital adaptations to evolve in the future, in response to these behavioral traits. Female snakes may acquire a genital adaptation that allows them to gain control over copulation in response to this forcible insemination.

Cryptic Female Choice

Cryptic female choice is the final hypothesis proposed to explain causes of genital coevolution. This hypothesis suggests that females select for certain male genitalia that stimulates them in a way that indicates mate quality (Brennan & Prum 2015; Eberhard 2010). Generally, the evidence for cryptic female choice driving genital coevolution in snakes is quite weak. Traditionally, spines have been thought of as a possible stimulator, allowing females to select for certain males that provide that stimulation; however, this idea has been refuted. As discussed earlier under the sexual conflict hypothesis, spines cause damage to the female. Female red-sided garter snakes were observed bleeding during and after copulating with a male with spined hemipenes (Friesen et al. 2014). Additionally, in seed beetles, hemipenile spines are also damaging, showing damage to the female reproductive tract after just one instance of mating (Ronn et al. 2007). Under cryptic female choice, a male trait that stimulates the female in a way that enables her to exhibit choice will not cause damage.

Further, weak evidence also presents a possible instance of female choice occurring in snakes. Friesen et al. (2013) demonstrated that red-sided garter snake females do not appear to be inherently receptive to sperm, as when males were prevented from depositing mating plugs, sperm leaked out of the female cloaca. This seems disadvantageous, but it may be a female adaptation for exhibiting choice. If females do not want sperm from a certain male, they may allow it to leak out, instead of contracting it up into the reproductive tract. However, it is unknown if in a different case, perhaps with a high genetic quality male, the outcome would be different. To test this, a quality male and a low-quality male would need to be bred with a female to observe her behavior of sperm uptake and leakage. If the female allowed less sperm from the quality male to leak, the evidence would provide support for a female genital adaptation driving genital coevolution. Moreover, as mentioned prior, the intraspecific variation observed in *Calamaria lumbricoidea* by Inger & Marx (1962) may be due to female choice. Intraspecific variation in genitalia suggests that sexual selection is occurring via female choice, specifically, cryptic female choice (Gilligan & John 2006). In intraspecific populations, males vary in quality and females choose among them, driving variance in primary and secondary sex characteristics, such as genitalia. Thus, it is quite possible that this intraspecific variation in *C. lumbricoidea* is due to female choice for a specific genital characteristic. Further research would be beneficial to confirm if this species needs stimulation in order to become pregnant, as seen in domestic pigs (Bonet et al. 2013). If stimulation is needed in snakes, that would be substantial evidence for this hypothesis.

Conclusion

As addressed in this review, significant and convincing evidence exists to provide reasoning behind genital coevolution and corresponding variation in snakes. The sexual conflict and lock-and-key hypotheses appear to have the most substantial evidence behind them, with cryptic female choice having the least support; however, it appears that each unique species and situation has a different hypothesis that strongly supports it. Certain species have very convincing evidence for one hypothesis, but others present evidence that refute that same hypothesis. There may not be one answer for all snakes that explains genital coevolution and corresponding variance. We should, however, before coming to a definite conclusion, investigate female genitalia in greater depth, as many unanswered questions lie in the female genitalia. Nonetheless, this review highlights important areas of biology, and sheds light on how studying genitalia may be useful in other areas of ecology, conservation biology, as well as evolutionary biology. Studying genitalia has implications in defining species, which serves purposes in conservation biology. Determining if species are reproductively isolated or not may yield them a better chance at conservation, as the Endangered Species Act employs the Biological Species Concept, a concept which defines a species based on reproductive isolation (Wheeler & Meier 2000). Additionally, studying snake genitalia in particular may shed light on the evolutionary origins of the hemipenis, and help gain a better understanding of the vastness of reptile genitalia in general. Most importantly, future studies are needed to investigate female snake genitalia in greater depth, as it is overall still widely understudied (Ah-King et al. 2014). Studying female genitalia will allow further confirmation of these three hypotheses, either for the whole group of snakes, or for individual species or families of snakes. Furthermore, future studies should be completed on the genitalia of snake species living in sympatry, as further evidence of interspecific variation in sympatric species would help support the lock-and-key hypothesis.

Red hue in Tadpole tails decreases based on predator presence

Lea Wong, Frances Rice, Joy Shelton and Flavia Barbosa
Lake Forest College
Lake Forest, Illinois 60045

Introduction

Plasticity refers to an organism's ability to display differential phenotypes according to its environment (Xuea and Leibler 2018). Such capability of change and adaptation allows for many organisms to have higher fitness. Many organism's physical environments continuously vary due to climate change, which leads to higher temperatures and elevated sea levels. Furthermore, organisms must also display plasticity in the presence of predators for survival. An organism's change in shape and color is typically linked to predator-induced phenotypic plasticity (Arnett and Kinnison 2017). Predation risk becomes higher if organisms do not adjust to different predatorial signals present in their environment. Adjustment to these signals can be size, as a smaller size could allow an organism to escape quickly or color to decrease predator visibility. Further, predator-induced phenotypic plasticity has been studied in tadpoles, specifically in *Dendropsophus ebaraccatus*. Scientists who collected data from tadpoles in ponds near Gamboa, Panama examined the plasticity of tadpoles from shaded environments containing predators and compared them to their study ponds (Touchon and Warkentin 2008). This allowed them to analyze the predator's likelihood of consuming an egg from a particular environment. Touchon and Warkentin mainly observed the phenotypic differences shown on the tails of the tadpoles, not specific color quantities. Through detailed examination of Touchon and Warkentin's research, we investigated how predators influence the value of red color on the tails of tadpoles, *Dendropsophus ebaraccatus*. Hence, we aimed to determine if the average red value was influenced by predators through image analysis. Based on this information, we hypothesized that if tail color is plastic within the tadpoles, the tadpoles with redder, thus darker, tails will be more prevalent in an environment with predators. Thus, their tails will have an increased average red value. The darker color would allow the tadpoles to decrease their visibility to predators and become less likely to be consumed compared to tadpoles with more transparent, less red tails. Since the red color is similar to foliage and sludge found at the bottom of the ponds, the tadpoles will be able to camouflage themselves and potentially avoid any ambush attacks. To test our hypothesis, we compared the average red color value of those tadpoles in the presence of a dragonfly predator and to a control group comparing mean values of the control group to the insect predator group.

Methods

The goal of Touchon and Warkentin's experiment was to investigate phenotypic plasticity in tadpoles of tree frogs (*Dendropsophus ebraccatus*). Specifically, they were looking at how the tadpoles develop under control conditions, with dragonfly predators, or with fish predators. Their methods consisted of taking seven mating pairs of the tree frogs from the Quarry Pond in Gamboa, Panama. Then, mating pairs were placed in plastic bags with water, and they were left to breed overnight. There were 2100 eggs, and they were returned to the pond the following day. A couple of days later, they collected 20 of each predator from the same pond. Then, the tadpoles were divided into groups containing 20 tadpoles in each container; the container had another section with either a predator or no predator for the control group. They documented the phenotypes of the tadpoles by taking photographs of them, in groups of 5. For conducting our experiment, we measured average redness in tadpole tails in an insect predator environment compared to the control environment. We looked at 40 tadpoles from each environment. To prevent measurer bias, we divided the image analysis evenly to ensure not just one person was measuring the control, for example. Using the software ImageJ, combined with the tadpole images given to us from the experiment above, we were able to analyze average redness. First, we measured the tails. We defined the tail as the distance from the base of the iridescent mouth bubble to the tip of the narrow tail tip (Figure 1). Then, we divided the length in half for a controlled area (Figure 1). By doing this, we determined the area of the tail proportional to body size. We outlined the second half of the tail using the "polygon" tool, and then we used this area to measure the RGB, specifically, the average red value. Lastly, we conducted a one-tailed t-test

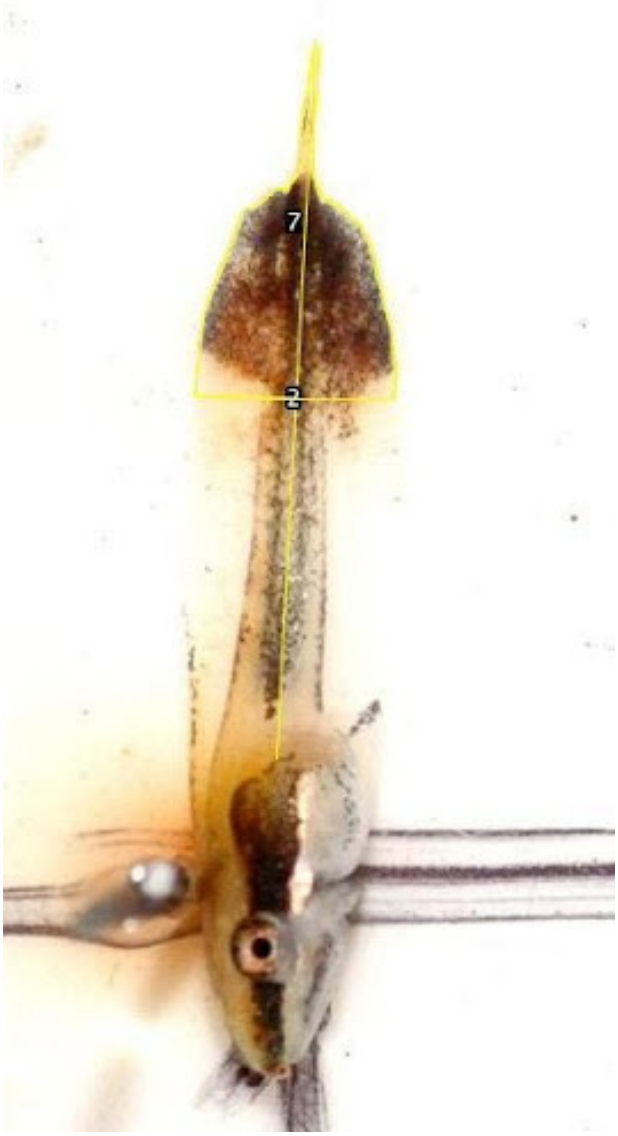
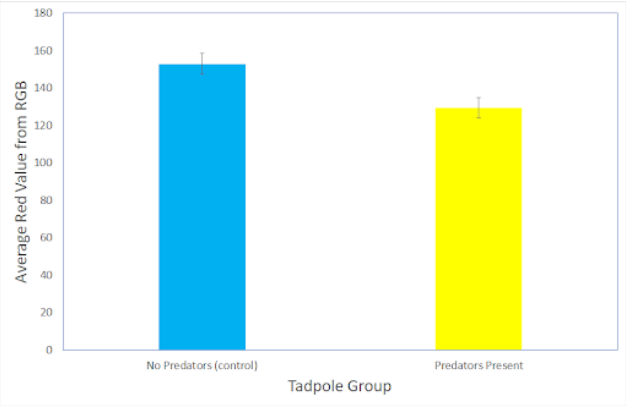


Figure 1:
Measurement of the tail. Line 2 represents the length of the tail. The halfway point of the tail is where the 2 is marked, and measurement 7 represents the area that was used to measure the colored part of the tail.

Results

Our data shows that the average red value decreased when the tadpoles were present with insect predators (Graph 1). Average red value was highest when tadpoles were not present with predators (Graph 1). The mean red value for the control group was 152.91, and the mean value for the insect predator group was 129.36. Our t-test determined that there was a significant difference between the control group and the predator group ($T78= 3.069$, $p < 0.0015$). et al., 2014). These neurodegenerative diseases, along with some other representative examples, are showcased in Figure 3a.

Figure 1:
Average Red Color Present on Tadpole Tails in Environments with Predators and without Predators: The x-axis represents the types of tadpole groups (n=40). The blue bar represents the No Predators (control) tadpole group, and the yellow bar represents the Predator tadpole group. The y-axis represents the average red value from RGB measurements,



with 255 being the reddest value and 0 being no red present. The standard error for the blue bar is 5.45, and the standard error for the yellow bar is 5.42.

Discussion

We report a significant difference in red color on tadpole tails in *D. ebraccatus*. Specifically, we observed a higher average red value on the tails of the tadpoles in the control group, and a lower average red value on the tails of the tadpoles in the group containing dragonfly predators. Although significant, these data do not support our hypothesis, as the tadpoles in the predator-laden environment did not have tails with a higher average red value as predicted. However, our data does support the presence of predator-induced phenotypic plasticity in these tadpoles, as the difference in tail color based on predator presence was significant. Our results align with previous studies that have demonstrated that such a shift in phenotype in the presence of a predator is due to plasticity (Arnett and Kinnison 2017).

Discrepancies between our results and our hypothesis may have been due to improper color analysis; average redness may have not been the best measure. The tadpole tails in the predator-laden environment presented a reddish, brownish tail color, whereas the control group tails presented a brighter and lighter red orange color. Because of the color composition of brown, the RGB values may have been skewed, yielding semi-equal quantities of each color, and in turn, decreasing the average red value. Furthermore, the vibrancy of the tail color may have skewed the average red value. The control group tails were much more vibrant and had higher average red values. This indicates that vibrancy may equate to a purer red color. In a study investigating predator-induced color change and camouflage in horned ghost crabs, *Ocypode ceratophthalmus*, researchers observed changes in both color and saturation, a measure of brightness (Stevens et al. 2013). Saturation may be more important in color analysis than previously thought. Thus, in the future, measuring saturation may be a better way to analyze the chromatic differences in the tails of *D. ebraccatus* tadpoles.

Although this plastic trait may be beneficial in predator avoidance, it may have negative implications in energy and resource allocation, as predator-induced phenotypic plasticity has been shown to be quite costly. McCollum and Leimberger (1997) demonstrated how costly this plasticity can be by providing evidence of tadpoles only showing a color change in a predator-laden environment when they were well fed. This evidence demonstrates that plastic traits are so costly that they require a surplus of resources. If *D. ebraccatus* tadpoles were to not require a surplus of resources to induce this plasticity, they may suffer stunted growth if energy is allocated to plasticity instead of development. It is unknown whether *D. ebraccatus* tadpoles allocate their energy and resources in a similar way, and so, this would be a worthwhile avenue for future research.

Moreover, this study has important applications in other areas of research, specifically, amphibian conservation. Plasticity in tail color may have benefits in protection from harmful UV radiation in *D. ebraccatus* tadpoles. UV radiation is known to be detrimental to frogs, as a review documenting amphibian decline cited numerous cases where increased UV radiation has caused stunted growth, malformations, and slowed development (Alton and Franklin 2017). As tadpoles are at the most critical post-embryonic developmental stage, it can be assumed that UV radiation is most detrimental for them. Having plasticity in tail color can be extremely beneficial in these cases, as a darker tail yields a higher melanin content, equating to increased UV radiation protection. This trait, although currently present for predator avoidance, may be co-opted for UV radiation protection in the future, especially as the ozone layer continues to thin. Future studies should investigate the differences in UV ray protection between tadpoles reared without predators and tadpoles reared with predators to determine if the co-option of this trait for UV ray protection could be a possibility for *D. ebraccatus*.

Hypothetical Protein PA5198 (Yestervin) indicated to be an LD-Carboxypeptidase within Pseudomonas Aeruginosa

Mathieu Norcross, Co: William Conrad, Makenzie Kamm, Tate Rosenhagen, Allyson Richards, Kayenath Khan, Leslie Gonzales, Hannah Kobienia, Sergio Sosa, Anthony Sullivan, George Vladimirov. Bomby Ahuja-Kittiphanh
Lake Forest College
Lake Forest, Illinois 60045

Abstract
The present experiment investigates the function of protein PA5198 found in *Pseudomonas Aeruginosa*, known as Yestervin, using a series of different bioinformatic methods. Preliminary research from the protein data bank provides an enzyme consortium number of 3.4.17.13. These findings led to the hypothesis that Yestervin acts as an LD-Carboxypeptidase in biological systems. Bioinformatic tools used to confirm the function of Yestervin are as followed: PyMol identification of active sites within Yestervin using motif comparison, BLAST/SMART BLAST sequence comparisons between Yestervin and proteins of known function, Dali global alignment comparing carbon backbone structures of known proteins and Yestervin, Pyrx autodocking identifying ligand binding to a Yestervin-like protein's active site amino acid residues, protein purification and kinetic evaluation through PNPA enzymatic assay. Results from these bioinformatic tools indicate that Yestervin is indeed a LD-Carboxypeptidase used to cleave amide bonds between L and D amino acids occurring naturally in bacterial peptidoglycan. These findings could play an important role in treatment development of *Pseudomonas Aeruginosa* infections, as well as provide mechanisms for fighting antibiotic-resistant bacteria in a hospital setting.

Introduction
The vast gap of knowledge surrounding discovered proteins and their function serves as a setback for advancing knowledge in many fields of Biology. To date, around 20% of protein in even the most well-studied organisms have no descriptions on their roles in biological systems (Wood et al. 2019). These proteins could serve to explain a multitude of mechanisms that have been previously misunderstood and could open doors to many new discoveries in the field of Biology.

The hypothetical protein PA5198, also known as Yestervin, found in *Pseudomonas Aeruginosa*, is a protein of unknown function requiring further inquiry. The unknown protein has an R-free value of 0.125 with 0.2% Ramachandran outliers, a total of six unique ligands (L(+)-Tartaric Acid, Di(Hydroxyethyl)ether, Glycerol, 1,2-Ethanediol, Potassium and Sodium ions), and PDB ID labeling it protein 1ZL0. Little is known about the protein of unknown function, however, it is suggested that the protein is possibly an LD-Carboxypeptidase. The enzyme consortium number (EC 3.4.17.13), which matches that of the unknown protein, suggests that the unknown protein is a LD-Carboxypeptidase known for the ability to cleave amide bonds between L and D amino acids occurring naturally in bacterial peptidoglycan. This severing of L and D amino acids takes tetrapeptides, truncates them into tripeptides, and then to peptidoglycan building blocks for the bacteria. LD-Carboxypeptidases have been the target of antibiotics due to their importance. The sensitivity to lactam antibiotics varies widely between antibiotics of the same class but correlates with the chirality of the amino acids of the antibiotic used.

The catalytic class of LD-Carboxypeptidases is unknown, being named the U61 family. In *Pseudomonas aeruginosa* this U61 enzyme was found to have LD-Carboxypeptidase activity. A study by Korza & Bochtler (2005) found that the *Pseudomonas aeruginosa* recombinant converted tetrapeptides to tripeptides and had a structure of a serine peptidase with a Ser-His-Glu catalytic triad. The Ser115 and His285 of *Pseudomonas aeruginosa* were ruled out as part of the enzyme as the activity was not homologous to LD-Carboxypeptidase, further narrowing down the structure of the unknown protein in question. This carboxypeptidase in question was further investigated by Xu et al. (2020) focusing on the Carbapenem Resistance in *Pseudomonas aeruginosa* infections. Carbapenem is used to treat *Pseudomonas aeruginosa* infections in a clinical setting, but recent resistance to Carbapenem has led to further investigation into the mechanism behind the resistance. It was found that the *ldcA* (PA5198) gene encoding for murein tetrapeptide carboxypeptidase negatively influences *ampC* expression in *Pseudomonas aeruginosa*. The *ampC* expression is an important mechanism in bacteria that helps them develop resistance to many anti-bacterial drugs. With murein tetrapeptide carboxypeptidase expression leading to decreased expression of *ampC*, a mechanism using carboxypeptidases to combat drug resistance in bacteria could arise (Xu et al. 2020). The resistance to Carbapenem is due to a multitude of overexpressed and under-expressed genes that produce varying amounts of proteins. One of these overexpressions that allows Carbapenem resistance is the overexpression on the chromosomal gene *ampC* encoding for intrinsic cephalosporinase in *Pseudomonas aeruginosa*. This overexpression links carboxypeptidase to Carbapenem resistance, allowing for further research into therapeutic treatments regarding the carboxypeptidase in *Pseudomonas aeruginosa*.

Understanding the unknown protein Yestervin in *Pseudomonas aeruginosa* is important because of the possible clinical implications it could have. *Pseudomonas aeruginosa* is the most common-gram negative bacterium found in nosocomial infections. The bacteria is responsible for 16% of nosocomial pneumonia cases, 12% hospital-acquired urinary tract infections, 8% of surgical wound infections, 10% of bloodstream infections, and 38% of ventilator-associated pneumonia deaths (Bodey et al. 1983). The bacterium is most problematic in immunocompromised patients, as it is an opportunistic infection because of the bacteria's presence throughout the natural environment. Once in the body, the infection is fast at causing problems throughout the body, from endocarditis in the heart, to eye problems, to urinary tract infections. The bacteria produces several extracellular products responsible for the extensive bodily damage and has been resistance or poorly treated with a number of different antibiotics throughout history (Van Delden & Iglewski 1998). The Yestervin protein could be the missing link in effective therapeutic treatments for *Pseudomonas aeruginosa*, which is why further investigation is a necessary process.

In this experiment, the Yestervin protein (1ZL0) was investigated using a series of different structural and sequential bioinformatic databases. These databases compared the hypothetical protein's structure and amino acid sequence to proteins of known function. From these results, it is hypothesized the Yestervin protein is acting as a LD-Carboxypeptidase within biological systems.

Methods
PyMol/ProMol Methods:
To identify proteins with similar structural active sites, PyMol/ProMol structural alignment was performed. The Pymol program was accessed through Microsoft Remote Desktop app. Once opened, the Promol application was accessed through the Plugin tab. Under the Promol interface, the Motif Finder tab was accessed, and the PDB name (1ZL0) was entered in the query box. Once a PDB name was entered, a set including all motifs is selected and RMSD values were calculated. The Motif Finder search parameters were unaltered, and a full protein structure was generated for Yestervin. The result pane under the motif finder lists all the active sites found on the protein entered. The results list Levenshtein distance, identity of the motif template, RMSD values, and an EC class for the motif selected. Using the RMSD values found under each motif, find the motifs with the lowest RMSD values. A motif with a low RMSD value is selected, and motif alignment is visualized through Promol. A visualization of active site alignment between the motif and the protein was generated and analyzed. The molecules generated are then identified for their residues. Clicking on each residue of the alignment shows the amino acid linked in the motif and how many atoms are in the respective alignment in the Pymol GUI interface. All values and visual representations of motifs were recorded, and values were analyzed to see if the unknown protein being studied best fit the motif of certain EC classes.

BLAST/Pfam Methods:
To identify proteins with similar sequences and domains compared to Yeservin, we performed a BLASTP and Pfam sequence search. The FATSA sequence for the Yestervin protein was obtained from https://www.rcsb.org/. This FASTA sequence was put into the BLAST query box and submit-

ted with no changes to the original BLASTP search criteria and the protein sequence was analyzed. Once the sequence was analyzed, a graphical summary is presented showing the top 100 sequences in the BLAST databank that overlap with the sequence of the query protein. The graphic summary was captured and analyzed. The top three alignments of each protein were looked at in detail and examined for their E-values and percent overlap. Once a general BLAST was run, a SMART BLAST was run Yestervin. The SMART BLAST provided a phylogenetic tree that showed how each protein relates to proteins found in other organisms, and where common ancestors arose between Yestervin and known proteins in specific organisms. Once all information is collected, the data is analyzed, and a conclusion regarding the possible function of the unknown protein was reached. The FASTA sequence for Yestervin was then put into the sequence query box (https://pfam.xfam.org/search#tabview=tab0) and run based on default search parameters. Once the protein's sequence was analyzed, all the protein superfamilies that match up with query protein sequence were displayed. Clicking on each protein superfamily gives a summary of the family with an example structure, and literature where the protein superfamily is discussed. Under the "domain organization" tab, common sequence architecture found within the protein superfamily is detailed. Under the "HMM logo" tab, the conserved amino acid residues are detailed on each sequence position. Once all Pfam data is collected, the data is analyzed. From this data, the possible function of the unknown protein is deduced and compared to data and conclusions from the BLAST and ProMol data collected earlier.

Dali Global Alignment Methods:

To identify proteins with similar 3D carbon backbones, we performed a Dali 3D Global alignment. The Dali search query was accessed at http://ekhidna2.biocenter.helsinki.fi/dali/. From there, the PDB search tab was selected and the PDB ID for each respective protein was added into the search bar. After Dali search was completed, the matches against full PDB were selected. These results were downloaded and put into raw data storage for further analysis following this lab. A summary of all matching proteins is presented with RMSD, Z-score, LALI, NRES, and %ID values. A description of each protein is provided at the end of each matching chain. From this summary page, three proteins of differing function were selected for further analysis. These proteins with different functions were analyzed using a 3D image with cartoon view and structural conservation. Each protein and its overlap with the query protein were captured and added to their respective tables for further data analysis. Following the analysis of 3D image superposition, the three proteins of differing functions were compared to the active sites of Yeservin found in the previous ProMol lab. Each protein of known function was selected, and the "structural alignment" tab was selected. Using the previously determined active sites for Yestervin, each protein of known function was analyzed to see if the active sites of our unknown proteins were conserved in the proteins of known functions. This was done by determining the first three amino acids before the conserved active site amino acid from the PDB ID of our unknown sequence. Using this four-letter amino acid sequence, a search was performed on the "structural alignment" tab. The four-letter amino acid sequence was found for the query protein and was compared with the protein of known function in the "structural alignment" tab. The four-letter amino acid sequence for the known protein that matched up with the query protein was recorded to see if the active site amino acid was conserved. Using all this data, the proteins of known function were determined to be good fits or not, and a hypothesis on both protein 2O14 and 1ZL0's function was created.

Autodock Pyrx Methods:

A hypothetical reaction for the Yestervin protein has been hypothesized using previous experiments. The AutoDock program was used to look at what ligands are used in the hypothesized reaction. The protein used in the AutoDock experiment is protein 5Z01 which is Murein Tetrapeptide Carboxypeptidase. This protein was used because Yestervin could not be loaded into the AutoDock program, so the protein 5Z01 was used because it has a similar carbon backbone and similar hypothetical function to the Yestervin protein. The PDB file for protein 5Z01 was downloaded from the protein data bank, and was uploaded to the AutoDock program. The E.C. Class 3.4.17.13 was put into the protein data bank search bar and the ligands that are commonly found for that EC class were observed. A total of five different ligands that were found in that EC class were selected (TLA, PEG, DMU, DEU, and ABU). Each ligand was saved as a .sdf file onto the computer and added into the AutoDock program. Once the protein and the ligands were loaded into the AutoDock program, Vina Wizard was run with all the ligands and protein 5Z01 selected. Once the program was run, the analysis for each ligand compared to protein 5Z01 was displayed. The list of all the ligands and the statistical analysis for each ligand were saved and stored in raw data storage. Once data was saved, the free energies of binding for each conformation of each ligand were observed. The ligand conformation with the most negative binding energy was visually observed in the AutoDock program and saved as a PDB file. Once the PDB file is saved, PyMol is opened and both the ligand and protein PDB files are added into the program. The PyMol visualizes the ligand inside the protein and the surface function shows the ligand fitting into binding sites on the protein. The amino acid residues that bind to the ligand at the binding site are visualized within PyMol and recorded in the results section. Pictures of the ligand within the protein binding site and the amino acid residues that help bind the ligand to the protein were recorded as figures within the results section.

Nickel-His purification of Yestervin:

The purpose of the Nickel-His purification is to separate the Yestervin protein from other non-specific proteins in order to further study the properties of Yestervin. A column with nickel ions present is used to separate Yestervin from other proteins. A binding buffer composed of 50mM Na2HPO4, 300mM NaCl pH 8.0 supplemented with 10mM imidazole is added to the Nickel-His column along with the protein solution. This buffer solution reduces the non-specific interactions between other proteins and the column, allowing our protein of interest to bind to the column specifically while also allowing Yestervin to remain in its native state. The pH of the buffer allows for Nickel-His binding to occur within the column, while also not denaturing the protein because of its neutral pH level. A wash buffer composed of 50mM Na2HPO4, 300mM NaCl pH 8.0 supplemented with 20mM imidazole is then added to the column in three successive additions. This wash buffer specifically elutes all proteins that are not of interest, leaving the POI attached to the nickel ions within the column. Finally, an elution buffer composed of 50mM Na2HPO4, 300mM NaCl, and 250mM imidazole at pH 8.0.A is added to the column. The excess amount of imidazole binds to the nickel ions, forcing the POI off the column, causing it to elute into fractions. A total of 3 elution additions are added to the column until there is no POI being eluted from the column anymore. The different fractions are run through a Gel Electrophoresis to see the contents of each of the fractions. The lysate shows all proteins present within the protein solution, including the Yestervin protein. The flow-through shows proteins that are eluted during the addition of binding buffer. The three different wash additions elute the proteins that remain in the column that are not the Yestervin protein. The elution fractions contain all of the purified Yestervin proteins separated from all other proteins. These separated Yestervin proteins are set aside for further research in order to determine their function in biological systems.

Protein yfkn and 2O14 purification through 6X-His protein spin column:

The purpose of the 6X-His protein column purification was to purify the protein of interest, yfkn, along with protein 2O14 for positive control. The procedure for this experiment was performed by Dr. William Conrad at Lake Forest College. Professor Conrad followed the HOOK protein purification spin protocol to purify protein yfkn and 2O14. The BL21 (DE3) E. Coli with six different plasmids were transformed and the bacteria was grown overnight in LB broth containing 100 ug/ml ampicillin at 37 C shaking at 240 rpm. Once grown, a total of 1 ml of bacteria was transferred into autoinduction media which contained lactose that induced bacterial expression. The HOOK bioscience protocol was then followed by Professor Conrad as directed, except no protease inhibitor was added into the samples. Once samples were created following the HOOK bioscience protocol, protein expression was observed in bacterial strains by placing 15 ul of bacteria in 5 ul 4X SDS PAGE loading buffer, which breaks the bacterial cells and linearizes the protein. The buffer also contains 1 ul TCEP which breaks any disulfide bonds. The samples were then denatured at 95 degrees C and 10 ul of both protein samples were added to a 15-lane 16% tricine gel. A 10 ul bio-rad precision protein was run through the gel to ensure identification of molecular weights of the proteins. The proteins were then lysed according to the HOOK purification kit and all fractions were saved and loaded into the gels. The bacterial cells were harvested from a 50 ml bacterial culture by centrifugation at 5,000xg for 10 minutes. The bacterial pellets were then resuspended in 2mL bacterial PE LB until the suspension was deemed homogenous. The sample was vortexed and 5-50 ul PEL-B™-Lysozyme was added before the sample was incubated at 37 degrees C for 30-60 minutes. The sample was vortexed again for 30 minutes after

incubation. This vortex occurred before soluble proteins were separated and was centrifuged at 25,000xg for 15 minutes. The now clarified lysate was transferred to a 15 ml centrifuge tube and was swirled until a homogenous slurry formed. A 0.8 ml 50% resin slurry solution was then transferred to the lysate before being incubated for 15 minutes at room temperature. The tube was then centrifuged at 1,500xg for 5 minutes to pellet the resin. The supernatant was discarded, and the resin was suspended in a 250 ul wash buffer. The 6X-His spin column was then added to a collection tube and then centrifuged for 2 minutes at 2,000xg. The flow-through was collected and the spin column was returned to the collection tube where 500 ul of wash buffer was added to wash away any unbound or nonspecific proteins. Once the wash buffer was added a second time, the column was eluted. The elution of the 6X His tagged proteins occurred through the addition of 0.5 ml elution buffer to the resin and the incubation of the column for 5 minutes at room temperature. The spin column was then centrifuged for 2 minutes at 2,000xg. The flow through was transferred to a 1.5 ml centrifuge tube and the spin column was then returned to the collection tube. This process was repeated three times to create three total elution trials. All of the proteins from the collection tubes were then added to a 15-lane 16% tricine gel where the results of the purification were analyzed to determine if protein yfkn and 2O14 were properly purified (methods were obtained from Professor Conrad's description on lab archives and through the HOOK 6X His Protein Spin Purification Protocol provided).

Protein Kinetics analysis:

The purpose of this experiment is to determine enzyme kinetics such as Vmax and Km values for both protein 2O14 and yfkn 3GVE. This was done using an assay measuring absorbance of the conversion of substrate to product of the hydrolysis of PNPA. This hydrolysis occurs in the presence of hydrolase enzymes, therefore high absorbance and Vmax values indicate an enzyme is a hydrolase. A 2O14 elution 1 and 3GVE (yfkn) elution 1 sample was created after being dialyzed into 50mM sodium phosphate buffer. The samples were spun in a Pierce 3 kDa MWCO centrifiugal filter concentrator three times using 20 mL of buffer and 1 mL of elution. The samples' protein abundance was measured using absorbance at 280nm with a path length of 1 cm, while the extinction coefficient for each protein was found through https://web.expasy.org/protparam/. Using a 96 well plate, each well was filled with 160 ul of 50mM sodium phosphate buffer. Then in each column, 10mM PNPA was added in the wells with a volume of 0-20 ul. A total of 20 ul of 2O14 and 3GVE were added to Rows A and C respectively and observed for changes in absorbance over time. The data was recorded into an Excel document and Michaelis-Menten plots, Lineweaver-Burke plots, and other protein kinetic values were calculated using the absorbance data. Substrate concentration and initial velocity values (calculated from absorbance) were calculated and used to create the Michaelis-Menten graph. The substrate concentration and initial velocity values were then used to find the 1/V0 and 1/[S] values used to create the Lineweaver-Burke plot. The lineweaver-burke plot was used to calculate the Km and Vmax values for each protein, which then was used to calculate other protein kinetic values that quantitively showed the protein's enzymatic activity.

Results

PyMol/ProMol active site structural analysis suggest EC range for Yestervin protein:

The Yestervin protein is a protein of unknown function from *Pseudomonas aeruginosa*. The protein is a hypothetical protein with a sequence length of 311 and an EC= 3.4.17.13 (Source: https://www.rcsb.org/structure/1ZL0). Research into the EC class 3.4.17.13 suggests the protein is a LD-Carboxypeptidase known for the ability to cleave amide bonds between L and D amino acids occurring in bacterial peptidoglycan (Korza & Bochtler 2005). The EC class suggested in the PDB was tested through Promol analysis, which is a local structure-based program comparing active site residues between known proteins and Yestervin. The best fit criteria used in this experiment were motifs with a Levenshtein value of 0 and a RMSD value below 2. The Yestervin protein was expected to have active site alignment with motifs around EC = 3.4. The best fit active site for Yestervin protein was suggested to be motif 2ace. The 2ace motif has a Levenshtein value in the range of 0-4, RMSD All: 1.5116, RMSD alpha: 1.5092, and RMSD alpha and beta: 1.3696. The 2ace motif showed 3 alignments with similar residues in those alignments (His 285 Yestervin- His 440 2ace, Glu 217 Yestervin- Glu 327 2ace, and Ser 115 Yestervin- Ser 200 2ace) (*Figure 1*). The alignment visualization shown in *Figure 1* shows differences between the motif and Yestervin structures. The motif 2ace has an EC class of 3.1.1.7, which is not in the range of the expected EC of 3.4 for the protein Yestervin. The second-best active site was determined to be 1o2u. The 1o2u active site has a Levenshtein value of 0, RMSD All: 1.4783, RMSD alpha: 0.058, and RMSD alpha and beta: 0.6694. The overall motif alignment with the Yestervin protein is shown in *Figure 1*. The 1o2u motif was chosen as the second-best active site because it had less residue-matches compared to 2ace, but still had a Levenshtein value of 0 and a RMSD value below 1.5. The residue alignment between the 1o2u motif and the protein were found to be similar (His 51 Yestervin- His 57 1o2u, and Asp 69 Yestervin- Asp 102 1o2u) (*Figure 1*). The motif 1o2u has an EC class of 3.4.21.4, which falls in the predicted range of the Yestervin protein. The two best fit active sites found were from differing EC classes. The best fit active site belonged to the EC class of 3.1, while the second active site belonged to the predicted EC class of 3.4. Research into the difference between EC classes 3.1 and 3.4 shows the differing functions the two motifs that have significant active site overlap with Yestervin have. While both EC classes are hydrolases, EC class 3.1 are esterases acting on ester bonds, while EC class 3.4 are peptidases that act on peptide bonds. The two best fit active sites suggest that the Yestervin protein is either an esterase acting on ester bonds or a peptidase acting on peptide bonds. More analysis is needed to confirm Yestervin's EC class and its function in biological systems. In conclusion, the protein Yestervin needs further analysis in order to properly determine its function, as the active sites show conflicting EC values and functions in biological systems.

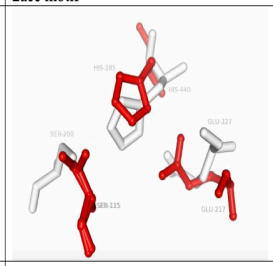
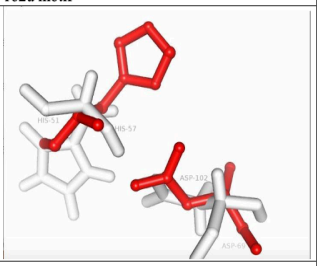
Motif	2ace motif	1o2u motif
Image of motif from PyMol program		
EC class	3.1.1.7	3.4.21.4
RMSD All	1.5116	1.5855
Levenshtein value range	0-4	0
# of residues	3	2
Yestervin residue	His 285, Glu 217, Ser 115	His 51, Asp 69
Matching motif residues	His 440, Glu 327, Ser 200	His 57, Asp 102

Figure 1: ProMol active site alignment between Yestervin (red, 1ZL0) and the 2ace and 1o2u motifs (white). The 2ace-Yestervin amino acid residue overlap occurred between three residues (a His, Glu, and Ser). Yestervin and the 2ace motif showed significant overlap with an RMSD value of 1.5116, meaning the motif and protein are closely aligned and thus may share similar functions. The 1o2u-Yestervin amino acid overlap occurred between two residues (a His, and Asp). Yestervin and the 1o2u motif showed significant overlap with an RMSD value of 1.5855 between the two amino acid residues suggesting the motif and Yestervin may share similar functions.

BLAST/SMART BLAST sequence alignment results indicate LD-Carboxypeptidase as a significant match to protein Yestervin.

From PyMol/ProMol analysis, protein Yestervin could not be conclusively determined to belong to a specific EC class. To help narrow down the possible functions, sequence alignment through BLAST programs were run to determine possible proteins with similar sequences. The protein Yestervin had significant BLAST findings that helped narrow down the function. The expected results for both the BLAST experiment were that the Yestervin protein would have significant sequence alignment with LD-Carboxypeptidases and any close relatives to the LD-Carboxypeptidases. The BLAST sequence alignment tool found significant overlap with LD-Carboxypeptidases. Both the BLAST and SMART BLAST showed that protein Yestervin had significant sequence overlap with LD-Carboxypeptidases found in a multitude of different bacteria, which is consistent as LD-Carboxypeptidases belong to prokaryotic bacteria and not in eukaryotic or archaic or-

ganisms (*Figure 2*). The BLAST experiment also indicated the superfamily Peptidase_S66 as the potential protein family that the protein Yestervin may belong to. The BLAST superfamily analysis showed a conserved catalytic triad (Ser-Glu-His) between the Yestervin protein, and multiple LD-Carboxypeptidases were found within different organisms in the Peptidase_S66 Family (*Figure 3*). These findings suggest that the protein Yestervin matches sequentially with LD-Carboxypeptidases. These LD-Carboxypeptidases have a function of hydrolyzing the amide bond that links dibasic amino acids to C-terminal D-amino acids. These LD-Carboxypeptidases have an EC: 3.4.17.13, which is the same EC value as Yestervin. From this data, we can hypothesize that the protein Yestervin shares the same function as LD-Carboxypeptidase and indeed does belong to the EC class 3.4.17.13 .

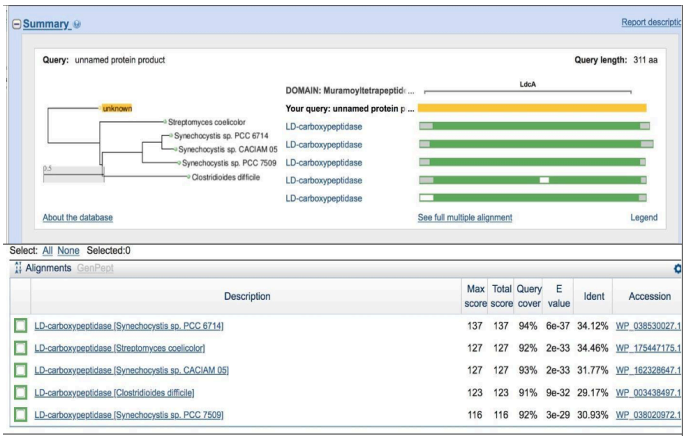


Figure 2: A SMART BLAST sequence search showing a phylogenetic tree of closely related evolutionary relatives between Yestervin and proteins from a multitude of proteins from different bacteria. This phylogenetic tree compares Yestervin to proteins of known function in a series of different model organisms. Yellow band shows the Yestervin sequence, while the green bands show sequence hits from different bacteria species. All significant hits for Yestervin show LD-Carboxypeptidases from different bacteria species.

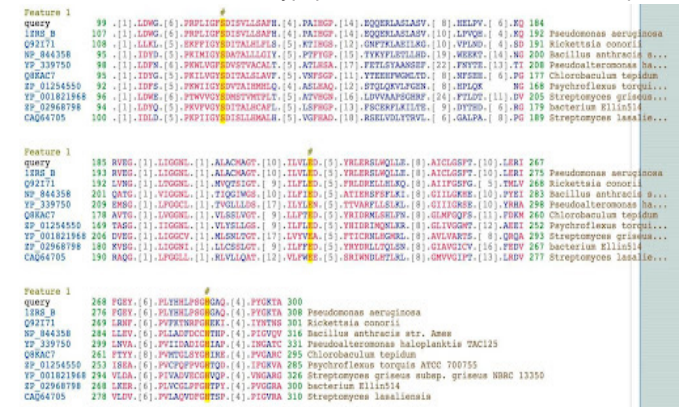


Figure 3: Conserved domain alignment from BLAST search between different bacteria species showing conserved catalytic triad between the Yestervin and LD-Carboxypeptidases from a multitude of different species. The Ser-His-Glu Triad was found in previous research by Korza & Bochler (2005). Highlighted amino acids indicate the conserved Ser-His-Glu triad. Red amino acids indicate conserved amino acids throughout all the different bacteria species, while the blue amino acids indicate amino acids that are not conserved throughout all observable bacteria species.

Dali Global Structural alignment shows the protein Yestervin's significant overlap with the Murein Tetrapeptide Carboxypeptidase protein 5Z01.

By BLAST sequence alignment, Yestervin was observed to have significant sequential overlap with known LD-Carboxypeptidases. These results were followed by searching for homologous structures rather than primary sequences using Global 3D alignment through the Dali search engine. This Dali search compared proteins of known function with similar alpha carbon backbone structure to Yestervin. These results allowed for both carbon backbone and active site structural site alignments to be examined between proteins of known function and Yestervin. The 3D superposition alignment between Yestervin and three proteins of known function provided more insight into Yestervin's function. The gap in knowledge surrounds Yestervin's function. The expected results are that Yestervin lines best with LD-Carboxypeptidases as indicated in previous BLAST experiments. The best fit carbon backbone alignment with Yestervin is protein 5Z01-A. The protein has a Z-score of 33.6, RMSD value of 2.1, and a 27% ID. The overlap between the two proteins is significant, with multiple coils and middle carbon backbone chains being conserved between 5Z01-A and Yestervin. However, there is no overlap with the Yestervin ligand groups and some of the outer carbon backbone chain of the 5Z01-A (*Figure 4*). The significant overlap is underscored by the high Z-score, low RMSD, and significant LALI value of 284. From these values and the visual, it can be concluded that Yestervin has significant overlap with protein 5ZO1-A which is found to be a Murein Tetrapeptide carboxypeptidase (*Figure 4*). Another significant alignment came from protein 3TYX-B. This protein had a Z-score of 33.3, an RMSD of 2.3, and percent ID of 20%. The 3D superposition image shows significant overlap between the protein's carbon backbone, but no overlap in the middle ligands of Yestervin. There also is lacking overlap with some of the outer carbon backbone of protein 3TYX-B. Besides the missing overlap on these structures, the alignment looks significant and is underscored by the high z-score, low RMSD, and high LALI of 332. The protein 3TYX-B is a Microcin immunity protein MCCF, which goes against the expected results (*Figure 4*). The third protein with significant overlap was protein 4INJ-A. This protein had a Z-score of 33.2, an RMSD of 2.1, and a percent ID of 26%. The carbon backbone shown on the 3D superposition image shows significant overlap between the middle of the carbon backbone for both proteins. However, the proteins don't match at the Yestervin middle ligands (green ball-like structures in *Figure 4* images), and at the top part of the Yestervin carbon backbone. The proteins also do not overlap at middle ligands of protein 4INJ-A and at the outer carbon backbone of protein 4INJ-A. Despite these discrepancies in overlap, the significant overlap between the middle backbones is underscored by the high z-score, high percent ID, low RMSD, and high LALI value of 326. Protein 4INJ-A is described as a LMO1638 Protein which is described as a hydrolase (*Figure 4*). This goes against the expected results of the matching protein being a carboxypeptidase. The active sites of the proposed catalytic triad within Yestervin were then compared to the Dali fit proteins to see if there was conserved active site alignment. The goal of this was to determine if the Dali proteins were good fits when it came to Yestervin's active site. To determine this, the active site residues for Yestervin were compared to each of the Dali fit protein sequences to see if the active site residues were conserved. In protein 5Z01-A, 2 of 3 active sites found in Yestervin (His 285, Ser 115) were conserved. The Glu residue in Yestervin was replaced with an Asn in protein 5Z01-A. These findings, along with 3D superposition data, suggest that Yestervin and 5Z01-A have similar structures and thus similar functions (*Figure 4*). The protein 5Z01-A is a Murein Tetrapeptide carboxypeptidase, which suggests that Yestervin acts as a carboxypeptidase in biological systems. Protein 3TYX-B had 2 of 3 conserved amino acids when compared to protein 1ZL0. The active site residue Glu 217 in Yestervin is replaced with a phenylalanine in protein 3TYX-B. Despite this discrepancy, all other data suggests that protein 3TYX-B is a good fit to Yestervin (*Figure 4*). However, the protein 3TYX-B does not match as well as protein 5Z01-A does, meaning the function of Yestervin is more likely to be a carboxypeptidase rather than a microcin immunity protein MCCF. The protein 4INJ-A showed little active site overlap when compared with Yestervin, having only the His 285 active site conserved. Using this data, protein 4INJ-A was determined to not be a good fit for Yestervin, and therefore does not share the same function as Yestervin. From all of this data, we can hypothesize that Yestervin is a carboxypeptidase. Yestervin fits best with protein 5Z01-A, which is a Murein Tetrapeptide carboxypeptidase. This data, along with data collected in BLAST and ProMol sequencing, suggest that Yestervin is a carboxypeptidase. Therefore, it is hypothesized that Yestervin acts as a carboxypeptidase within biological systems.

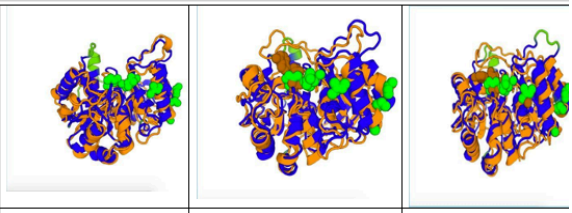
Chain/Description	5Z01-A Murein Tetrapeptide carboxypeptidase	3TYX-B Microcin immunity protein MCCF	4INJ-A LMO1638 Protein
Image Captured (Cartoon with Structure Conservation view)			
Active Site: His 285 PSGH	DFGH	DCCH	DFGH
Active Site: Glu 217 EDVG	EDIN	EDSS	EDDF
Active Site: Ser 115 IGFS	CGHS	IGYS	CGFA
# of active site matches	2	2	1
Good Fit?	Yes	Yes	No
Z-Score	33.6	33.3	33.2
RMSD	2.1	2.3	2.1
LALI (length of alignment)	284	287	280
NRES (Number of Residues)	305	332	326
%ID	27	20	26

Figure 4: Dali Global Alignment results showing significant carbon backbone and active site matching between Yestervin and proteins 5Z01-A and 3TYX-B. All three proteins (5Z01-A, 3TYX-B, 4INJ-A) were compared to Yestervin through protein superposition. The blue ribbons represent overlap between the protein in question and Yestervin. The orange ribbons represent the protein in the database that doesn't overlap with Yestervin, while the green ribbons represent Yestervin structures that don't overlap with the proteins in the database. The green balls within image represent Yestervin ligands not found on query proteins. After sequential comparisons, Protein 5Z01-A and 3TYX-B were found to be good matches with 2 out of 3 active site amino acids being conserved. Protein 4INJ-A was determined not to be a good fit because of the lack of overlap between Yestervin and 4INJ-A active site amino acids. Red letters show amino acids conserved between Yestervin and query protein while black letters show amino acids that are not conserved. The highlighted amino acids are the active site residues in Yestervin.

Pyrx Autodocking show ligands DEU and TLA binding to expected active site in protein 5Z01

Following Dali Global alignment matches, the ligands involved in the potential Yestervin reaction were found. The goal of this Autodock experiment was to determine specific ligands/substrates that bind to Yestervin and possibly play a role in the enzymatic reaction. The ligand binding of the Murein Tetrapeptide Carboxypeptidase 5Z01 was used to provide conclusive results into what the ligands are involved in the hypothetical reaction of the protein of interest Yestervin. The protein Yestervin was unable to be used for this experiment, as the AutoDock program was unable to load the protein into the program. As a result, protein 5Z01 was used, as it was the best fit protein found in the Dali sequence which compared carbon backbones of the proteins. This switching of the protein being observed led to changes in the expected amino acid residues involved in the ligand binding. The Yestervin protein had an active site composed of His 285, Glu 217, and Ser 115. However, in protein 5Z01, the Glu 217 was not conserved and instead had an Asn at that site. Therefore, we would expect ligands to bind with or near a His, Asn, and Ser amino acid (*Figure 5*). Protein 5Z01 had the greatest affinity for ligands DMU and TLA, with binding affinity of -5.9 and -4.9, respectively (*Figure 5*). The ligands DMU, TLA shared the same binding site on protein 5Z01 and share similar amino acid residue interactions (*Figure 5*). This binding site shared by both ligands was also shared by the other two analyzed ligands ABU and PEG. The ABU and PEG ligands were not analyzed in detail because their binding affinity of above -4 suggested they were not the strongest fit for the active site. Another ligand DEU was analyzed as well but did not interact with the expected active sit and was thus not analyzed. The ligand TLA has a binding affinity of -4.9 with interactions with 2 expected amino acid residues. The TLA ligand has two amino acid residue interactions with Ser 106 and Asn 176, which is consistent with the proposed active sites. The visualization of these amnio acid residues interacting with TLA are found in *Figure 5*. Both the Ser and Asn were involved in the binding of the TLA ligand, suggesting that the ligand is involved in the hypothetical reaction for Yestervin. The DMU ligand has similar results to that of the TLA ligand. With a binding affinity of -5.9, the DMU ligand binds a little stronger to the active site compared to TLA. The DMU ligand also has active site amino acid residue interactions, having close interactions Asn 176, Ser 106, and His 270. DMU differs from TLA in the fact that it shares a close interaction with His 270, which is the final expected amino acid residue involved in the active site. The visualization of amino acid residues interacting with ligand DMU are found in *Figure 5*. These results indicate that the ligand DMU is involved in the hypothetical reaction of the Yestervin protein as it has interactions with the catalytic triad of amino acids typical of the Yestervin protein. The DMU ligand is only present in 4 currently known hydrolases, but the significant alignment with the expected amino acid residues at the active site suggests that the ligand is involved in this hypothetical hydrolase reaction. In conclusion, both TLA and DMU have significant binding affinities under -4 and have significant overlap with the expected amino acid residues, showing that they bind to the expected active site. TLA has a less significant binding affinity and only binds to 2 of 3 expected amino acid residues, but is a prevalent ligand in hydrolases, which is the expected reaction for the Yestervin protein as Yestervin is a hydrolase. The ligand DMU has a more significant binding affinity and binds to all 3 of the expected amino acid residues. While it is not often found in hydrolases, the binding affinity and amino acid residue interactions suggest that it is an important ligand for the Yestervin reaction.

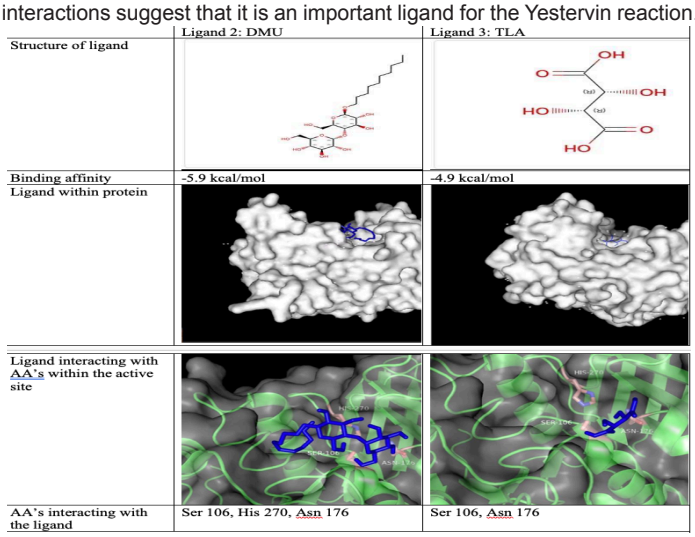


Figure 5: Pyrx Autodocking of two ligands that had significant binding with protein 5Z01. Ligands DMU, and TLA structures from the PDB were provided and their binding affinities indicated they bind significantly to 5Z01 as they have a binding affinity under -4. Both DMU and TLA share the same active site. Both DMU and TLA are found within the same active site and interact with expected amino acids within that active site. The ligand DMU (left side in blue) interacts with all three amino acid residues (Ser 106, His 270, Asn 176 in pink) attached to the green carbon backbone, while TLA (right side in blue) only interacts with two of the active site residues (Ser 106, Asn 176 in pink).

Protein purification successfully purified target protein yfkn

Following Autodocking experiments, where the protein active site and amino acid residues were identified and observed, the protein yfkn was purified in solution for further investigation into Yestervin's unknown function. The protein yfkn replaces Yestervin for this experiment as yfkn was the closest available protein to Yestervin that was purified by Dr. William Conrad. The goal of the protein purification experiment is to transform **E. Coli** with plasmid expressing yfkn, then purify the protein from the

E. Coli using the HOOK 6X His Protein Spin protocol which performs rapid purification of 6X His tagged proteins from bacterial cultures. To accomplish this goal, a His protein Spin protocol was implemented in which a figure showing the expected kDa for each protein was created and then the protein of interest was purified with nickel chelating resin to elute and purify the protein. Protein yfkn was purified in a 15 lane 16% gel and took up lanes 9-15. The expected results would show protein yfkn at approximately 80 kDa and would show pure purification in the last elution fraction. The protein yfkn shows a band in between 75-100 kDa, which is the expected result as protein yfkn has a molecular weight of 80 kDa. *Figure 6* shows protein yfkn solution running through a 15 lane 16% tricine gel. The protein lysate lane shows an abundant presence of yfkn. This abundance of yfkn causes bands to appear in the flow through and wash lanes (*Figure 6*). These results occur because there is such an abundance of protein that there is not enough nickel to bind to within the column, and thus some of the protein cannot bind to the column and is washed out. The elution trials show a strong band at 80 kDa, which shows the appearance of yfkn but the first two elutions show a protein at 10-15 kDa. Elution 3 shows the protein yfkn pure within its fraction, which is the expected result (*Figure 6*). In conclusion, the protein yfkn was purified in the final elution trial as the band at 80 kDa in the elution 3 fraction indicates that the only protein present within that fraction is the protein yfkn. This means that the protein was successfully purified and thus can be examined further in future experiments.

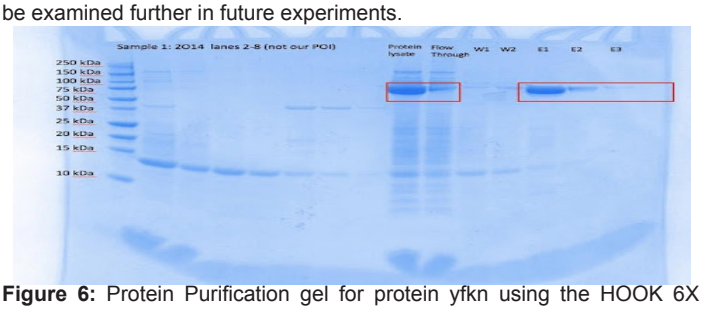


Figure 6: Protein Purification gel for protein yfkn using the HOOK 6X His Protein Spin protocol which performs rapid purification of 6X His tagged proteins from bacterial cultures. Lanes 2-8 show the successful purification of 2O14 that was used as the positive control for protein kinetics experiments. Lanes 9-15 show significant portions of purified protein yfkn with significant bands within the protein lysate lane each elution trial at the expected 80 kDa. So much yfkn was purified that there was not enough room for all purified protein to bind, explaining the band on the flow through. The red boxes show the lanes where yfkn was purified.

Protein yfkn demonstrated no activity in enzymatic assay when compared to positive control 2O14

After successful purification of both protein 2O14 and yfkn, both proteins were added to an enzyme activity assay in order to determine protein kinetic values such as Vmax, Km, and Kcat among other values. The goal of this experiment was to quantify both proteins' enzymatic activity with the values of Vmax, Km, Kcat. The technique involved included adding both purified proteins into a 10mM PNPA solution and watching for changes in color of the solution. The color changes were measured in absorbance, which indicated enzymatic activity and was used to calculate all figures and values for both proteins. The expected results for each protein were that a Vmax, Km, and Kcat value would be calculated, and that the proteins would have quantified enzymatic activity values. For this experiment, the discussion of protein 2O14 will be used as a positive control to indicate that enzymatic assay errors or other variables are not what caused the results for protein yfkn. For protein 2O14, enzymatic activity was determined through the assay's absorbance calculations and initial velocity, Michaelis-Menten, and Lineweaver-Burke plots were used to quantify this enzymatic activity. The initial velocity plot for protein 2O14 demonstrates that as substrate concentration increased, the product concentration also increased (*Figure 7*). This increase in product as substrate increases demonstrates that enzymatic activity is present in the reaction. This initial velocity plot was followed by a Michaelis-Menten plot for protein 2O14. The Michaelis-Menten plot was calculated and further demonstrated enzymatic activity. The plot showed that as substrate concentration increased, so did the initial velocity of the reaction. The reaction did not meet a Vmax as the peak did not level off within the given substrate concentration range (*Figure 7*). This inability to distinguish Vmax and Km from the Michaelis Menten led to the production of a Lineweaver-Burke plot. The Lineweaver-Burke plot demonstrated quantitative values that allowed for the calculations of Vmax and Km. The lineweaver-burke plot demonstrated a y-intercept of 0.0415, which was used to calculate a Vmax value of 24.1 (*Figure 7*). This high Vmax explains why the Vmax was not seen on the Michaelis-Menten graph as the graph did not go as high as 24 for the initial slope. The slope of the plot was found to be .1238, which was used to calculate a Km value of 2.98 since slope=Km/Vmax. The Vmax and Km values demonstrate that there is indeed enzymatic activity for protein 2O14. From these two values, the Kcat which is the turnover number, or the number of substrate molecules each enzyme site converts to product per unit of time, was found to be 4075361, demonstrating that the 2O14 protein is a catalytic enzyme within the assay. Enzyme efficiency was determined to be 1366024, demonstrating that 2O14 is an efficient enzyme. The units of microliter per enzyme and activity of enzyme were found to be 0.00241 and .120 respectively, further quantifying the enzymatic activity of 2O14. The assay was effective in determining protein kinetic values for protein 2O14, and thus showed that protein 2O14 is indeed able to catalyze the hydrolysis reaction of PNPA. The Vmax, Km, and Kcat values give a quantitative measure of the protein 2O14's enzymatic function, which were used as a positive control for the experimental protein yfkn.

For the protein yfkn, the results showed that the protein had no enzymatic activity within the assay. The protein yfkn was used instead of the Yestervin protein because of restraints in time and the availability of protein yfkn as a purified protein. Protein yfkn was the closest protein available to Yestervin and was thus used as a substitute for Yestervin. The protein yfkn showed no enzymatic activity within a PNPA assay as shown in *Figure 7*. The initial velocity plot shows that there is no enzymatic activity as there is no product formation no matter how much substrate is present in the system (*Figure 7*). This lack of increases in product as the substrate increases demonstrates that there is no enzymatic activity for protein 3GVE in the PNPA assay. The Michaelis Menten graph for protein 3GVE further confirms these findings (*Figure 7*). The Michaelis-Menten graph shows no consistent increase in initial velocity with substrate. This inconsistent trend further demonstrates that there is no enzymatic activity for 3GVE in the current assay. The lineweaver-burke plot was unable to find a distinguishable Vmax, Km, or any protein kinetic values (*Figure 7*). The lineweaver-burke had a negative slope and no distinguishable y-intercept meaning that protein kinetic values like Vmax, Km, and Kcat were not distinguishable. These inconclusive graphs lead to the conclusion that protein 3GVE is not involved in hydrolysis and may be a catalyst for other reactions. The current hydrolysis assay shows no function for protein 3GVE, and thus we can conclude that protein 3GVE is not a catalyst for the PNPA hydrolysis reaction and may be a catalyst for other types of reactions. Different assays with differing reactions should be performed in order to determine protein 3GVE's function and its protein kinetic quantitative measures such as Vmax, Km, and Kcat.

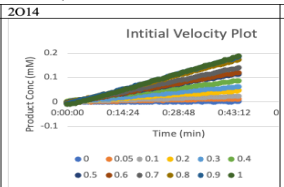
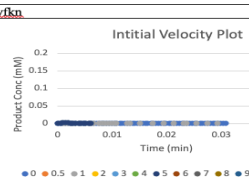
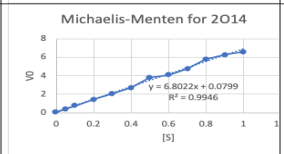
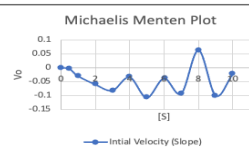
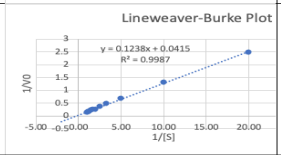
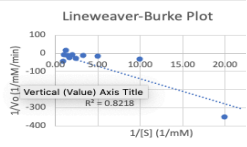
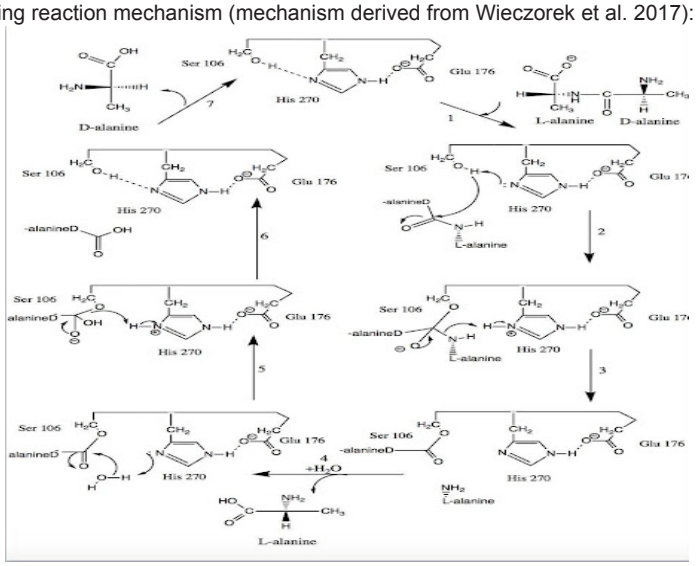
Protein being analyzed	2O14	yfkn
Initial Velocity Plot		
Michaelis Menten plot		
Lineweaver-Burke Plot		
Vmax	24.09639	N/A
Km	2.983373	N/A
Kcat	4075361	N/A
Enzyme efficiency	1366024	N/A
Units per microliter of enzyme	0.00241	N/A
Activity	0.120482	N/A

Figure 7:
Comparison of protein kinetic measures between positive control protein 2O14 and protein yfkn. The enzymatic assay where hydrolysis of PNPA

occurred was used for both proteins, and absorbance values were used to determine protein kinetic values. Protein 2O14 provides a positive control, showing the expected initial velocity, Michaelis-Menten, and Lineweaver-Burke plot from which kinetic values like Vmax and Km were derived. The initial velocity plot looked at product concentration in mM over a given time within the reaction system. This allowed for initial velocities measures to be calculated which were used to create the Michaelis-Menten and Lineweaver-Burke plots. Protein yfkn showed no activity as evident from its initial velocity plot and thus kinetic values were unable to be derived.

Discussion

Using all the data from the different bioinformatic tools, the hypothesis that Yestervin is an LD- Carboxypeptidase can be supported. The PyMol active site analysis implicated Yestervin as either belonging to EC class 3.1 or 3.4, while also suggesting the possibility of a Ser-His-Glu catalytic triad through the amino acid residue comparison between Yestervin and 2ace. The sequential comparison through BLAST/SMART BLAST indicated Yestervin was likely a LD Carboxypeptidase belonging to the Peptidase_S66 superfamily while further supported the presence of a Ser-His-Glu catalytic triad through catalytic triad conservation among different bacteria species with similar sequences as Yestervin. Running Yestervin through a Dali Global alignment found a significant carbon backbone match with the Murein Tetrapeptide Carboxypeptidase protein 5Z01, which further supports the hypothesis that Yestervin is a LD carboxypeptidase. The protein 5Z01 also had significant overlap with the active site amino acid residues in Yestervin. The protein 5Z01 was used in Pyrx autodocking and showed that ligands DMU and TLA bind within the expected active site and interact with the expected Ser-His-Glu catalytic triad (in the case of 5Z01, the Glu was replaced with an Asn meaning the catalytic triad observed was a Ser-His-Asn triad). The ligand TLA is a very common ligand among hydrolases, with involvement in 110 known hydrolase proteins. With the TLA ligand being so common in hydrolases along with the interaction with the expected active site, it can be concluded that TLA is an important ligand involved in the hypothetical reaction of the Yestervin protein. A possible function for the ligand TLA in Yestervin is the function of inhibitor. The TLA molecule shown in *Figure 5* shows that the TLA has two carboxy groups but no amine group. This presence of no amine group means the cleaving of L and D amino acids by Yestervin would not be possible as there is no amine group that connects to a carboxy group that would thus be cleaved by Yestervin. The Yestervin protein was then replaced by similar protein yfkn due to the unavailability of Yestervin, which was purified through the HOOK 6X His Protein Spin protocol as it showed up at the expected 80 kDa within the 15 lane 16% tricine gel. The purified protein yfkn was unable to be accurately assessed for protein kinetic measures due to inactivity within the assay used. The positive control of 2O14 did show activity, meaning the lack of activity of yfkn was not due to a systematic error, but rather that yfkn did not react with PNPA in the assay. All of these key findings support the hypothesis that Yesetervin is indeed an LD-Carboxypeptidase within biological systems. The Ser-His-Glu catalytic triad within Yestervin acts to cleave L and D amino acids through the following reaction mechanism (mechanism derived from Wieczorek et al. 2017):



The results showing the Ser-His-Glu catalytic triad within Yestervin support the hypothesis that Yestervin is an LD-Carboxypeptidase as well. A review of different serine proteases showed that a Ser-His-Glu triad has been found in the LD-Carboxypeptidase (PDB: 1ZRS) within the organism *Pseudomonas Aeruginosa*, which is the same organism Yestervin has been found in (Korza & Bochtler 2005) (Ekici et al. 2008). With the protein 1ZRS being an LD-Carboxypeptidase within the same organism as Yestervin and containing the same Ser-His-Glu catalytic triad as Yestervin, the hypothesis that Yestervin is a LD-Carboxypeptidase just like 1ZRS can be concluded from the research performed in this study along with the research from other studies previously cited. The Yestervin protein has the potential to be an alternative protein than the suggested LD-Carboxypeptidase. The PyMol active site alignment suggested the possibility that Yestervin belongs to EC class 3.1. The LD Carboxypeptidases belong to EC class 3.4, while EC class 3.1 are esterases that cleave ester bonds. The PyMol bioinformatic program suggests the possibility of Yestervin belonging to the class of esterases, as 2 of the 3 best fit active sits belong to proteins that are known esterases. These results can be explained by the similarities between esterases and peptidases. Both enzymes use hydrolysis to break bonds within proteins. Both of them belong to the same EC class, meaning both are hydrolases in nature. Therefore, it is possible that there is some overlap between the amino acid residues between esterases and peptidases as they both cleave bonds because they are both hydrolases. The Dali global alignment also suggests the possibility that Yestervin could be a Microcin immunity protein MCCF. This immunity protein that matched with Yestervin showed similar Z-score values with the Dali best fit Murein carboxypeptidase (33.3 vs 33.6 respectively) and higher RMSD values than the matched carboxypeptidase (2.3 vs 2.1 respectively). Both Dali protein matches also have two matching active site residues, with the Glu active site amino acid not being conserved in both cases. From these findings, it is plausible to consider an alternate function of Yestervin as an immunity protein in biological systems. The reason for these findings has to do with the similar functions between Microcin immunity proteins and Carboxypeptidases. Uniprot analysis of the Microcin immunity protein indicated it had molecular functions indicative of hydrolase activity, which is the same molecular function as a carboxypeptidase. The biological process of these Microcin immunity proteins is to support bacteriocin immunity. Bacteriocin immunity is the resistance to bacteriocins, which are polypeptide antibiotics that could harm bacteria. This bacteriocin immunity is similar to carboxypeptidases' role in the *ampC* mechanism, which is a mechanism for antibiotic resistance in bacteria. The role of carboxypeptidases in antibiotic resistance is further discussed in future studies. Both the Microcin immunity protein and carboxypeptidases have similar hydrolase molecular functions, and both play a role in antibiotic resistance in bacteria. These similarities between the two proteins can explain why Yestervin had results that suggested the protein may be a Microcin immunity protein. The data from these experiments could be refined on a multitude of different fronts. The data obtained from the Pyrx Autodocking lab used the protein 5Z01 which was a known protein with the function of a Murein Tetrapeptide carboxypeptidase. This caused the amino acid residues within the active site to change when looking at ligand binding through Autodock (Glu residue changed to Asn). A better refinement of the data would be using Yestervin within the Pyrx program to show how the ligands interact with the Ser-His-Glu catalytic triad instead of looking at how the ligands using Yestervin within the Pyrx program to show how the ligands interact with the Ser-His-Glu catalytic triad instead of looking at how the ligands interact with a similar Ser-His-Asn catalytic triad. Another refinement of the data would be using Yestervin within the purification and protein kinetic studies. A limitation of this study was the unavailability of Yestervin within solution that could be purified. Instead, the protein yfkn was used, which is a different EC class compared to Yestervin and has a completely different structure when compared to Yestervin. The protein yfkn has an EC class of 3.1, which means it is an esterase, while Yestervin has an EC class of 3.4, which is the class of peptidases. The use of yfkn was out of availability and thus the results were different than if Yestervin was purified. Yestervin contains a different kDa and pI value than yfkn and may have reacted differently than yfkn acted within the protein kinetic assay. The lacking availability of Yestervin and the use of the different proteins 5Z01 and yfkn in the Autodocking, protein purification, and protein kinetics experiments is a glaring limitation of this study. In future studies, the Pyrx program will be run using Yestervin and Yestervin will be purified, and the kinetics of the protein will be analyzed to provide further evidence that supports or refutes the hypothesis that Yestervin

is an LD-Carboxypeptidase. This future study would be performed using all the same methods as previously described within this report, but the protein Yestervin would be used in all experiments instead of substitute proteins like 5Z01 and yfkn. The expected results would be that the protein Yestervin is indeed a LD-Carboxypeptidase in all experiments. Another future study revolves around the ligand TLA, which was implicated to act as an inhibitor to Yestervin's active site. TLA will be further studied and its function within the active site will be determined. This study would be performed using an assay with TLA present in the assay. The TLA would bind to the Yestervin protein and the activity of Yestervin would be analyzed in a similar fashion seen in the protein kinetics experiment. A positive control would be used to ensure that the system was not the issue. The expected results would be that TLA is indeed an inhibitor because its structure with two carboxyl groups indicate it cannot partake in the LD-Carboxypeptidase proposed mechanism. If TLA was found to be an inhibitor from these experiments, more studies would need to be performed looking into the application of TLA during Pseudomonas aeruginosa infections. The LD-Carboxypeptidases within Pseudomonas aeruginosa act to recycle peptidoglycan. If TLA is implicated to be an inhibitor of Yestervin, future studies could be centered around TLA's role in medical intervention fighting Pseudomonas aeruginosa infections by stopping peptidoglycan recycling leaving the bacteria vulnerable and weakened. The ligand DMU should also be investigated in future studies to see if it acts as a substrate for the Yestervin active site. This would be performed in a similar fashion to the future study regarding the ligand TLA. There are no current expected results, as DMU is a rare ligand within hydrolases and its function within the active site is currently unknown. This unknown interaction between DMU and Yestervin active site amino acid residues is the reason that this future study should be performed, as there is a gap in knowledge that needs to be filled. Another future study should focus on the potential of pharmaceutical manipulation of Yestervin activity. The importance of understanding how to manipulate Yestervin is large as it has implications for fighting antibiotic resistant bacteria. Carboxypeptidases within bacteria play a role in antibiotic resistance, as their presence leads to decreased expression of ampC. The expression of ampC is an important mechanism involved in developing antibiotic resistance in bacteria. Therefore, the presence of a carboxypeptidase could lead to a mechanism of using carboxypeptidases to combat drug resistance in bacteria (Xu et al. 2020). The hypothesis that Yestervin is an LD-Carboxypeptidase has been supported by multiple bioinformatic tools and previous studies, meaning that Yestervin can be further studied and possibly targeted for medical interventions that could save thousands of lives from in hospital antibiotic resistant infections that often end fatally. Antibiotic resistance bacteria are an impending global health crisis that could cause major problems in the future. Studying Yestervin, its role within antibiotic resistance bacteria, and how it can be manipulated to weaken antibiotic resistant bacteria is necessary research that could be the beginning of solving the major issue of antibiotic resistant bacteria.

Looking at the human body's microclimate in a biogeographic context

Kerry Mullen
Lake Forest College
Lake Forest, Illinois 60045

Introduction

Species diversity varies across the globe and different environmental conditions. Despite the differences in the number of species between locations, there are measurable patterns relating to species diversity. For example, moving from the poles to the equator, there is an increasing gradient of the number of species present (MacArthur 1965). Typically, the tropics are believed to contain the greatest species diversity because they are composed of many habitats in a relatively uniform climate. The stability of the tropics, along with high habitat complexity, high productivity, and greater competition, are thought to be the driving forces behind the increase in diversity (Pianka 1966). On top of this, it is hypothesized that more uniform environments will have more effective barriers to dispersal than environments with greater fluctuation. This is because organisms that exist in fluctuating environments are more likely to adapt to a greater range of conditions. They are also less likely to encounter unbearable conditions than their counterparts from a stable environment with a smaller range of conditions present (Jazen 1967). As such, organisms from tropical environments are more restricted in their ability to migrate than organisms from temperate climates, allowing diversity to build over time. Establishment of a species in a new location is a rare and difficult phenomenon that gets increasingly unlikely as the distance between locations grows. Dispersal, especially over long distances, is characterized by difficulties involving isolation, ecological opportunities, and climatic differences. Typically, species that are good dispersalists are less competitive and weedier than their counterparts (Carlquist 1966). These species have adapted for movement rather than being able to outcompete other individuals and species. Dispersalist species would be unlikely to successfully establish a new population in a location already inhabited by other species. Rather, they would need to find an empty or sparsely populated area to colonize, which are few and far between. Meanwhile, species that are adapted for survival rather than dispersal are unlikely to survive the journey to a new location and will not migrate or expand from their original range without some form of intervention.

On average, the human body is home to trillions of individual microbes and thousands of species (Hulcr et al. 2012). The human body can be considered a planet with each individual harboring unique microbial communities that live in a range of physiologically and topographically distinct niches. Much like how the Earth is broken down into different biomes based on environmental conditions, human skin is broken down in different regions based on physiological characteristics. Skin is generally characterized as one of three types: sebaceous, wet, or dry (Byrd et al. 2018). Therefore, the human body can be thought of as at least three different biomes. These biomes can then be compared to preexisting environments on Earth. Moist, hairy underarms can be considered analogous to tropical rainforests, while smooth, dry forearms are comparable to desert areas (Grice et al. 2009). Known patterns about Earth's species diversity can then be applied to the human body to hypothesize how microbe richness and abundance may vary between each body part. To explore and test the dynamics of microbes on the surface of the human body, species presence and amounts were recorded on three different parts of the human body: the armpit, the elbow crease, and the back of the knee. We hypothesize that the armpit is a warmer and more constant environment compared to the knee and the crease of the elbow. Since the armpit is a more constant environment, we predict that there will be greater species diversity. This hypothesis is based on the tropics being a highly diverse ecosystem with which the armpit shares many qualities, such as high humidity, complex environment, and relatively constant conditions. Our second hypothesis is that isolation between body parts would decrease similarity in microbe species. Therefore, we expect the elbow to have more similar microbes to the armpit because they are closer in proximity to each other than the back of the knee is to either of them.

Methods

For this study, we collected swab data from 15 participants, sampling their armpit, back of the knee, and elbow crease for a total of 45 samples. The participant pool we sampled from were males at Lake Forest College who use antiperspirants and do not shave their armpits. Additionally, all participants were questioned on their shower frequency per week, how recently they showered and applied antiperspirant, and their amount of regular contact with chlorine chemicals. The 15 participants were swabbed following the lab handout except for the duration of the Q-tip swab. Rather than 3 rotation cycles, participants swabbed each location for 15 seconds. For each sample location, participants swabbed an area of 2.5 cm at the center of each site, approximately the area of a quarter. Each sample was recorded with either the letter A for the armpit, E for the elbow crease, or K for the back of the knee. This was followed by numbers 1 through 15, which corresponded with the participant's number. Participants' names were recorded along with their number on a separate document for anonymity. Additionally, the initials KM were added to each label to differentiate the samples from other experiments.

Each participant conducted the swabbing themselves following the instructions outlined above. The amount of time between sample collection, application to the petri dish and storing the samples in the incubator was approximately one hour. We followed the inoculation protocol provided for each sample and stored the plates in the 36°C incubator for 3 days before taking them out and wrapping the edges of the sample plates with parafilm to be stored in the fridge. Each day within the 3-day span, we checked the plates at about 6:00 PM to observe growth in the incubator. After one day of cooling in the fridge to ensure no residual growth, the samples were photographed for analysis and placed back into the fridge. The photographs of each petri dish were examined to measure the number of species and number of microbes present in each. Overall, we differentiated species using morpho-species characteristics of size, shape, texture, and color. All the data were recorded in an Excel spreadsheet and compared in averages using a one-way ANOVA of three body parts for statistical analysis. We ran separate ANOVA tests for each of the following measurements: species abundance, diversity, and richness from the data. Diversity was measured using the Shannon Weiner Diversity Index and the results were analyzed for significance using a one-way ANOVA. We also completed six t-tests to examine the statistical difference between our two most plentiful species in each location.

Results

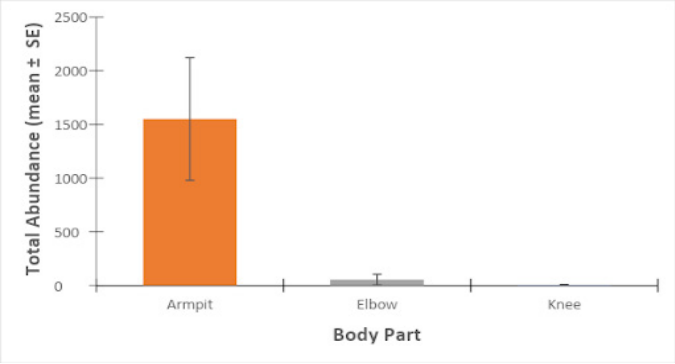


Figure 1. **Total average abundance of species located at each of the three body locations.** The mean was calculated for each body location (armpit, elbow crease, and back of the knee). Error bars measure the standard error of each total average abundance. Statistically significant by ANOVA: $F_{2, 42} = 7.03$, $p^* = 0.002$.

Total average abundance was calculated to determine which of the three locations measured had the most overall microbes. This data was found to be significant (ANOVA: $F_{2, 42} = 7.03$, $p^* = 0.002$) with greater microbe growth located in the armpit compared to the elbow and knee, which is reflected in Figure 1. Total abundance was calculated by taking the average of the counted microbes on each body part. Species richness (ANOVA: F2, 42 = 1.84, p= 0.171) and species diversity (ANOVA: $F_{1, 4} = 0.42$, p= 0.550) were also calculated, but both data were found to be insignificant.

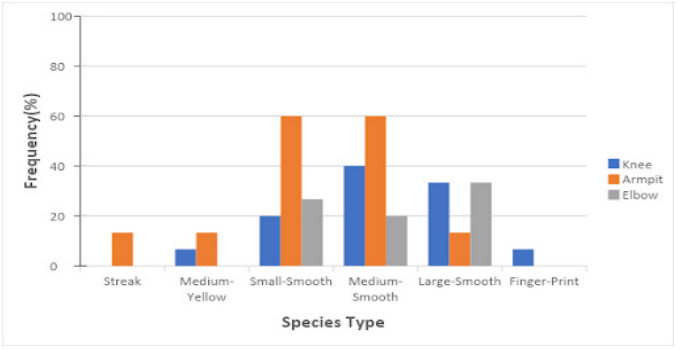
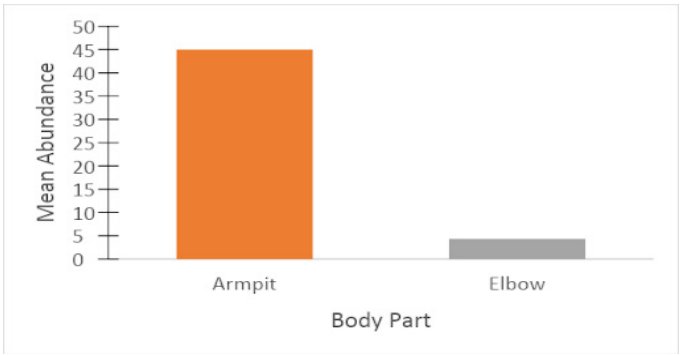


Figure 2. **The frequency of each species recorded from counting the number and type of species from 45 samples.** Descriptions of the distinguished species are along the x-axis based on the researcher's view of distinct morpho-characteristics. The y-axis denotes frequency [%].

Frequency analysis of the six species present in the forty-five samples taken showed that the armpit and the knee had the maximum species types (5 out of 6), while the elbow samples only contained 3 out of 6 (Figure 2). The most common species across all three locations were the small-smooth, medium-smooth, and large-smooth circles. Large-smooth circle distribution was found to be insignificant (ANOVA: F2,42 = 0.05, p= 0.949) while the medium-smooth circle distribution (ANOVA: F2,42 = 3.38, p*=0.044) and the small-smooth circle distributions (ANOVA: F2,42 =6.88, p*= 0.003) were found to be significant. Three t-tests were performed between each of the locations on the body for both the medium-smooth and small-smooth circles. Each of the medium-smooth location comparisons were not found to have significance (Paired t-testAE: tdf = 1.73, p= 0.105, Paired t-testAK: tdf = 1.92, p= 0.076, Paired t-testEK: tdf = 1.92, p= 0.076), indicated by the mean abundances measured in Figure 3. Of the small-smooth circle data, comparisons between the armpit and the elbow (Paired t-test: tdf = 2.54, p*= 0.024), as well as the armpit and the knee (Paired t-test: tdf = 2.68, p*= 0.018), both were found to be significant, while the compared abundances between the elbow and knee were insignificant (Paired t-test: tdf = 0.97, p= 0.346), as shown in Figure 4. The small-smooth circles had greater preferential abundance on the armpit than both the armpit and the knee.

A



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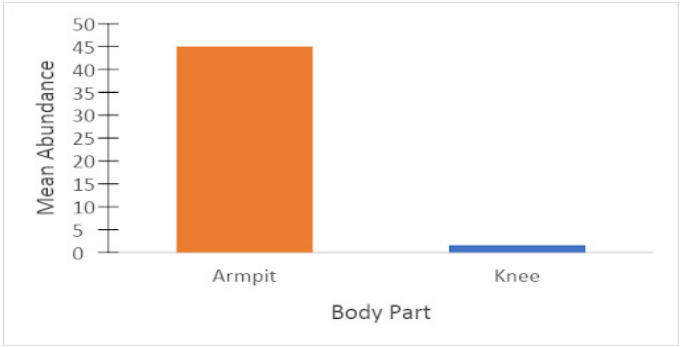

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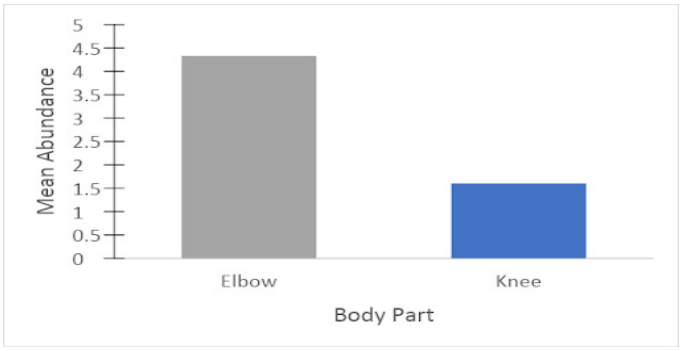
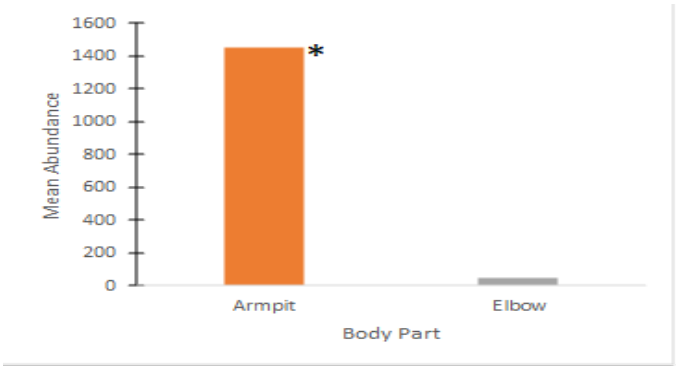


Figure 3. **Mean abundance of medium-smooth circular species.** Comparison of medium smooth species between (A) armpit and elbow, Paired t-test: tdf = 1.73, p= 0.105; (B) armpit and knee, Paired t-test: tdf = 1.92, p= 0.076; (C) elbow and knee, Paired t-test: tdf = 0.63, p= 0.542.

A



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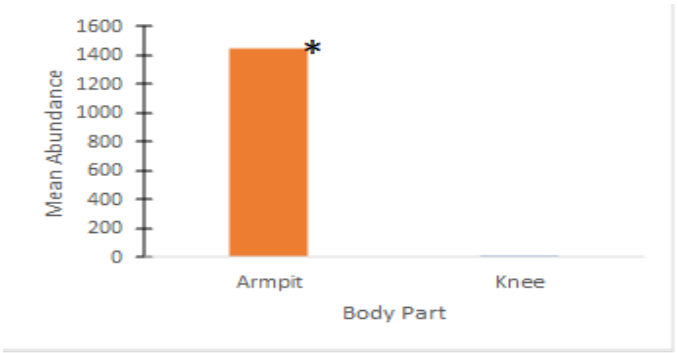

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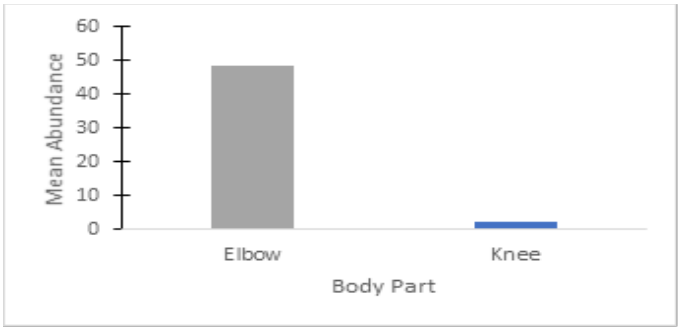


Figure 4. **Mean abundance of small-smooth circular species with significant data indicated by *.** Comparison of differences between (A) armpit and elbow, Paired t-test: tdf = 2.54, p*= 0.024; (B) armpit and knee, Paired t-test: tdf = 2.68, p*= 0.018; (C) elbow and knee, Paired t-test: tdf = 0.97, p= 0.346.

Discussion

While the hypotheses posited that the armpit would have the greatest amount of species diversity, species richness data were not found to be

significant. However, despite the insignificant species richness value, the armpit was found to have the highest frequency of species (Figure 2) and the greatest total abundance (Figure 1) of the three sampled locations. Additionally, the armpit did have the greatest calculated diversity index. However, this also was not found to be statistically significant. Based on these results, we reject the hypothesis that the armpit would have the greatest species diversity due to its warmer and more constant climate. We also cannot accept the hypothesis that isolation between body parts would decrease similarity in species diversity. This is due to a lack of significant results when comparing species richness and abundance between locations. The mean abundance only produced significant results for small microbes between the armpit and elbow and the armpit and knee. All other comparisons between locations and other species produced nonsignificant results. While much of the data proved to be statistically insignificant, an interesting trend involving the frequency of certain species was discovered. It was observed that the medium-smooth and small-smooth circular microbes had a greater presence than other species found in the samples. Hucler et al. (2012) found a similar trend with the bacterial phylotypes present in the human belly button, with the most frequent and abundant phylotypes being present across independent populations. These frequent and abundant phylotypes were termed oligarchs, the same as species found to be both predictably frequent and abundant in tropical rainforests. While oligarchs were present in multiple samples, not one oligarch was present in every sample, much like what we found with our medium-smooth and small-smooth circular microbes. Hucler et al. (2012) determined that these oligarchs allow for some degree of predictability while the rest of the system is made up of randomly determined microbes.

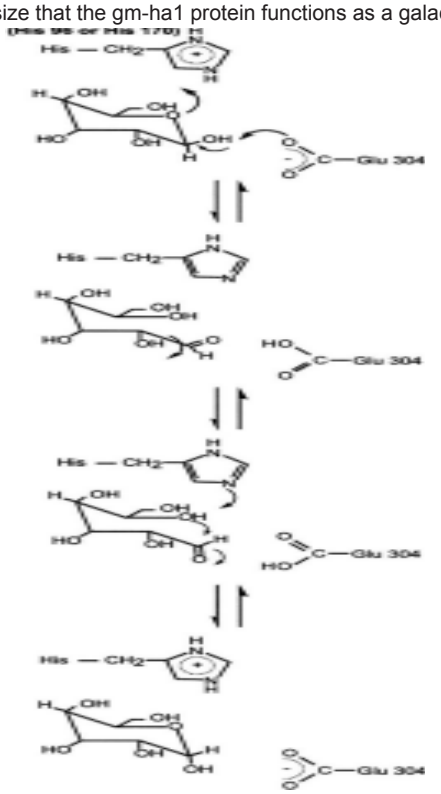
This study was highly restricted due to a variety of limitations as well as sources of error. The chief limitation was the limited sample population swabbed, with only 15 individuals being tested, resulting in a total of 45 samples. More participants and a larger sample size may have produced more microbe variety. Our gut microbial communities stabilize around three years of age, and the strains within the gut likely come from close contact and family members, which are then maintained throughout life (Byrd et al. 2018). The skin may undergo a similar process, and therefore individuals from different regions may have different microbial communities on their skin. Additionally, while shaving practices and the use of antiperspirants were controlled for, the type of antiperspirant and use of other chemical products were not controlled. The differing chemical makeup, addition of other products, or time of product application could have affected the sample's microbe makeup. Finally, relying on self-designed morpho-characteristics to distinguish species type most likely affected the total number of species recorded and therefore skewed the diversity data. Further research into the microbe diversity of human skin remains both an intriguing scientific venture and medically beneficial. Characterizing the microbes that inhabit specific body parts may provide more insight into the balance between human health and disease. Human skin has high variability at different points in time, though there are some common areas of the human body where certain microbe taxa and diversities are consistently located (Ursell et al. 2012). This variability limits what we know overall and could be causing different medical and health issues. Antibiotic exposure, modified hygienic practices, and lifestyle changes have the potential to alter the skin microbiome selectively (Grice et al. 2009). The altered skin microbiome has many results, such as an increased frequency of human skin conditions or removal of a protective barrier unknown to us. Understanding the ecosystem that lives with us every day can also provide insight into the condition that led to the emergence of antibiotic-resistant organisms.

4RNL-A (gm-ha1) Structure and Sequence Shows Homologies to Galactose Mutarotase Enzymes

Haaris Ahmed, Bomby Ahuja, Dr. William Conrad, Leslie Gonzales, Kayenath Khan, Nathaniel Kregar, Karina Mora, Mathieu Norcross, Tate Rosenhagen, Max Smith, Sergio Sosa, Anthony Sullivan, and George Vladimirov
Lake Forest College
Lake Forest, Illinois 60045

Abstract
Gm-ha1 is a protein of unknown function with the PDB ID: 4RNL. This protein is found in *Streptomyces platensis*, which is a bacteria species that produces two highly effective antibiotics. Despite the protein being of unknown function, it is hypothesized that this protein functions as a galactose mutarotase. Galactose mutarotase, also known as an aldose-1-epimerase, is an enzyme that commits the first step in the metabolism of galactose. In order to investigate the function of gm-ha1, various bioinformatic methods were used. ProMOL revealed that gm-ha1 had a homologous active site to PDB: 1snz, which is a human galactose mutarotase enzyme. Moreover, Pfam showed that gm-ha1 had conserved residues with the aldose-1-epimerase family and likely had a similar sequence to other proteins in that family. Further, Dali showed gm-ha1 had a high level of global alignment with other aldose-1-epimerase enzymes. Besides, Autodock and PyMOL showed that NAD was most likely an important ligand in relation to gm-ha1 enzyme catalysis. Lastly, purification was performed on a different protein (c8orf32) and confirmed the presence of purified protein in the gel. However, the kinetics experiment of the same protein proved that the data was invalid and could not be used in this study. The bioinformatic data obtained in this study do not reject the hypothesis that gm-ha1's function as a galactose mutarotase enzyme. This was expected according to the data of the PDB profile of gm-ha1 (Tan et al. 2014). If the function of gm-ha1 is confirmed, it can give more knowledge of the *Streptomyces platensis* bacteria strain which can ultimately result in increased production of important antibiotics.

Introduction
The galactose mutarotase enzyme is a common protein found in both prokaryotes and eukaryotes. It is essential for the metabolism of galactose as it converts beta-D-galactose to alpha-D-galactose, which is the first step in normal galactose metabolism. This interchange can occur spontaneously in pure water *in vitro*, but organisms require the enzyme to perform this conversion *in vivo* because this reaction requires catalysis (Bouffard et al. 1994). In *Escherichia Coli*, galactose mutarotase is coded in the *gal* operon. This operon is induced by D-galactose, which indicates that the galactose mutarotase has some importance when D-galactose is present (Lee et al. 2008). Galactose mutarotase is essential for many prokaryotic cells as galactose is an abundant sugar and an excellent source of energy for bacteria. The PDB ID: 4RNL protein is found in *Streptomyces platensis* and is proposed to be a galactose mutarotase. However, this has not been confirmed as its function is unknown (Tan et al. 2014). It may prove beneficial to understand the function of the proteins in *Streptomyces platensis* as this species has a very important use in society. *Streptomyces platensis* is responsible for producing two key antibiotics, platensimycin and platencin. These two antibiotics have been shown to be effective against bacteria strains, such as MRSA and Streptococcus pneumoniae, that have developed resistance to many antibiotics (Falzone et al. 2017). Some researchers experimented and found a way for *Streptomyces platensis* to overproduce these two antibiotics tenfold the normal amount (Smanski et al. 2009). Learning how to overproduce antibiotics is a huge benefit to the medical field as antibiotics are in high demand, especially potent ones such as platensimycin and platencin (Smanski et al. 2009). A study that endeavored to determine the nutrients that increase *Streptomyces platensis* growth used a medium with high levels of glucose and lactose, which had growth of the bacteria on it. This indicates that *Streptomyces platensis* most likely utilizes galactose in its metabolism and growth. Therefore, determining the function of this protein may prove beneficial for providing media for culturing *Streptomyces platensis*. The protein was not named, so in this manuscript, the protein will be called gm-ha1. However, this study focused on the 4RNL-A chain for all sections of this report, so this name will be used when necessary. The original gm-ha1 study used *E. coli* which is one of the most common bacteria used for biochemical studies. One experiment had cloned the mutarotase gene of *Acinetobacter calcoaceticus*, which is another type of bacteria. This was performed by a complicated process of purifying the proteins, creating probes for the gene, cloning the gene, inserting the gene into a plasmid, and inserting the plasmids into *E. coli* (Gatz et al. 1986). In theory, this same process should be able to be performed on *Streptomyces platensis* to produce the same resulting *E. coli* with the mutarotase gene present. Another study that examined the mechanisms of galactose mutarotase had identified the amino acids and their positions that are crucial in performing the mutarotase functions. For example, it identified that Glu 304 and His 170 are the key peptides for catalysis (Thoden et al. 2003). The sequence of the 4RNL structure is listed in the PDB, so perhaps the sequence can be examined to see if the key peptides listed in this study are present in the same location in gm-ha1. We hypothesize that the gm-ha1 protein functions as a galactose mutarotase enzyme.



Schematic 1. This is the catalytic mechanism of galactose mutarotase. It is therefore the proposed mechanism of the gm-ha1 protein. This image shows the conversion of beta-D-galactose to alpha-D-galactose. This schematic was obtained from (Thoden et al. 2003).

Methods and Materials
PyMOL/ProMOL
ProMOL was used for examining the active site homologs for protein gm-ha1 as a plugin for PyMOL. 4RNL-A was examined by using the Motif Finder in ProMOL and searching"4RNL" in the query box. The template libraries selected were A set and P set. A list of structural homologs came up and the protein that had the lowest RMSD value was selected because this was the closest active site structural match. Important data were obtained such as the Levenshtein distance, EC class, and the RMSD value. This allowed a closer look at the homology of the two proteins because a Levenshtein distance of zero means that all of the residues of the active site homolog are found in the 4RNL-A. A low RMSD value means that the distance between the alpha and beta carbons are very close between the two structures. These values were all considered in finding the closest active site alignment for gm-ha1. The full protocol is located in the student guide in the Labarchives notebook at mynotebook.labarchives.com.
BLAST and Pfam
BLAST and Pfam were used to compare proteins with similar sequences to gm-ha1 to further the examination of the function of these proteins. For BLAST, the graphic summary and alignment were looked at and for Pfam, the HMM logo and the alignments were used. For BLAST, the FASTA sequences of the POIs were searched in Protein-Protein BLAST to find the top "hits" of the proteins, or the proteins that had the best alignment scores. The E-values and percent identities of the top three hits for each POI were recorded. These matches can provide insight into the function of the POIs as the three matches have the function of the protein in the name, so this allows easy comparisons. A SmartBLAST search was used to compare the POIs to proteins in other organisms and the E-values and percent identities were

also compared to determine whether the other proteins were good matches. For Pfam, the sequence search option was used. This was performed by pasting in the FASTA sequence for the POIs. This search gave the superfamily the POIs most likely belonged to, which was selected to examine the HMM (Hidden Markov Model) logo graph. The HMM logo was used to determine highly conserved amino acids, as it is a statistical algorithm that predicts the sequence based on previous data, which can be compared to the alignment of the sequences of the POIs. Peptides that appear large on the HMM logo would be highly conserved, so a small section would be chosen in the HMM logo to compare amino acids residues. In the search results page of Pfam, there is an option to hide/show alignment, so this would be selected to show the alignment, and the area selected from the HMM logo would be searched to determine whether the POI sequence showed these highly conserved amino acids. This is supporting evidence that the POIs belong to the families that they were listed in. The full protocol is located in the student guide in the Labarchives notebook at mynotebook.labarchives.com.

DALI

Since protein structure is key to function, Dali was used to visualize and quantify the global alignments of the POIs and backbone structural homologs to give insight into the function of the POIs. The PDB search was used by entering the PDB ID and chain letter of the POIs. This search was performed for "4RNL-A". When the search was finished, the "matches against full PDB" option was selected to observe the alignments against the entire POI structure. This option was selected because it compares the structure of the entire backbone of the protein minus the side chains. To view the structure comparisons, the "3D superimposition" was selected and the cartoon image option was used for the screenshots in the table (Table 1). The active site residues were found on PDB and a few peptides in sequence were used to compile a search of the POI sequence active site against the structural homologs in order to determine whether the active sites were conserved among the homologs. These results, alongside the z-score, RMSD, the length of the alignment (Lali), Nres, and % ID were all used to determine whether the homologs were actually good fits compared to the POI. Alignment and fitness can be determined through analyzing z-scores and Lali values, where greater values of either measure reflect greater alignment. Examining the functions of the good-fit homologs gives insight into the function of the POIs despite the fact that the side chains were not included in this search. The three proteins that were compared to gm-ha1 were 1SNZ-B, 1NSS-A, and 1SO0-A. The full protocol is located in the student guide in the Labarchives notebook at mynotebook.labarchives.com.

Autodock

This lab was started by first downloading a .pdb file of the POIs and loading the molecules in PyRX. These molecules were made to be macromolecules, which converts the file into a .pdbqt, which is required for Autodock. The ligands were found by searching the EC class of the POIs (5.1.3.3) in PDB and selecting the unique ligands that were larger in size than single molecules or very small structures. The five specific ligands chosen for this lab were 4QQ, MID, NAD, MIT, and MKY. These ligands were downloaded as one "ideal SDF" file as this is compatible with PyRX. This file was imported to PyRX, and the ligands were all minimized and were converted to .pdbqt files to correctly autodock the ligands to the protein. The ligands were docked using Vina Wizard in PyRX by first clicking start under Vina Wizard. The ligands were selected, and the forward option was selected. Then, "analyze results" was selected to provide a list of the ligands and their RMSD values to determine the best fit mode for each ligand, which was mode 0 for all ligands. The ligand NAD_A_352 was selected for visualization because it had a high binding affinity. The protein with the docked ligand was saved as a .pdb file to import it into PyMOL to compare the location of the ligand binding site to the active sites of the POIs. In PyMOL, the visualization settings were set to lines for the 4RNL-A structure and sticks for the ligand. The surface setting for 4RNL-A did not work because the ligand could not be seen. To compare the active site and ligand binding site, the 4RNL-A had everything hidden except for the two active site residues and the ligand, which were both as sticks. This visualization allowed for easy comparison of ligand binding site and the active sites to determine if the ligands may play a role in catalysis of the reaction or is a substrate. The full protocol is located in the student guide in the Labarchives notebook at mynotebook.labarchives.com.

Protein Purification

Column purification is used to isolate a purified protein of interest, which allows the protein to be manipulated for future experiments. The purification used in this experiment followed the Hook™ G-Biosciences protein purification spin column protocol. The bacteria were lysed with Bacterial PE LB™ and *PE LB*™-Lysozyme so that the 6X His tagged proteins were accessible. The tagged proteins were purified using immobilized metal affinity chromatography (IMAC). 0.4 mL of immobilized metal affinity resin is added to the protein lysate. Then, the resin is transferred to a spin column, where the His tagged proteins were washed twice with Tris-NaCl and eluted three times with the imidazole buffer. The bacteria before lysis, the protein lysate, the flow through, the two washes, and the three elutions were all run through a gel to determine if the protein of interest was correctly isolated and purified. The full protocol is located in the student guide in the Labarchives notebook at mynotebook.labarchives.com, and these methods were performed by Dr. Will Conrad.

Protein Kinetics

First, the POI elution samples were dialyzed into a 50 mM sodium phosphate buffer of neutral pH (7.6) using the Pierce 3kDa MWCO centrifugal filter concentrator. It was spin-concentrated three times using a 20 mL buffer per 1 mL elution. Protein abundance was measured using absorbance at 280 nm and 1 cm path length. This was converted in mg/mL using Beer's Law. Each well was filled with 160 uL of 50 mM sodium phosphate buffer, X uL (0-20) of 10 mM para nitrophenol acetate (PNPA) and Y uL of acetonitrile so that X and Y equal 20 uL total. Baseline absorbance was read at 405 nm to determine product concentration without enzymatic activity. Then, 20 uL of enzyme was added to each well and absorbance was read every 41 seconds at 405 nm. The full protocol is located in the student guide in the Labarchives notebook at mynotebook.labarchives.com and these methods were performed by Dr. Conrad.

Results

ProMOL search indicates active site homolog

Active site homologs give key insights into protein function since protein activity takes place in the active site. The active site of gm-ha1 was searched in ProMOL to find and compare active site homologs of gm-ha1. Figure 1 shows the closest active site alignment where there is almost complete overlap between gm-ha1 and 1SNZ. It had a very low RMSD value in ProMOL of 0.12 for the alpha and beta carbons in the structure and it had the same EC as gm-ha1. The 1SNZ active site is homologous in structure to the active site of gm-ha1. 1SNZ is a human galactose mutarotase enzyme, which indicates that gm-ha1 is homologous to a human galactose mutarotase.

Pfam sequence comparison shows conserved residues in aldose epimerase protein family

While local active site alignment is key to protein function, examining the protein sequence is also an important aspect of determining protein function. Comparing a protein sequence to a family of proteins can indicate the function of said protein may be similar to the protein family. The sequence of gm-ha1 was searched in Pfam and the HMM logo graph was used to compare the gm-ha1 sequence with the predicted sequence of the HMM logo graph. Since the suggested function of gm-ha1 is a galactose mutarotase enzyme, it would be expected that gm-ha1 would be a part of the aldose 1-epimerase family, as the galactose mutarotase enzyme is a type of aldose 1-epimerase. In Figure 2, the sequences in the bottom image show that the HG residues are 5 peptides before the uncertainty line and the W residue is 3 Peptides after the uncertainty, so the highly probably amino acids in the aldose 1-epimerase family are conserved in the correct placements. This indicates that the sequence of gm-ha1 is homologous to proteins in the aldose 1-epimerase family.

DALI data shows good global alignment with Aldose 1-Epimerase enzymes

While sequence comparisons are important in investigating protein function, global structure is arguably the most important aspect of protein function. DALI shows global alignment of proteins both visually and statistically, which is a powerful tool in the investigation of proteins of unknown function. The data from Pfam gives the indication that gm-ha1 will most likely match closest to aldose 1-epimerase and galactose mutarotase enzymes. The data from DALI confirms this as the three closest matches were all aldose 1-epimerase and galactose mutarotase enzymes. The image capture from Table 1 shows very close 3D alignment between the gm-ha1 among the three proteins and this is confirmed with 1SNZ-B and 1So0-A both showing the highest z-score of 53.6 and a very low RMSD of 1.0. Both active site residues were also conserved in all three homologs, so gm-ha1 shows very close global structural alignment for aldose 1-epimerase enzymes.

Autodock and PyMOL shows NAD ligand binding near the active site

Ligands with high binding affinities near the active site indicate mol-

ecules that are most likely essential for enzyme catalysis. PyRX was used to determine the binding affinity of the ligands to find tightly binding ligands as these typically play an important role in the function of an enzyme. PyRX gave numerical and visual data when searching the ligands and docking them to the POIs. Autodock revealed that the NAD_A_352 ligand was bound in the 4RNL-A structure with a high binding affinity. This high binding affinity indicates that this ligand may play an important role with gm-ha1, such as a substrate or a cofactor. PyMOL was used to visualize the binding site of the ligand with the protein structure and compare the location of the ligand binding site with the active site residues. The protein and the ligand were imported to PyMOL, and the active site residues were highlighted. Since the ligand has a high binding affinity, it is expected that the ligand most likely plays a role in the enzyme (gm-ha1) catalysis. Therefore, it is expected that the ligand should be bound near the active site. Figure 3 shows that the ligand is bound very closely to the active residues which further indicates that the ligand plays a role in the enzyme function. Therefore, the ligand is most likely a substrate or cofactor that supports the enzymatic reaction.

Nickel His purification yielded purified c8orf32 protein

Moving away from bioinformatics, proteins of interest were purified to obtain the pure protein. In order to determine the function of a specific protein, it must be purified and isolated. The POI was purified using the HOOK 6X His Protein Spin Purification Kit. The molecular weight of the c8orf32 protein with the Maltose Binding Protein tag was 65,971 kDa. The black circle in Fig 4 shows the protein of interest in the gel. These lanes were the three elutions, which is where the protein is expected to be present. The protein bands are also between the 75 kDa and 50 kDa markers, which is expected because the weight was 65,971kDa. The elutions contain the protein of interest with some other bands that are most likely impurities. There are also some impurities present in the final wash, so there were most likely still some impurities still present in the column. In ideal condition, there should have been one band of pure protein present. Therefore, the c8orf32 protein was present and purified in the elution, however, there were some impurities present in the gel.

Protein kinetics data indicates invalid elution sample of c8orf92

Continuing on after purification, eluted protein samples were tested to determine if the protein is present. Protein kinetics data is used to determine if an eluted sample has a specific protein of interest by monitoring whether it catalyzes a reaction and shows product formation. This was performed using absorbance vs time, then plotting the data with Michaelis-Menten and Lineweaver-Burk plots. The Michaelis-Menten graph (Fig 5) failed to show a burst phase as the data was linear from 0-10 on the x-axis. The data then showed a peculiar curve and a possible plateau, but not likely valid data. The Lineweaver-Burk plot (Fig 5) showed a negative y-intercept, which means that the data is invalid. It is invalid because a negative y-intercept of -0.0171 which would yield a negative Vmax. This would lead to other variables, such as Km and Kcat being negative, which is not possible. Therefore, this enzyme data is not valid and cannot be used for protein c8orf32.

Discussion and Conclusion

This study coincided with the expected results for each bioinformatic section that examined gm-ha1. ProMOL revealed that the A chain of gm-ha1 had a homologous active site to 1snz, which is a human galactose mutarotase. This supports the hypothesis that gm-ha1 is a galactose mutarotase. The active site is where the enzyme catalysis takes place, so having a homologous active site indicates a very similar, if not the same reaction taking place. However, only having a similar active site does not confirm a relation between the homologies. For the sequence of gm-ha1, the HMM logo graph from Pfam highlighted that the gm-ha1 protein sequence had conserved some common residues in the same location as proteins of the aldose-1-epimerase family. This also coincides with the hypothesis of gm-ha1's function as a galactose mutarotase because some common residues in the sequence are the same. However, this section only examined three residues among an entire polypeptide sequence, so this is very little data in comparison to the entire sequence. Dali had compared the global alignment of gm-ha1 and showed that the closest matches were all aldose-1-epimerase enzymes. This also supports that gm-ha1 is a galactose mutarotase enzyme because its overall structure is most similar to aldose-1-epimerase enzymes. Since structure determines protein function, this is sound support for gm-ha1's hypothesized function. Autodock was used to find ligands that bound to the A chain of gm-ha1 and NAD was found to be a high affinity ligand. It was confirmed in PyMOL that NAD also binds very close to the active sites, so NAD is most likely a key ligand in the catalysis of gm-ha1. The purification and kinetics experiments were not of gm-ha1, but of different proteins. However, the proteins used in this study could be substituted with gm-ha1 to obtain the corresponding data for this POI. The purification experiment confirmed the presence of purified c8orf32 protein in the gel, but the kinetics experiment data was invalid and could not be used in this study. This may have been due to impurities present in the protein elution, which could have hindered the protein activity. Since the purification and kinetics experiments were not able to be performed for gm-ha1, this would be a reasonable future path to take in determining the function of gm-ha1. With purifying the protein, one experiment that could be performed is to put an eluted sample of the protein in a solution of gel that contains beta-D-galactose. I hypothesize that gm-ha1 is a galactose mutarotase enzyme, so there should be alpha-D-galactose product forming if my hypothesis is correct. I believe that this would be a conclusive study on the function of gm-ha1 when combined with the bioinformatic data provided by this study. For the bioinformatics, only the A chain was used for searches in this study, so repeating the same bioinformatics for the other three chains would help confirm the results of this study. This study was performed remotely due to COVID-19, so the gm-ha1 protein was never physically experimented on. All of the experiments were bioinformatic, or a different protein was experimented on by Dr. Conrad. This limits the scope of this study as the protein was never physically worked with. However, this study provides multiple bioinformatic sources that support the hypothesis of gm-ha1's function. This study was aiming to better understand a protein in the *Streptomyces platensis* bacteria species, as this is an important bacteria strain in producing antibiotics. It is still inconclusive whether the gm-ha1 protein is a galactose mutarotase, but the data from this study gives both structural and sequential indications that gm-ha1 is a galactose mutarotase enzyme.

Figures

ProMOL depicts good active site alignment with 1SNZ motif

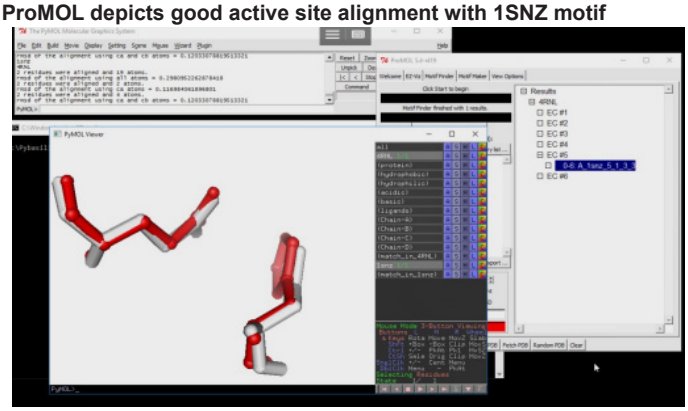


Figure 1. This image is a screenshot from ProMOL that shows the active site structural alignment of 4RNL-A (red) and the active site homolog motif, 1SNZ (white). The image on the right shows the EC of 1SNZ (5.1.3.3) as well as the Levenshtein distance (0-6).

Pfam shows conserved amino acid residues of gm-ha1 among the aldose epimerase protein family

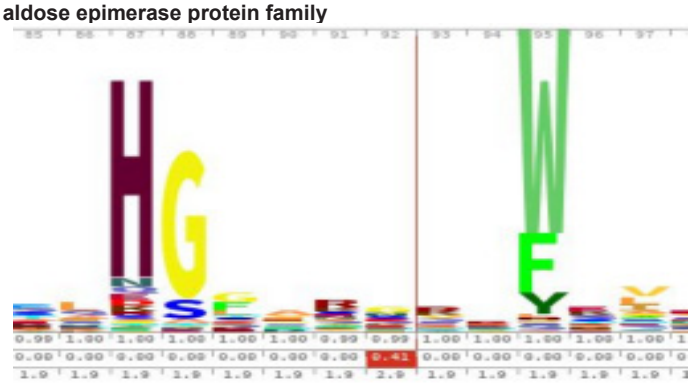
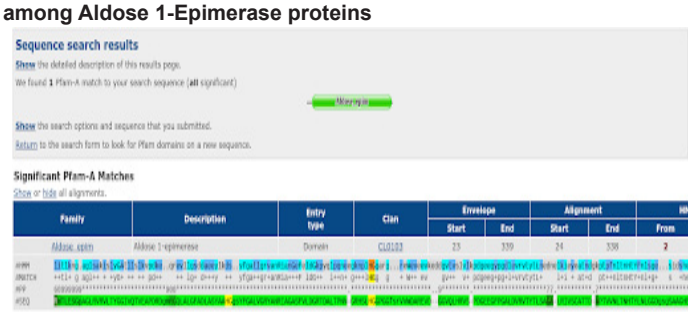
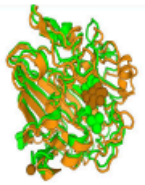
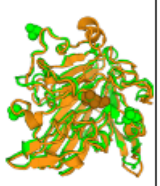
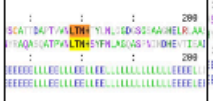
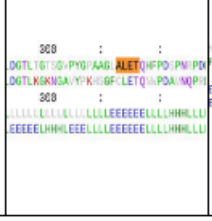
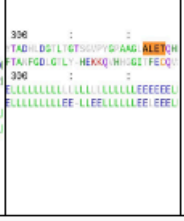


Figure 2. The top image is of the HMM logo from Pfam for the aldose epimerase family (PF01263) where the larger the letter, the higher probability that the amino acid residue is present at that location. The red line indicates uncertainty in the sequence. The bottom image compares the sequences of the HMM and the sequence of gm-ha1. The highlighted section was the result of searching for the consecutive HG peptides as seen in the HMM logo.

Dali shows good global alignment and conserved active residues among Aldose 1-Epimerase proteins



PyMOL shows NAD ligand in 4RNL-A near active residues

Chain/ Description	1SNZ-B Aldose 1-Epimerase	1NSS-A Galactose Mutarotase	1So0-A Aldose 1-Epimerase
Image Capture (Cartoon with Structure Conservation View)			
Active Site: Histidine (H)-182 LTNH			
Active Site: Glutamate (E)-308 ALET			
# of active site match	2/2	2/2	2/2
Good Fit? (Y/N)	Yes	Yes	Yes
Z-Score	53.6	44.3	53.6
RMSD	1.0	1.6	1.0
LALI (length of alignment)	333	319	335
NRES (Number of residues)	342	339	344
%ID	44%	29%	44%

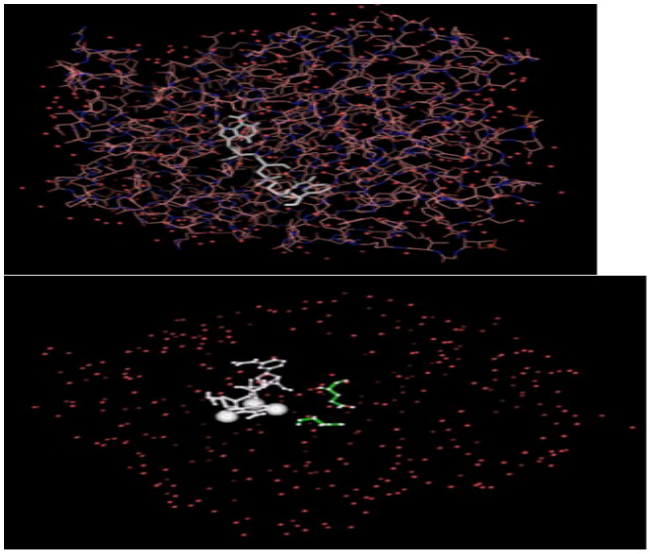


Figure 3. These images were screenshots from PyMOL after importing the ligand from Autodock. The left image shows where the NAD ligand is bound in the 4RNL-A structure where the 4RNL is shown in red and blue lines and the NAD ligand is shown in white sticks. The image on the right shows the same ligand, but the 4RNL-A structure is hidden, except for the two active sites residues of gm-ha1, shown in green. The top residue is glutamate, and the bottom is histidine.

Nickel His column purification shows presence of purified POI

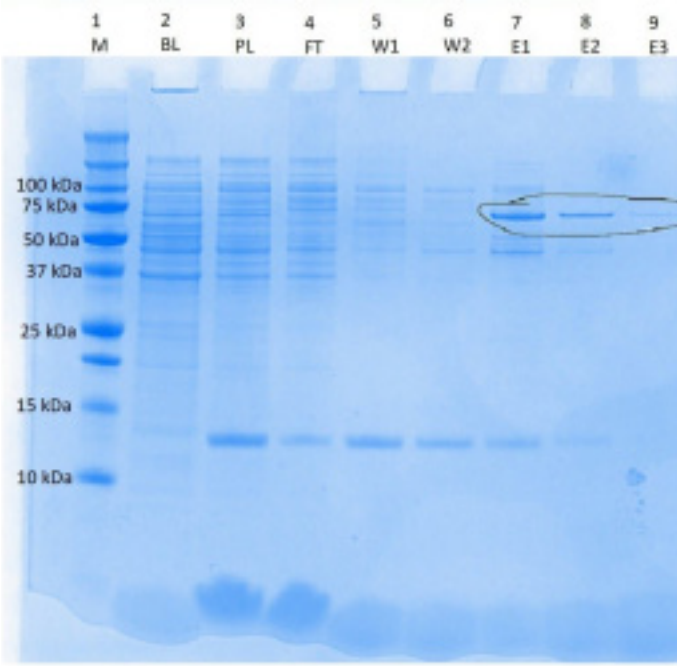


Figure 4. The proteins in this gel were purified using the HOOK 6X His Protein Spin Purification Kit. The first lane is the marker with relevant weights labeled. The second lane was the bacteria before lysis. Lane 3 was the protein lysate and lane 4 was the flow through. Lanes 5 and 6 were the two washes. Lanes 7, 8, and 9 were the elutions, and the dark bands in the circle are c8orf32.

Protein kinetics plots for c8orf32 protein show invalid data for

c8orf32 kinetics

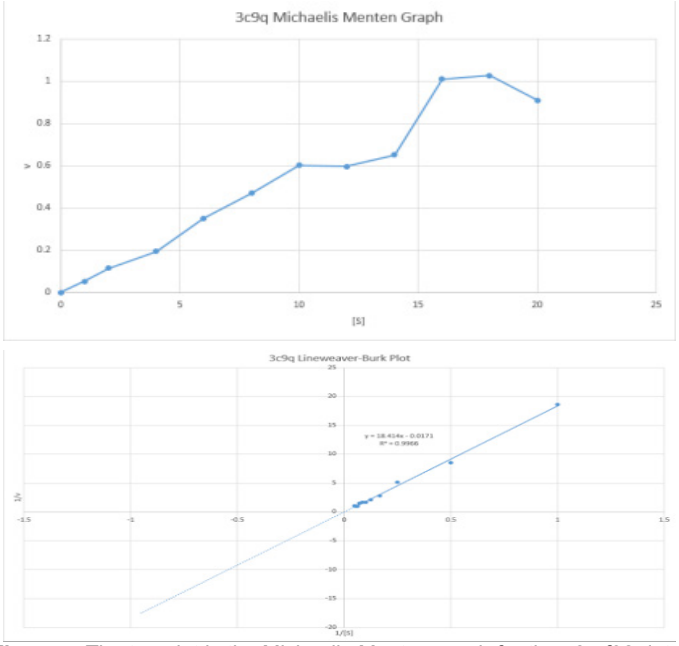


Figure 5. The top plot is the Michaelis-Menten graph for the c8orf32 data, generated using Excel. It depicts the velocity vs substrate concentration from 0-20 uL of substrate. The bottom plot is the Lineweaver-Burk plot for the same c8orf92 data from the Michaelis-Menten graph. The equation of the best-fit line of the data is shown in the graph above the trendline. Inverse velocity vs inverse substrate concentrations were the axes in this graph.

Habitat heterogeneity of the human skin microbiome: A comparison of the dorsal & ventral forearms

Megan Truman
Lake Forest College
Lake Forest, Illinois 60045

Introduction

The human skin is the largest organ of the human body and provides a microbiome ecosystem for bacteria. Human skin is composed of 1.8 m2 diverse habitats made up of creases, folds, and specialized niches that support various microorganisms (Grice and Segre 2011). Its variety of regions offers a wide array of habitats for microbial bacteria to reside. These various habitats make up environments with different chemical and physical properties. The chemical and physical make-up of the human body also dictates the type of bacteria residing in these habitats. Physiochemical skin features select for different sets of microbial communities whose niches are specialized to these habitats (Grice and Segre 2011). Biogeography of the human body also determines the composition of bacterial communities (Costello et al. 2009). Human skin is divided into biogeographical habitats via the effects of temperature, moisture, and density of hair follicles.

As the human skin provides a diverse combination of regions, the habitat complexity of these "geographic" areas plays a significant role in the bacterial composition. Since the skin varies topographically amongst regions, differing habitats are recognized to support distinct assemblages of microorganisms (Grice and Segre 2011). The topography of human skin is based on skin thickness and density of hair follicles and glands (Grice and Segre 2011). As skin thickness and the density of hair follicles and glands vary throughout the human body, there is a distinction between habitat heterogeneity. Hair density shapes habitat heterogeneity, as it may provide bacteria with more coverage from elements like the sun or temperature variation (Busse et al. 2018). The combination of hair follicles and sebaceous glands affects habitat structure as the gland sits at the base of the follicle and secretes oils that alter moisture and pH levels (Grice and Segre 2011). The density of these two structures ultimately influences the differentiation between habitats as regions with a high density of glands are moister and harbor more bacteria, whereas regions with more hair follicles shelter a wider variety of species (Grice and Segre 2011). These characteristics are important to consider when studying the biogeography of the skin microbiome because they differentiate between areas like the face and the arm. Ultimately, the variation in habitat creates niche-specific bacteria with physiological differences that affect community diversity, composition, and biomass (Oh et al. 2014). We must also consider habitat complexity and its effect on niche specification, and therefore species richness, to accurately map the habitat heterogeneity of the human body.

Comparable to our planet's diversity rules, the human body also has diversity rules that make up our skin microbiome which we can use to predict areas of diversity hotspots. Recent studies have discovered a link between habitat complexity and species richness, and we can use these findings as grounds to formulate a hypothesis on the human skin microbiome. Regions of high topographic complexity like the tropics and the benthos of marine environments contain high species diversity because they harbor challenging abiotic factors that lead to speciation. Speciation is driven by the partitioning of niches via factors like temperature, precipitation, and variability. Topographic complexity drives the increase in niche space, allowing more species to coexist and therefore more richness within the habitat (Allouche et al. 2012). A recent study on the benthos of marine environments provides evidence for this trend since they discovered that coral reefs harbored high species diversity and primary productivity because these areas were more topographically complex (Zawada et al. 2010). Taking these biodiversity rules into account plays a significant role in the composition of human microbial communities as regions of topographic complexity contain biodiversity hotspots and shape species traits, biotic interactions, and range distributions (Badgley et al. 2017). Therefore, the connection between habitat complexity and species richness will assist in the prediction of microbial diversity.

To study the diversity and richness of microbial communities on the human skin, we will compare topographically varying environments throughout the body. A recent study provides evidence for this comparison as they displayed that the human skin offers an opportunity to study the taxonomic and functional compositions of our microbial communities (Oh et al. 2014). As this composition differs across regions, looking at the density of hair follicles and sebaceous glands creates topographically varying regions as dissimilar as rainforests and deserts. (Oh et al. 2014). To display this compositional relationship between habitats, we will study the dorsal and ventral forearms as they offer differing temporal and topographic environments (Grice et al. 2009). The dorsal and ventral forearms are ideal for the study of varied bacterial composition between regions as they offer different spectrums of habitat heterogeneity. The dorsal forearm offers more heterogeneity as there tends to be a higher density of hair follicles and sebaceous glands (NYU Medical Center and School of Medicine 2007). The dorsal forearm also experiences greater exposure to sun and temporal variability. The ventral forearm harbors less topographic complexity and hair density along with decreased environmental exposure (NYU Medical Center and School of Medicine 2007). As these regions vary drastically in habitat heterogeneity, they are the ideal subject for the purposes of this study.

We aim to characterize the relationship of habitat complexity and the level of species richness on the human body. We hypothesize that topographically complex environments produce greater species richness. Using human forearms, we predict that more hairy forearms are more topographically complex environments than less hairy forearms. We also predict that the hairier environments produce more bacteria growth (richness) than less hairy environments.

Methods

The study included 40 total samples of bacteria cultures from human forearms. We collected two samples from 20 individuals, one from the dorsal forearm and one from the ventral forearm. The dorsal forearm was swabbed in a 2.5 cm2 site, 2 in. from the elbow joint. The ventral forearm was swabbed in a 2.5 cm2 site, 2 in. from the elbow crease. We also collected an equal number of samples from men and women, 10 from men and 10 from women. For data collection, we used random sampling where we did not characterize the heterogeneity of individual samples until after data collection. The samples were plated on agar and placed in the incubator within 1-2 hours after collection. All samples were collected and inoculated according to the *Sampling/Inoculation Procedure* within the Human Biogeography Lab document. We assigned each sample with a number between 1-30 and either letters "A" or "B" (i.e., 1A & 1B). The number corresponds to the individual in which we took the sample, while the letter corresponds to whether the sample was taken from the dorsal or the ventral forearm. The letter "A" corresponds to a sample collected from the dorsal forearm, and the letter "B" corresponds to a sample collected from the ventral forearm. The assigned number and letter will also correspond to the photograph taken for categorization of the hair density. Hair density was determined by estimating hair length in centimeters and the percentage of hair coverage within the 2.5 cm^2 sample site. We achieved these measurements by placing a 2.5 cm by 2.5 cm grid on the sample sites and photographing the area. Hair length and density were both estimated after collection via photographs displaying the 2.5 cm^2 sample site. Each sample was categorized on a continuous scale, but we ultimately determined that a more "hairy" and topographically complex environment contained a hair length of 1 cm or greater and a hair density greater than 50%. A less "hairy" and topographically simple environment harbored a hair length of 1 cm or less and a hair density less than 50%. We also determined different species of bacteria by their color, size, edge shape, and shininess. We determined species richness from the total number of morphologically distinct bacteria species on the plate. The total abundance of bacteria grown was determined by the total number of colonies present on the plate. We ran two linear regression tests to better understand our hypothesis and the relationship between species richness and habitat complexity on the human body. A linear regression compared hair density to the total abundance of bacteria. To correlate the dorsal and ventral forearms, we ran a linear regression for both data sets. In both regressions, we performed a log transformation of total bacterial abundance to standardize the data from outliers. Comparing the amount of bacteria growth per hair density on the dorsal and ventral forearms provided better grounds to test our hypothesis and display the relationship between species richness and habitat complexity of the human body. The male and female samples were also plotted separately to look for trends in hair density and bacterial abundance between the two sexes. An ANO-

VA test was performed within a regression analysis to obtain the *p*-value (*Significance F*), degrees of freedom (*df*), and the variation between samples (*F*). This test helped us determine the significance of our results.

Results

A total of 10 different bacterial morphospecies were discovered. This morphospecies set included *large and small yellow, small and shiny pink, large and small white, large brown, rough-edged white with yellow center, smooth-edged with a brown center, rough-edged white,* and *rough-edged brown*. The *large yellow* and l*arge white* bacterial species were most abundant as they occurred in 80% of our plates containing bacterial growth. The rarest species was the *rough-edged white with a yellow center* as it only grew one colony on a single plate. The plates with the highest abundance of bacterial growth were sampled from males. Our results contained three plates with overgrowth, all of which occurred on male samples (9A, 43 colonies, 5 morphospecies), (20A, 100 colonies, 6 morphospecies), (20B, 27 colonies, 4 morphospecies). Linear regression of the dorsal forearm revealed that the relationship between hair density and *log* abundance was insignificant ($F_{1,18} = 0.684$, $p = 0.419$). The dorsal forearm data points also did not significantly fit the linear regression and varied from the linear trendline ($R^2 = 0.366$). Linear regression of the ventral forearm revealed that the relationship between hair density and *log* abundance was insignificant ($F_{1,18} = 0.214$, $p = 0.649$). The ventral forearm data points also did not significantly fit the linear regression and varied from the linear trendline ($R^2 = 0.117$). In the ventral samples, there were seven plates with one or more colonies grown while the dorsal samples only had four. Although both datasets do not show a significant ($p = 0.419$, $p = 0.649$) relationship between bacterial abundance and hair density, the ventral forearm did produce more bacterial growth overall.

Figure 1. Log_{10} of total bacterial abundance as a function of hair density on the dorsal forearm.

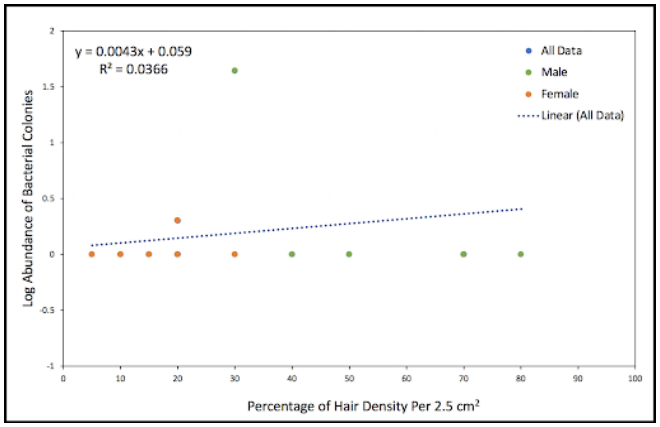


Figure 1 displays the total Log_{10} abundance of bacterial colonies grown amongst varying hair densities on the dorsal forearm. The percentage of area covered by hair per the 2.5 cm^2 sample site is displayed on the x-axis. The Log bacterial abundance grown in each plate is displayed on the y-axis. Male samples tended to have greater hair density and slightly more bacterial growth. Female samples tended to have less hair density and less bacterial growth. The model does not explain much of the variation ($R^2 < 1$) and bacterial abundance as a function of hair density is insignificant ($p > 0.05$).

Figure 2. Log_{10} of total bacterial abundance as a function of hair density

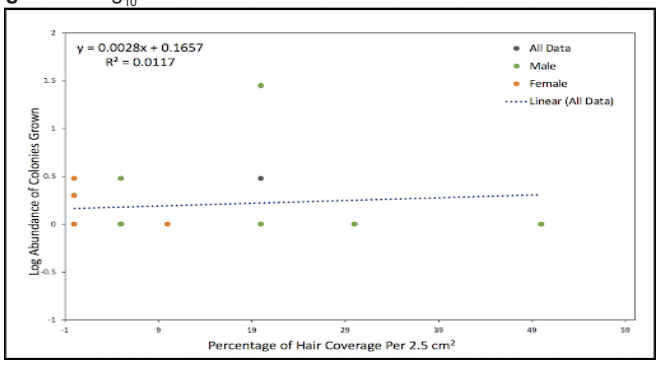


Figure 2 displays the total Log_{10} abundance of bacterial colonies grown amongst varying hair densities on the ventral forearm. The percentage of area covered by hair per the 2.5 cm^2 sample site is displayed on the x-axis. The Log bacterial abundance grown in each plate is displayed on the y-axis. Male samples tended to have greater hair density and more bacterial growth. Female samples tended to have less hair density and less bacterial growth. The model does not explain much of the variation ($R^2 < 1$) and bacterial abundance as a function of hair density is insignificant ($p > 0.05$).

Discussion

We studied the relationship between bacterial abundance and habitat hair density and found no significant correlation between the two variables. Our results from sampling the dorsal and ventral forearms do not support our hypothesis that there will be increased species richness within habitats of high complexity as there was no correlation between the density of hair coverage in 2.5 cm^2 and bacteria richness. Fitting the total bacterial abundance to a Log_{10} transformation did provide different results and did not further support our hypothesis. Our insignificant results may be due to differences in patterns o Although our results did not display a correlation between bacterial abundance and hair density, we did observe patterns between samples taken from the dorsal versus ventral forearm. Analyzing Figure 1 and Figure 2, we see that samples taken from the ventral forearm yielded more plates with bacterial growth than the dorsal region. Since the dorsal region harbors more complexity and habitat heterogeneity, this trend is contradictory as these characteristics are also linked to species richness (Pausas et al. 2013). An explanation for increased growth in the ventral region may be due to immigration from the dorsal forearm. The dorsal forearm is exposed to more sun and therefore more climatic variability, so it may force bacteria to look for a darker and moister region to accumulate. In biogeography, evidence for immigration is provided by the finding that climatic warming leads to range shift of species and pushes them to cooler lowlands (Badgley et al. 2017). This biogeographic trend offers an intriguing insight as our data displayed a similar model on the human body. Looking at the trendline of both Figure 1 and Figure 2, we observe more plates with bacterial growth as hair density increases from moderate to high. Although our data revealed these variables as insignificantly related, the amount of coverage from hair may explain this trend. A recent paper on bromeliad microfauna revealed that canopy cover significantly influenced richness within habitats. They discovered that increased canopy cover led to decreased daily fluctuations in habitat temperature, which allowed for an increased microfauna richness (Busse et al. 2018). Our results align with their findings as we found an increase in bacterial abundance with an increase in hair density. The survival of bacteria is protected from elements like sun and heat because greater hair density provided more coverage.

The majority of our samples taken from both the dorsal and ventral regions did not develop any bacterial growth. The widespread lack of growth may contribute to the insignificance of our data when comparing species richness and habitat complexity. Of the forty samples, only 25% produced one bacterial colony or more. The large lack of growth may be due to the selected habitat. When comparing areas of the body, occluded areas like the groin, toe web, and ear canal tend to have more bacterial growth because they are higher in temperature and humidity. When contrasted with areas like the forearm and leg that are drier and experience more temperature variations, they are found to have significantly less growth than moist regions (Grice and Segre 2011). The discrepancy between these two habitats explains the lack of growth as our samples were taken from a drier, more variable habitat. We also found that we had a fraction of plates with substantial overgrowth as compared to the rest of the samples. Plates that exhibited growth contained 5-10 times more colonies than the other plates, and we discovered that all overgrowth samples came from males. This discrepancy may be accounted for by the biological differences in males and females. As males and females produce different levels of sweat, sebum, and hormones, overgrowth may be because men tend to sweat more and therefore harbor more bacteria (Grice and Segre 2011). Men also tend to have greater hair density than females, which could also contribute to increased levels of sweat and sebum as greater hair density equals more follicles and sebaceous glands (Grice and Segre 2011). Looking at Figure 1 and Figure 2, our data agree with this suggestion because both the dorsal and ventral forearms have greater bacterial abundance with increased hair density in males rather than females. Our results also revealed that our samples produced a wide variety of bacterial morphospecies. This trend is supported by the idea that

habitats containing a greater diversity of microorganisms are typically less stable in member and structure over time. One of these temporally unstable environments includes the volar forearm (Grice and Segre 2011). As the forearm is exposed to sun, heat, and variability, it is more likely to harbor a wider variety of morphospecies because it interacts with more elements.

As a whole, our data reveals that habitat complexity does not play a significant role in skin microbial diversity, and the habitats we chose did not exhibit the level of bacterial abundance and diversity as we assumed. Our non-significant results could also be accounted for by the methods used. Recent evidence has revealed that the swab method we used does not yield an accurate representation of bacterial abundance and diversity (Favero et al. 1968). Some other issues with our methods may include the sample site area, time from collection to plating, and habitat selection. As we discussed above, our choice of the forearm habitat yielded limited growth because it is a drier and more exposed environment than occluded areas as they harbor better conditions for bacterial growth (Grice and Segre 2011). The chemical makeup of our habitats may have also contributed to limited growth because some studies revealed that the microbes residing in sebum and hair follicles are anaerobic. Bacteria of this type wouldn't be able to grow on our plates because they were in atmospheric oxygen conditions (Kong and Segre 2012). In the future, it may be beneficial for our study to compare habitats with more distinct contrast in heterogeneity, for example the armpit and the forearm. The study of skin microbe community composition and assembly is crucial to the identification of patterns in habitat heterogeneity, topographic diversity gradients, and niche specialization. f species richness in wet and dry habitats. Studies have shown that wetter habitats correlate with species richness and harbor significantly more species than drier habitats (Lengyel et al. 2016). This trend may explain our insignificant amount of bacterial growth on the forearm because it's categorized as a dry habitat within the human body.

Troxidoreductase, a protein of unknown function found in *Archaeoglobus fulgidus* shows EC 1 and EC 3 characteristics

Tate Rosenhagen and Dr. William Conrad
Lake Forest College
Lake Forest, Illinois 60045

Abstract
Troxidoreductase, a protein of unknown function found in *Archaeglobus fulgidus*, is commonly found in and believed to be responsible for the souring of oil fields via sulfur production. With a hypothesized EC 3 function we tested structure and function by using structural comparison (Pymol/Promol) and ligand binding analysis (Autodock) to examine similarities with proteins of known function and BLASTP and Pfam to search for families and genetic sequence similarities in the DNA. Results showed active site similarities between Troxidoreductase and some EC 3 proteins, but closes structural and ligand binding similarities with EC 1 proteins, leading to the hypothesis that Troxidoreductase is an Oxidoreductase that also has function of a Hydrolase, which could explain the souring of oil fields through the production of sulfur gas when water is present.

Introduction
Oil production is currently a vital industry, as many of the machines used every day around the world are powered by oil-based fuel. Thus, the efficient usage, but also efficient production, of oil cannot be understated, as an oil field turning sour can cost the producers and the customer money, as well endangering workers through the production of sulfur gas. Oil fields are an extreme environment in which few organisms can be found. Those surviving there are often considered extremophiles as they are also found exclusively in extreme conditions, such as on the ocean floor around hydrothermal vents. One such organism able to survive in these conditions is *Archaeoglobus fulgidus*, a sulfur-loving extremophile. This archaebacteria has been found in the depths of the biosphere, but also in hot deep oil reserves, and is hypothesized to cause the missing first step of hydrocarbon oxidization in oil fields, which then in turn leads to the souring of said oil field or reservoir (Khelifi et al. 2014). *Archaeglobus fulgidus* has been found to create a biofilm in response to stressors in the environment which may help it attach to metal in order to stimulate growth in a metal deficient environment, thus aiding in growth as it is a metal-reducing organism (Lapaglia and Hartzell 1997). As a hyper thermophilic sulfur reducing organism, there is reason to believe that *A. fulgidus* may be the first step of hydrocarbon oxidization in oil fields, which in turn leads to the souring of oil fields and reservoirs (Khelifi et al. 2014). The souring of oil fields leads to increased oil prices to the average consumer, as a high sulfide concentration can lead to rejection by the oil refiners, lowering the supply of oil in the market. It also produces hydrosulfide gas, an extremely toxic gas that lowers air quality and must be monitored during the oil mining process. Furthermore, it can corrode the infrastructure of an oil mining machine and plug the reservoir through the precipitation of iron sulfide, all increasing the cost of the production of oil (Gieg, Jack, & Foght 2011). In an effort to examine this possible reaction, we have chosen to examine hypothetical binding site Troxidoreductase on hypothetical protein AF_1432. AF_1432 is part of the HD superfamily of metal dependent phosphorylases and a hypothetical protein believed to be found in *Archaeglobus fulgidus* (Aravind and Koonin 1998). HD domains have been found to play a role in antiviral defense in the human protein SAMHD1 (Beloglazova et al. 2013). The SAMHD1 protein was found to limit replication of the HIV-1 genome, but not HIV-2, and the purified HD domain from humans and mice contained dGTP-stimulated tryptophohydralayse activity (Beloglazova et al. 2013). Troxidoreductase's enzyme consortium number is 3.1.3.89 where each number separated by periods signifies something about the protein. The 3. Signifies the enzyme is a hydrolase, and the 1 signifies it is an esterase, so it acts on ester bonds. 3.1.3 signifies phosphoric-monoester hydrolases, and the 3.1.3.89 specifies that Troxidoreductase shows specificity towards deoxyribonucleoside 5'-monophosphates ("ENZYME - 3.1.3.89 5'-Deoxynucleotidase.") From this information, there is reason to believe the *Archaeglobus fulgidus* may cause the vital missing step of the souring of oil fields. Due to the organism of origin, as well as the EC number, we hypothesize that Troxidoreductase will have characteristics of a metal dependent phosphorylase due to its origin in *A. fulgidus* but perform some sort of role of a hydrolase due to its EC 3 classification.

Methods:

Pymol/Promol Search
In this lab we used a VPN to access Lake Forest College's programs remotely via a remote desktop. This was necessary in order to use Pymol and Promol, as both of these programs have strict licenses and can only be run on Lake Forest's network. Once Pymol and Promol were opened, motif finder in Promol was selected and our two proteins, 2O14 and the unknown, were searched separately. The search can be narrowed by selecting different libraries to search your protein in, as well as limiting the search by EC number if your EC number is known. 2O14 has an EC number of 3, so 2O14's search was limited in terms of EC 3 for time's sake. Searches typically take 5-10 minutes. Pymol then shows a search result box, as well as an image of your searched protein, which can be compared against other proteins that have come up in your search. We then checked the show alignment box and calculate RMSD box and compared our searched protein with every protein result that had a Levenshtein score of 0. From there 3 RMSD values were calculated in the Pymol molecular graphics system box, the first being the RMSD of all atoms, the second being just alpha carbons, and the third being alpha and beta carbons. The RMSD of alpha and beta carbons will be of particular note to us this lab. Finally, after choosing the three matches with the lowest RMSD value (alpha and beta carbon) were selected to compare residue alignments. This was performed by clicking on the structures that lined up together, and the amino acid residues that matched would show up in the pymol graphic box. Alternatively, by clicking the small s in the pymol viewer window, and then clicking the structures that overlap again. The AA sequences will be displayed at the top of the viewer, with the overlap of the structure being highlighted in red.

BLASTP Search
First, the amino acid sequences for our unknown protein and 2O14 from rcsb.org. The sequences were run through the BLASTP search and the amino acids were broken into 3 amino acid 'words' compared to other protein sequences of known functions. Other proteins were sorted by number and percentage of alignments, and thus the proteins most similar to our proteins could be seen and their functions could be examined in hopes of understanding our proteins functions. A SMARTBLAST was also performed to see the organisms in which our proteins are found. Finally, a Pfam search was performed on our proteins amino acid sequences to determine the superfamilies and domains found within our amino acid sequence. The Pfam search was largely supplemental to the BLASTP search in our findings.

Dali Search
In order to compare our proteins on Dali, we searched proteins by name (2o14 and Troxidoreductase) on Dali, which found similar structures without taking into account their side chains in order to save space. The search took a few hours, and we then examined one protein from each molecule to amount to a total of three similar proteins and examined their amino acid structures and their 3D cartoon structure comparisons. We then used the active sites found in the previous lab, found their location in the amino acid sequence, and created a five-letter amino acid code by choosing the five amino acids before our active site. This was necessary as Dali and RCSB amino acid number does not line up exactly. We then searched this amino acid sequence in order to find our active sites location in the Dali search and checked to see if the similar proteins had the same active sites. This may give insight into the function of our protein.

Autodock search
To begin with, PyRx was loaded in order to run an autodock test on our proteins of choice. We then went to RCSB, downloaded the PDB file of our protein, and uploaded it into PyRx. This molecule was then converted to a macromolecule. After that, we went back to RCSB to search for ligands of interest in the same EC class. These ligands ideal SDF data files were downloaded and many had to be edited from a txt to SDF file. Once 4-6 ligands were downloaded and translated to SDF files, they were inserted into PyRX via the import chemical table file – sdf function. After they were all imported, they were right clicked to minimize them all, and right clicked again to convert all to autodock ligand. We then used Vina Wizard to run the docking function, whereas we clicked all the ligands and our one protein of interest (macromolecule). The processing time took a few minutes, and we then sorted by affinity to find our best binding site match, showing both the ligand and the specific conformation of the ligand. We downloaded the list

by saving a CSV file which can be used in Excel later, and then saved the whole workspace as a tar.gz file. Finally, we downloaded our best match, macromolecule and ligand, again as a PDB and our best match ligand as a PDB file. We then uploaded these files into Pymol for a better visualization of the binding site of our ligand and macromolecule. By selecting the show surface of the macromolecule and highlighting the binding sites found in the previous Pymol lab, we can change them a different color. Any errors and issues were resolved with assistance from Dr. Conrad.

Protein Purification

First, we bound our protein of interest with the binding solution, which bound specifically to our POI. The binding buffer was 50 mM Na2, HPO4, 300mM NaCl, and pH 8.0, supplemented with 10mM imidazole. This binding buffer was chosen to reduce non-specific interactions between our protein of interest and other proteins, thus ensuring our binding solution only binds to our POI. Then we used a wash solution to elute any proteins not bound to our binding solutions, thus leaving us with hopefully pure protein bound in our column. Finally, we used an elution solution to elute our bound POI, which contained an excess of imidazole to force our protein out of the binding column. We expected to see our protein in the fractions after the lysate, and in the elution fractions with the last elution fraction having no protein, meaning we got it all out. We collected every fraction to ensure we did not have our protein elute too early or have other proteins present in our wash solution.

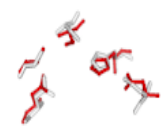
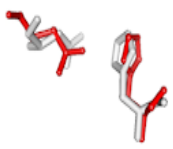
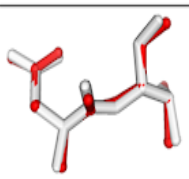
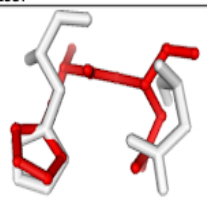
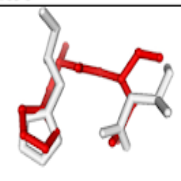
Protein Kinetics (2o14 and YfkN)

3GVE (YfkN) elution 1 was dialyzed into 50 mM sodium phosphate buffer (pH 7.6). This was done by using the Pierce 3 kDa MWCO centrifugal filter concentrator and spin-concentrated the samples 3x using 20 ml of buffer per 1 ml of elution. The protein abundance was measured using absorbance at 280 nm with a path length of 1 cm. The beer's law equation was used to calculate the concentration of the sample in molarity, and then converted to mg / ml. Using a 96 well plate, the following reagents were added with varying volume: 160 ul of 50 mM sodium phosphate buffer (pH 7.4) and X uL of 10 mM para nitrophenol acetate in acetonitrile. The baseline absorbance at 405 nm wavelength for each well was measured. 20 ul of enzyme was added to each well, and then proceeded to read A405 across the entire 96 well plate. The plate was read every 41 seconds in order to observe the change in absorbance over time in each well. This was repeated for 2o14 as well.

Figures:

Pymol and Promol shows EC 1 and EC 3 structural characteristics

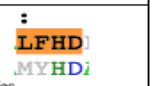
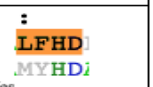
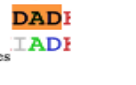
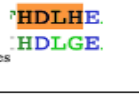
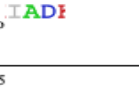
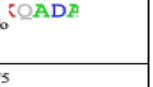
Table 1. Table containing three best alignments from a Pymol/Promol search with RMSD values for alpha and beta carbons. Troxidoreductase and 2O14 are shown in red, protein being compared shown in grey.

Alignment Ranking	2O14 (red) w/____ (grey)	2O14 RMSD (alpha and beta carbons)	1YOY (red) w/____ (grey)	1YOY RMSD (alpha and beta carbons)
1	 1BWR	0.712	 1S3I	0.65
2	 1A30	0.616	 1C9U	0.93
3	 2ACY	1.046	 1BG6	1.13

Dali search finds best match as another hypothetical protein in the same archaebacteria

Troxidoreductase

Table 2. Table of Dali search results and comparisons of Troxidoreductase (green) with known proteins (other colors). 3 best matches, each from a different organism, was chosen for comparison.

Chain/Description	4l1j-a HD DOMAIN-CONTAINING PROTEIN 2	1xx7-a OXETANOCIN-LIKE PROTEIN	2par-b 5'-DEOXYNUCLEOTIDASE YFBR
Image Capture (Cartoon)			
AS: His 76 (All residues off by two on fasta) LFHD	 Yes	 Yes	 Yes
AS: Asp 77 LFHD	 Yes	 Yes	 Yes
AS: Asp 126 DAD	 No Match	 Yes	 Yes
AS: Glu 80 HDLH	 Yes	 Yes	 No
AS: Asp 124 DAD	 No	 No	 No
# of active site match	3/5	4/5	3/5
Good Fit?	No	Yes	No
Z- Score	14.9	14.4	13.9
RMSD	2.2	2.3	2.6
LALI	138	132	136
NRES	188	172	178
%ID	23	33	21

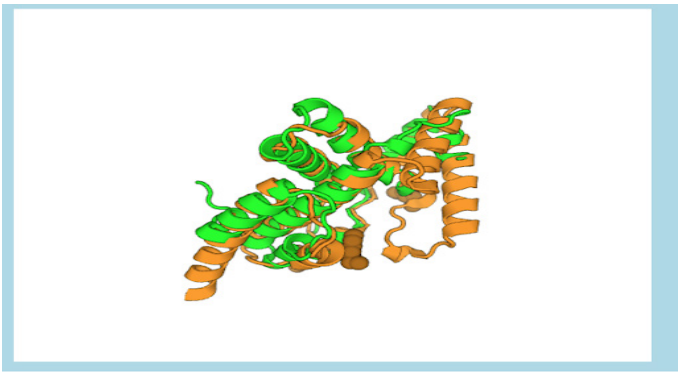


Figure 1. Dali structural comparison of Troxidoreductase (green) and 4L1J (orange). Structure is in cartoon format.

BLASTP search results point to HD domain presence

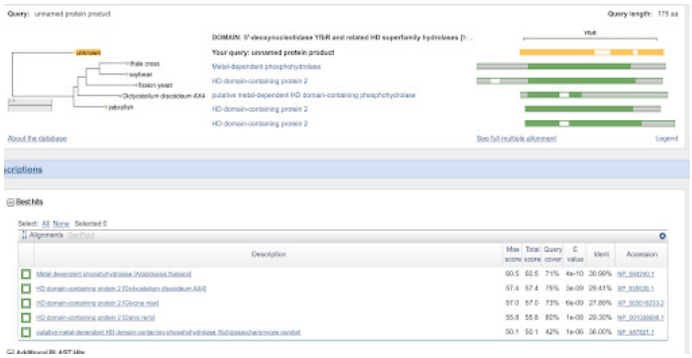


Figure 2. Phylogenic tree and family description from BLASTP search results of Troxidoreductase.

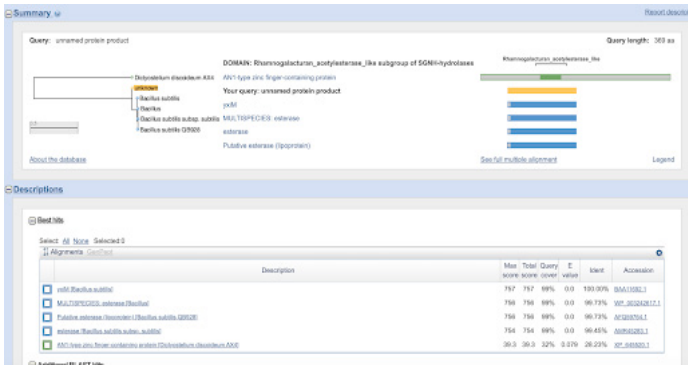


Figure 3. Phylogenetic tree for YXIM based on known protein sequences in organisms of origin. Here we see a large grouping of Bacillus species, suggesting a highly conserved function within Bacillus.

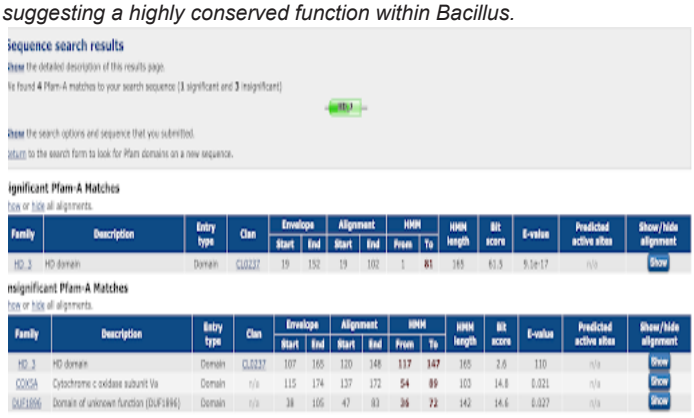


Figure 4. Family descriptions of Troxidoreductase from BLASTP search when compared with domains and families of known function.

Autodock results show lack of binding at hypothesized EC 3 binding sites, leading to EC 1 function hypothesis

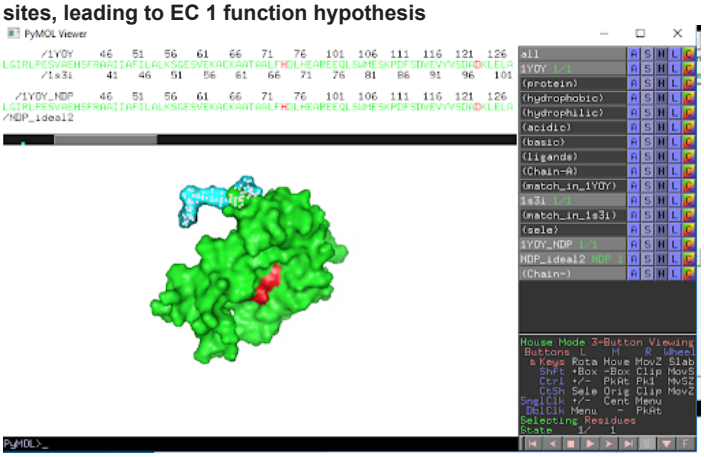


Figure 5. Pymol visualization of the Troxidoreductase and a ligand (NBD). AF protein Troxidoreductase is seen in green, previous binding sites seen in red, and NDB ligand shown in blue. The ligand does not bind to the hypothesized active site, suggesting another possible binding site and function.

Protein kinetics shows lack of enzymatic activity in unknown protein and unusable data with 2o14.

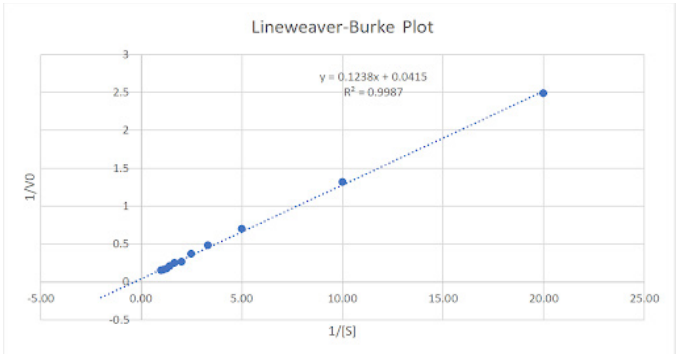


Figure 6. Line-Weaver Burke plot shows unusable data for 2014 elution 1. Line of best fit has a y-intercept of +0.0415, showing that we cannot use this data to draw conclusions of our protein's functionality.

Results:

Pymol/Promol search shows structural alignment with both EC 1 and EC 3 proteins.

After the initial research on RCSB, we examined Troxidoreductase in a Pymol/Promol search to compare structural similarities with proteins of known function. We did this as a first step to confirm that our hypothesized function of a hydrolase, due to its EC 3 classification, was correct. However, contradictory to our initial EC 3 classification, the three best matches were all proteins with an EC 1 classification. Initially, we believed we may have run this test incorrectly, but after running it with our positive control YXIM, we confirmed we were running our test correctly and had fairly good RMSD values as see in Table 1. After confirming the test was run correctly, we did more research on the best fit for AF_1432's Promol search and found that 1S3I, while classified as an EC1, had characteristics of an EC 3 as well, as it has functions of an oxidoreductase and a hydrolase. With this context, it does make sense that Troxidoreductase could show characteristics of an EC1 and EC3 type enzyme.

BLASTP search shows presence of conserved HD domain and few similarities with proteins of known function in sample organisms.

In an effort to add further details to explain the function of our protein, a BLASTPP search was performed on our protein of interest and YXIM, as a positive control. We hypothesized that our unknown protein would have domains of a hydrolase family and possibly an oxidoreductase due to the EC 1 classification in the Pymol/Promol lab. In Figure 2 we found that the phylogenetic tree did not produce good results, as can be seen with the highest identity score being a protein in Zebra fish with 30% match, followed by matches in Soybean and Thale Cress among other organisms. While a wide variety of organisms who point to a highly conserved mechanisms, the identity percentage of 30% at its highest instead points to a highly specific mechanism, as no other organisms in this databank had this mechanism. To ensure this was not simply an error run in the test, we ran the test with YXIM as well, and found a 100% match with 2014 in *Bacillus subtillis*, showing the test was run correctly (Figure 3). However, the most important result of this test was in Figure 4, where the HD domain was found to be present in Troxidoreductase. The HD domain is a known phosphohydrolase, which again helps us in hypothesizing that it is an EC 3 classification.

Dali search results are inconclusive as the only protein of good match was itself.

While the previous tests provide context as to the function of Troxidoreductase, finding the listed domains and structural similarities, we also decided to use a Dali search to look for other matches found through Dali's global alignment and ignorance of side chains. We hypothesized that the best match would again be a phosphohydrolase and, unfortunately, the best fit for our unknown protein was another hypothetical of protein of unknown function, 1xx7, but the hypothesized function was a metal dependent phosphorylase, which is what we believe our unknown protein to be (Table 2). The

next match with a protein of known function, which was a poor match to 4l1j, does provide some more context as it is a hydrolase found in homo sapiens, but it only had 3 out of 5 active site matches and a poor percent ID. Thus, we can continue with our hypothesis that our unknown protein is a metal dependent phosphorylase that may also some features of an oxidoreductase.

Autodock lab shows best binding ligands are ligands of 1xx7, an EC 1 protein that was most similar to Troxidoreductase in the Pymol/Promol lab.

Proceeding with our experiments, we performed an autodock test to examine ligand binding and compare then to the ligand binding sites hypothesized in the Pymol/Promol lab. In Figure 5 we see our protein Troxidoreductase in green, the proposed EC 3 binding site in red, and the ligand binding in blue. NBD, the ligand of best match, was found to be the best fit in our protein, and coincidentally is not an EC 3 ligand, but an EC 1 ligand from the protein of closest resemblance to Troxidoreductase, 1xx7, an EC 1 protein. This result lends itself to believing that Troxidoreductase has some features and an active site similar to a hydrolase, but the main active site, or the one who's ligands matched better in this search, was an oxidoreductase active site. Similar to its EC 1 counterpart, 1xx7, Troxidoreductase may help or perform two different reactions, but we can confidently say that a ligand of an EC 1 protein binds best to Troxidoreductase, thus suggesting EC 1 function.

Overall experiment is inconclusive, shows characteristics of EC 1 and 3, leading to hypothesis that Troxidoreductase is a water dependent oxidoreductase.

While we cannot make concrete conclusions from our data, we have been able to make some progress in understanding the function(s) of Troxidoreductase in these experiments. While not fitting simply into one EC class, Troxidoreductase appears to have active sites of an EC 3 protein as well as ligand binding affinity for EC 1 ligands (DMB).

Protein kinetics lab shows a reactive control enzyme but unusable data.

While we were unable, due to covid restrictions, to complete the purification and kinetics of our given protein, we were, as a class, able to complete the purifications and kinetic tests with 2o14, our class control. For our 2o14 sample, we saw enzymatic increase over time and an increase of enzymatic activity with an increase in concentration of the protein. However, as seen in Figure 6, the line-weaver burke plot had a positive Y intercept, meaning that the data cannot be used. In regard to our data, we would expect a very similar line weaver plot for our data to be successful, except for a negative Y intercept. From there we would be able to interpret their Km and Vmax.

Discussion:

While we cannot make concrete conclusions of our protein's functions based on our experiments, we can make a hypothesis based on the sum of our experiments. As previously stated, Troxidoreductase showed characteristics of EC 1 and EC 3 functions, with an HD domain similar to a hydrolase but structural similarities with an oxidoreductase. Upon further research, the EC 1 protein of best fit, 1S3I, which has a PDB classification of a hydrolase and oxidoreductase performs a catalytic reaction ending with the creation of carbon dioxide ((Bank, n.d.)). With this information and the data from our experiments, several assumptions can be made about the mechanism and role of Troxidoreductase. From our binding site comparison and our Dali search, we believe that the binding site seen in Figure 5 is used for a phosphohydrolase mechanism as the binding site falls within the HD domain. However, the ligand binding to the protein in Figure 5 is not binding to the active site highlighted, as this ligand of best fit is from an EC1 protein. This seemingly nullified our hypothetical phosphohydrolase mechanism, which was based on the EC3 hydrolase classification, and pointed towards a different mechanism all together. With Troxidoreductases similarities to 1S3I, and the ligand 1xx7 binding on a different region to our proposed active site, we also believe that our protein performs an oxidation reduction reaction along with its hydrolase function. This combination of reactions would make sense as our protein is believed to cause the souring of oil fields by producing sulfur gas, most likely by an oxidation reduction reaction as *Archaeglobus* bacteria are known to be sulfate reducing bacteria, and these oilfields tend to be soured with the addition of water, suggesting the hydrolase reaction may play some part in this reaction. However, we cannot make these statements with much confidence due to the fact that many of our lab results were not in agreement with each other and we were unable to take this specific protein, Troxidoreductase, to the completion of the lab, and thus are unable to propose a plausible mechanism at this time.

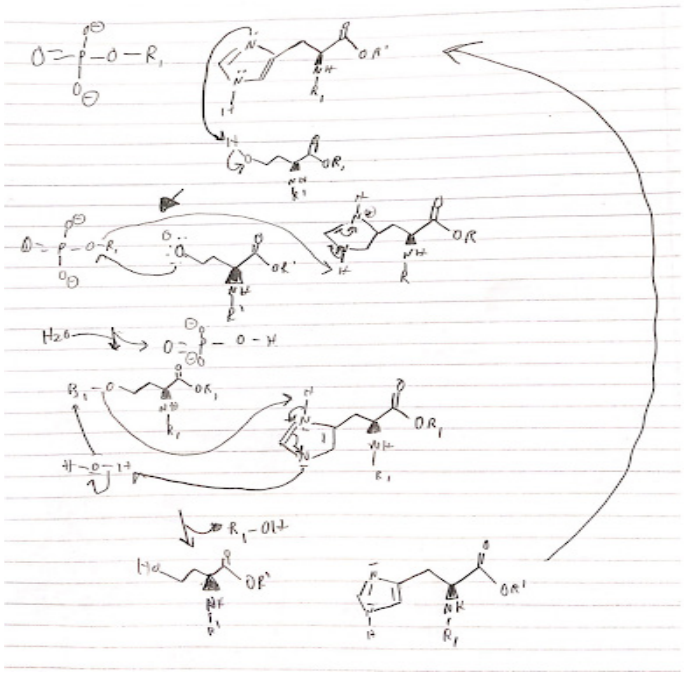


Figure 7. Hypothesized EC 3 mechanism for Troxidoreductase involving the Histidine 76 and Aspartic Acid 124 residues. This mechanism was not found in our experimental trials; thus, we cannot say that this mechanism is present. The mechanism found seems to be of EC 1 nature and more tests are required to find the correct mechanistic steps. Authors Note: Mechanism drawn by hand as Chemdraw failed to work.

If we were able to continue this lab, or study Troxidoreductase in the future, one possible experiment would be to test varying concentrations of our proteins in oil, both with and without water, and monitor each solution over time for production of sulfur, similar to our temperatures (83 degrees Celcius) to mimic the conditions within an oil field, as they are usually at high temperature and pressure (Beeder et al. 1994). If no wells had any production of sulfur, Troxidoreductase would not be the missing link in the oil souring chain of bacteria. If sulfur was produced in both the oil and oil + water wells, Troxidoreductase's oxidation-reduction reaction would not be water dependent, and if sulfur was produced in only the oil + water wells, then we would know the hydrolase reaction is necessary. It is our hypothesis that only the wells containing oil and water will produce sulfur, as in previous research oil fields were found to only sour with the addition of water, and that 1S3I requires the hydrolase reaction to produce carbon dioxide as previously stated. The next continuation of our future experiment would be to determine if inhibition of the HD domain found in the EC 3 active site would inhibit sulfur production from Troxidoreductase's reaction. This would have great significance on the oil industry, as the inhibition of sulfur production in an oil field would protect oil workers and lower the price of oil due to a decrease in unusable oil, or oil that needs to be refined even further, making it more costly. To do this, we would use the same experimental set up as in the previous experiment, but only with oil and water, and with the addition of varying amounts of six select SAMHD1 inhibiting compounds from the research paper *Identification of Inhibitors of the dNTP Triphosphohydrolase SAMHD1 Using a Novel and Direct High-Throughput Assay* (Mauney et al. 2018). The wells would contain increasing concentrations of each compound and monitored for production of sulfur gases. We expect if a compound successfully inhibits the HD domain in the active site, no sulfur would be produced in those wells. If a compound or compounds were successful in inhibiting the production of sulfur, we could then move on to studying the compounds effects on crude oil and seeing if the compound is a viable product for the oil industry to treat or prevent souring of oil fields. If none of the wells show inhibition of sulfur production, either the compounds did not successfully block the HD domain of Troxidoreductase, or the HD domain is not needed for the production of sulfur. In either case, more studies on Troxidoreductase are needed to determine what needs to be inhibited to limit the production of sulfur and if this organism is in fact the missing link in the souring of oil fields.

Distribution of *Rhinolophopsylla*, *Rhopalopsyllus*, and *Rhynchopsyllus*

Kerry Mullen
Lake Forest College
Lake Forest, Illinois 60045

Introduction

Siphonaptera, otherwise known as fleas, are an order of small external parasites that obtain nutrients by consuming blood from their host. With approximately 2,500 species, flea distribution extends to every continent, including Antarctica, and they can be found in habitats ranging from tropical rainforest to arctic tundra (Whiting 2008). This expansive range results from each species evolving a narrow range of tolerance for temperature and humidity. However, despite a narrow tolerance range, most adult fleas are not limited by microclimates. The seasonal and geographic distribution of fleas is primarily determined by larvae development's specific requirements (Hastriter 2009). The second stage of the flea life cycle, larvae, are typically not parasitic and most often develop off-host (Krasnov 2007). During this stage, the larvae, which are worm-like, blind, and legless, are exceedingly susceptible to life-ending conditions. After three molts, the larvae become pupae and spin into cocoons, where their appendages will begin to develop. The emergence of adults from these cocoons requires specific stimuli dependent on the species, such as temperature, vibration, or pressure (Hastriter 2009). As adults, fleas become permeant satellites of their hosts, alternating their time between periods on the host and periods in the host's burrow/nest (Krasnov 2007). As adults, a series of morphological adaptions allows fleas to attach and remain on their hosts. Laterally compressed bodies enable them to move through their host's fur or feathers. Strong claws and setae enable them to anchor themselves to their host's skin and prevent being dislodged. Their mouthparts have been modified for piercing skin and sucking. And despite not possessing wings, fleas have extremely strong hind legs adapted for jumping (Hastriter 2009). These strong legs allow individuals to jump from host to host or return to their original host after a period in the nest. Much of fleas' distribution and dispersal opportunities depend on what host species they reside on. Despite their strong hind legs, a flea's small size makes traveling great distances difficult, if not impossible, under their own power. Thus, in order for fleas to colonize new locations or new hosts, they must be carried there by another species. As such, a flea's habitat patch is their host, rather than their geographic location, with parasitic individuals distributed across host individuals who provide a place for living, foraging, and mating (Krasnov 2004). Thus, a flea's distribution can be expected to be influenced by the host's habitat and mode of transportation. Based on this information, two hypotheses were investigated. The first hypothesis is that fleas found on flying hosts will have a greater distribution than fleas found on non-flying hosts. Many bird and bat species have yearly migratory patterns that result in them traveling great distances between their summer and winter locations. Meanwhile, non-flying organisms, like rodents and deer, do not migrate great distances and therefore occupy smaller ranges than their flying counterparts. The second hypothesis is that the genus *Rhinolophopsylla* will have greater overall distribution than the genera *Rhopalopsyllus* and *Rhynchopsyllus* because *Rhinolophopsylla* contains more species within its genus. Because fleas have narrow tolerance ranges where their larvae can survive, the increased number of species within *Rhinolophopsylla* will allow the genus to cover a larger range of temperature and humidity than the two genera with fewer species.

Methods

The sample data of fleas was provided by the Chicago Field Museum in the form of specimen images that had been donated to the institution for research purposes. For each image, the collection location was imputed into a pre-formatted excel document also provided by the Field Museum. After recording the collection spots for every specimen, each location was run through GEOLocate to determine the latitude and longitude. Next, all the latitude and longitude sets were mapped onto the globe using ArcGIS mapping software (Fig. 1). Finally, distribution models were created using MaxEnt analysis software to determine where conditions would be suitable for species (Fig. 2-6). The original sample size consisted of 119 total specimen images; however, 7 images were excluded because they did not have a collection location and therefore could not be mapped. The functional sample size consisted of 112 specimen images which were then broken down either by host type (flying or non-flying) or by genus (Rhinolophopsylla, Rhopalopsyllus, and Rhynchopsyllus) to be mapped in MaxEnt. When broken down by variable 1, host type, the sample size consisted of 36 flying hosts and 76 non-flying hosts. When broken down by variable 2, genus, the sample size consisted of 30 Rhinolophopsylla, 76 Rhopalopsyllus, and 6 Rhynchopsyllus. These sample sizes were further cut down by the MaxEnt software as specimens with the same latitude and longitude were only counted once within each variable map. Additionally, when creating the MaxEnt models, the environmental variables of bio_5, 5b, 6, 7, 8, 16, and 17 had to be omitted in each of the models because configuration errors prevented the production of useable maps.

Results

Figure 1. ArcGIS map displaying specimen collection latitudes and longitudes of *Rhinolophopsylla*, *Rhopalopsyllus*, and *Rhynchopsyllus* fleas.

On the ArcGIS map shown in Figure 1, each point represents the latitude and longitude of at least one flea specimen. Locations with multiple specimens collected at the same latitude and longitude show up as a single point. This map's boundaries are separated by province, and the majority of the data points occur within Central/South America and the Middle East. The MaxEnt models displayed in Figures 2, 3, 4, 5, and 6 predict the probability that fleas of a given variable may survive around the globe. Areas of warmer color have a higher predicted probability because their conditions are more suitable. Meanwhile, areas of cooler color have a lower predicted probability that conditions are suitable. The scale to the right of each map associates each possible color with a given probability; areas of red have a probability of 1, and areas of dark blue regions have a probability of 0.

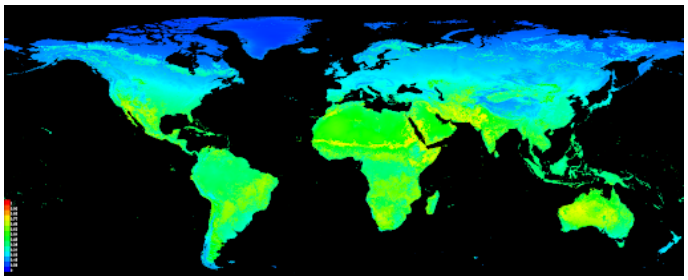


Figure 2. MaxEnt distribution model predicting suitable condition for Rhinolophopsylla, Rhopalopsyllus, and Rhynchopsyllus fleas with flying hosts.

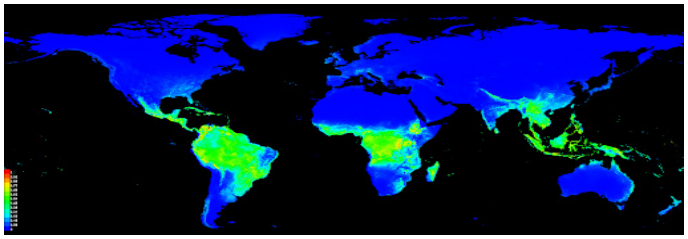


Figure 3. MaxEnt distribution model predicting suitable condition for Rhinolophopsylla, Rhopalopsyllus, and Rhynchopsyllus fleas with non-flying hosts.

As shown above, in Figure 2, *Rhinolophopsylla*, *Rhopalopsyllus*, and *Rhynchopsyllus* fleas with flying hosts have the possibility of encountering suitable conditions across much of the world. Most of North/South America, Australia, and Asia are colored lime-green, indicating approximately a .54 probability that those locations would have conditions suitable for fleas based on the sample data. Meanwhile, in Figure 3, a much smaller amount of the globe is colored lime-green, with only a portion of South America, Africa, and Southeast Asia having a .54 probability of suitable conditions for *Rhinolophopsylla*, *Rhopalopsyllus*, and *Rhynchopsyllus* with non-flying hosts.

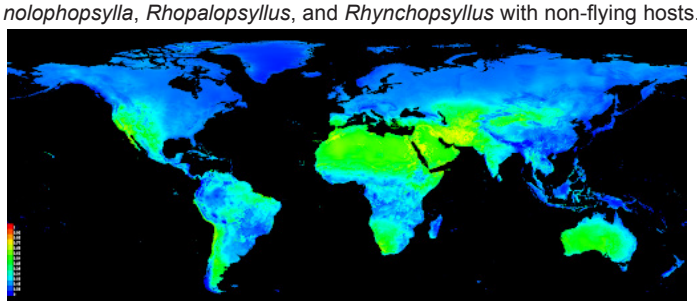


Figure 4. MaxEnt distribution model predicting suitable condition for *Rhinolophopsylla* fleas.

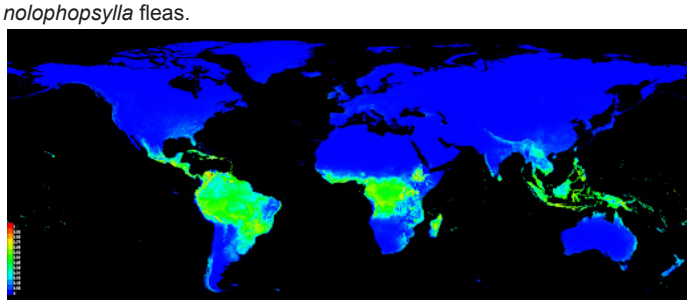


Figure 5. MaxEnt distribution model predicting suitable condition for *Rhopalopsyllus* fleas.

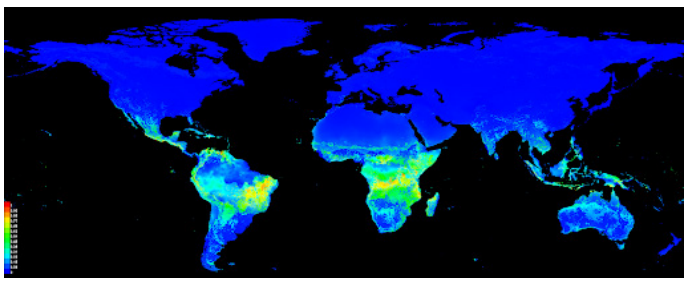


Figure 6. MaxEnt distribution model predicting suitable condition for Rhynchopsyllus fleas.

Figure 4 exhibits that the *Rhinolophopsylla* genus, which contained 9 species, has the possibility to occupy vast amounts of Africa, Australia, and the Middle East, as well as some stretches of land in North/South America and Asia. Meanwhile, in Figure 5, the genus *Rhopalopsyllus*, with 3 species, was only shown to have a smaller possible range than *Rhinolophopsylla* but a larger possible range than *Rhynchopsyllus*. This genera's possible range is restricted to tropical rainforest areas of South America, Africa, and Southeast Asia. Finally, in Figure 6, the genus *Rhynchopsyllus*, which has 1 species, is shown to possess the smallest potential range of genera, occupying smaller amounts of the tropical rainforest found in South America, Africa, and Southeast Asia than *Rhopalopsyllus* does.

Discussion

The greatest number of endemic flea genera are found in the Neotropical, Afro-Tropical, and Australian regions, with each region having greater than 50 percent of flea genera endemic. However, when looking at the Palearctic and Nearctic regions, the percentage of endemic species drops to below 45 percent endemic (Medvedev 1996). This distribution of endemic flea populations displays a similar pattern to the predictions given by MaxEnt in Figure 2-6. Throughout all the Figures, the two consistencies are that Neotropical, Afro-Tropical, and Australian regions are typically shaded lime-green, while the Palearctic and Nearctic regions are mostly shaded dark blue, which means that the Neotropical, Afro-Tropical, and Australian regions have a good chance of having suitable conditions to support flea life, while the Palearctic and Nearctic regions have almost no chance of having suitable conditions to support flea life. In general, it has been found that fleas survive better and develop faster under high ambient temperatures. This trend can explain the increased species richness and abundance of fleas in locations characterized by higher temperatures and lower altitudes, such as those found in the Neotropical, Afro-Tropical, and Australian regions (Linardi 2012). Overall, fleas depend on their hosts for almost everything. Their hosts' bodies provide food, shelter, and a place to reproduce, while their hosts' nests provide housing for their eggs. Due to this, generally, mammals with vast home ranges that do not inhabit dens almost always lack fleas of their own. Whereas hosts with nests exhibit more specific flea fauna than their non-nesting counterparts (Whiting 2007). Many non-flying hosts for *Rhinolophopsylla*, *Rhopalopsyllus*, and *Rhynchopsyllus* fleas are mammals that do not exhibit strong nesting behaviors. As such, it can be expected that fleas found on these non-nesting, non-flying mammals will have a smaller distribution than their flying, nesting counterparts because they lack a consistently protected place to lay eggs. This also contributes to fleas with flying hosts having a greater distribution because their hosts will nest in not one but two locations as they migrate with seasonal changes - Figures 2 and 3 display possible flea distribution that matches these restrictions, with fleas with flying hosts displaying a much larger distribution in Figure 2 than fleas with non-flying hosts does in Figure 3. Increasing specialization can be interpreted as an increase in the number of recognized habitats rather than reduced niche breadth within a habitat, thus defining habitats by how species use them (Krasnov 2004). Therefore, a flea genus could be considered more specialized than another if it contains more species that utilize a greater number of host species. Normally, when considering highly specialized species, one would assume that they have smaller ranges than their generalist counterparts. However, fleas are parasites and as such much exists on another organism to survive. Thus, fleas can either live on a specialized host with a small range or a generalist host with a wide range. With this in mind, flea genera with a greater number of species would have a better chance of being distributed farther than flea genera with a smaller number of species. In Figure 4, the *Rhinolophopsylla* genus, which contains 9 species, has the greatest possible distribution of the three genera evaluated covering vast amounts of land on every continent except Antarctica. Meanwhile, in Figure 6, the *Rhynchopsyllus* genus, which contained only 1 species, had the smallest distribution of the three genera. Overall, there was not much previous research about *Rhinolophopsylla*, *Rhopalopsyllus*, and *Rhynchopsyllus* fleas as much of the literature focused on human fleas, otherwise known as Pulex fleas. Additionally, more data mapping and specimen collecting should be done to increase our knowledge about other non-human fleas. This is important as fleas are often vectors of disease and common household pests, so expanding our knowledge on where these species and genera are located could help prevent infections and infestations in the future.

The widespread distribution and niche breadth of *Rhadinopsylla* fleas: via dispersal routes of small rodents

Megan Truman
Lake Forest College
Lake Forest, Illinois 60045

Introduction:

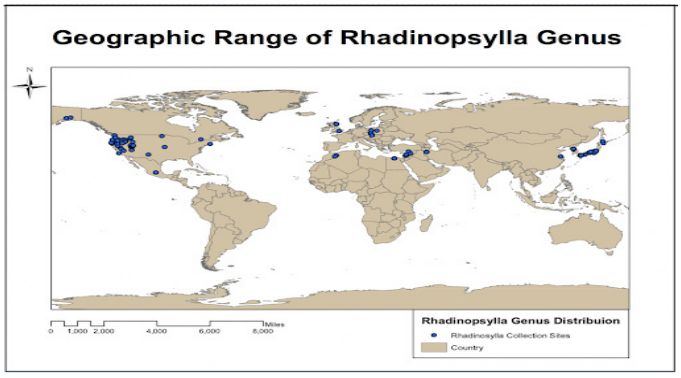
In biogeography, mechanisms of physical and environmental barriers shape the distribution of species. According to Darwin, these barriers also play an important role in the geographical ranges of organisms (Darwin 1873). The geographic range of a species reflects the species ecological niche (Brown and Lomolino 1998). An ecological niche is defined as the resources required for a species to persist in an environment. Two factors influence the breadth of an ecological niche: abiotic and biotic conditions. Abiotic factors include the physical space a species occupies, the temperature, and seasonality, whereas biotic factors include food requirements and the interactions between species (Hutchinson 1959). A species' niche breadth relies heavily on its specialization because its ecological niche is shaped by interactions with other species. The specialization of a species is its adaptation to a specific resource, function, or environment (Dictionary.com 2021). Species specialization influences the distribution of a species as it shapes the niche breadth of these organisms. A negative correlation has been discovered between the degree of specialization and the geographic range of a species (Krasnov et al. 2005). This correlation aligns with the niche breadth hypothesis which states that species able to tolerate a broad range of environmental conditions tend to have more suitable habitats to occupy, and therefore, have wider geographic ranges than species that tolerate only a narrow range of conditions (Brown 1984). This specialized relationship is frequently demonstrated through parasite-host interactions. This relationship determines species distributions as the parasite and host often coevolve (*Parasitic Relationships* 2021). The interaction of parasites living on hosts and adapting to their environment is due to the parasite's dependency on the host for food. As a result, the distribution of hosts determines the geographic range of many species of parasites (Shenbrot et al. 2007). Consequently, the niche breadth of the parasites is also determined by the geographic ranges of hosts. As discussed above, specialization shapes niche breadth. In parasite-host relationships, host-specificity is a determinant in the livable distribution of the parasite. Host-specificity falls into three categories: host-specific, host-opportunistic, and opportunistic . Host-specific parasites are highly specific and thus they will often have identical tolerances and geographical ranges as a single host species as their livability depends on having access to that specific host. Host-opportunistic parasites can have several different host species and thus are often distributed across the geographic ranges of several hosts. Finally, opportunistic parasites can exploit many hosts and therefore achieve a geographically scattered abundance (Shenbrot et al. 2007). The parasite-host relationship of fleas and small rodents offers the ideal model of this relationship, although these interactions occur in many other organisms as well. Due to the importance of host-specificity in the geographic ranges of organisms, we will be studying the parasite-host relationship of *Rhadinopsylla* subspecies and their small rodent hosts. Specifically, the *Rhadinopsylla* subspecies of *R. rectofrontia*, *R. heiseri*, and *R. syriaca*. These subspecies of *Rhadinopsylla* are categorized as "nest fleas"; nest fleas typically remain under host shelter for their entire lifetime (Medvedev et al. 2020). *Rhadinopsylla* subspecies reside in the Holarctic region, which includes North America and Europe. This genus of fleas offers a model for testing the distribution of fleas as they disperse via parasitized rodents like ground squirrels, gerbils, voles, and mice across the Holarctic region (Medvedev et al. 2020). Although host-specificity varies among the *Rhadinopsylla* subspecies, most can feed on many small mammal hosts (Medvedev et al. 2020). The level of host-specificity within *R. rectofrontia*, *R. heiseri*, and *R. syriaca* will vary amongst each subspecies, but it also reveals the niche breadth and therefore, the geographic range of these fleas. The parasite-host relationship between *Rhadinopsylla* subspecies and small rodents provides us with a better understanding of their distribution and geographic range. These interactions allow us to form two hypotheses for the pattern and mode of distribution for fleas. First, we hypothesize that *Rhadinopsylla rectofrontia*, *R. heiseri*, and *R. syriaca* have similar fundamental niches and geographic ranges despite their global distribution. Second, we hypothesize that these subspecies formed similar fundamental niches and distribution via dispersal of their small rodent hosts.

Methods:

This study included 216 samples from the *Rhadinopsylla* genus, collected in the field by outside sources within the last fifty years. This genus contains 18 different subspecies, but we compared the three subspecies of *R. heiseri*, *R. syriaca*, and *R. rectofrontia*. We chose these subspecies because they had large sample sizes and their samples had relatively few replicated coordinates. The *R. heiseri* subspecies contained 30 samples, and of those samples, there were 19 independent locations due to 7 samples with replicates. *R. heiseri* samples were collected from locations in Utah and California, U.S.A. The *R. syriaca* subspecies contained 18 samples, and of those, there were 6 independent locations due to 3 samples with replicated coordinates. The *R. syriaca* samples were collected from locations in Lebanon and Syria. The *R. rectofrontia* subspecies contained 19 samples, and of those samples, there were 10 independent locations due to 5 samples with replicated coordinates. *R. rectofrontia* samples were collected from locations in Japan. The coordinates that samples were collected from were used to graph the geographic range, and test the possible niche breadth, of the genus and each subspecies. These variables were used to test whether the *Rhadinopsylla* subspecies' range of distribution, mode of distribution, and niche breadths are similar. These tests were achieved via the use of MaxEnt software and the ArcGIS mapping system. MaxEnt software was used to map the geographic range and predict the niche breadth of R. heiseri, R. syriaca, and R. rectofrontia. The samples of the Rhadinopsylla genus and subspecies were run through MaxEnt to predict their global niche breadth. This process consisted of a total of four MaxEnt trials and four individual geographical maps. Each MaxEnt trial run was based on the BIO 374 Lab Assignment instructions and used the suggested settings. Along with MaxEnt, the ArcGIS mapping software was used to map the global locations of the entire *Rhadinopsylla* genus as a whole, along with the specific sites of *R. heiseri*, *R. syriaca*, and *R. rectofrontia*. We utilized ArcGIS because it accurately predicts and displays the geographic distributions of the *Rhadinopsylla* genus and subspecies. The coordinates of the *Rhadinopsylla* genus were entered into ArcGIS to produce a map of their precise locations. Then the coordinates of *R. heiseri*, *R. syriaca*, and *R. rectofrontia* were entered into ArcGIS to produce a map of their definite locations.

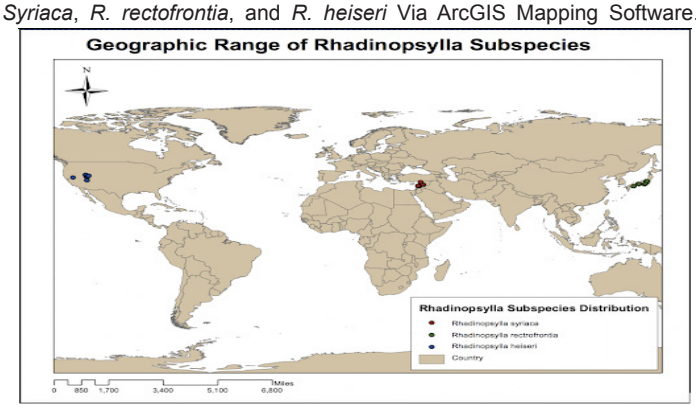
Results:

Figure 1. The Known Distribution of the Entire Rhadinopsylla Genus Via ArcGIS Mapping Software.



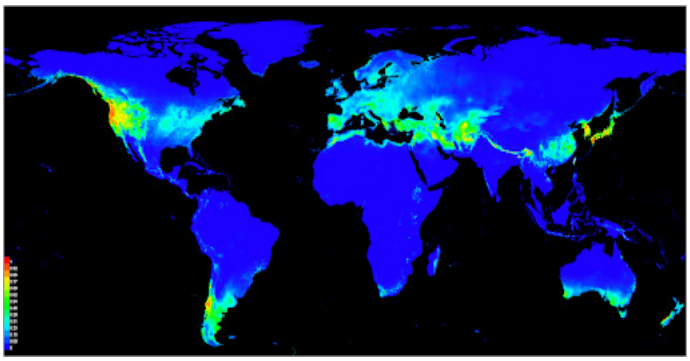
The precise localities of the sites in which the 18 Rhadinopsylla subspecies were collected. These samples made up a total of 216 collection sites, with 164 of those samples mapped due to replicated coordinates.

Figure 2. The Known Distribution of the *Rhadinopsylla* Subspecies, *R. Syriaca*, *R. rectofrontia*, and *R. heiseri* Via ArcGIS Mapping Software.



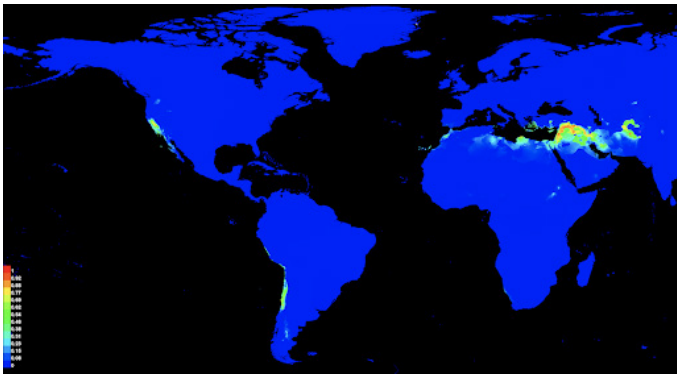
The precise localities of the sites in which the three Rhadinopsylla subspecies were collected. Of the 67 total samples for all three subspecies, 35 of those locations were mapped due to replicates. R. syriaca accounted for 18 total samples and 6 mapped locations within Lebanon and Syria. R. rectofrontia accounted for 19 total samples and 10 mapped locations within Japan. R. heiseri accounted for 30 total samples and 19 mapped locations within Utah & California, U.S.A.

Figure 3. Predicted Niche Breadth of the Entire *Rhadinopsylla* Genus Via MaxEnt.



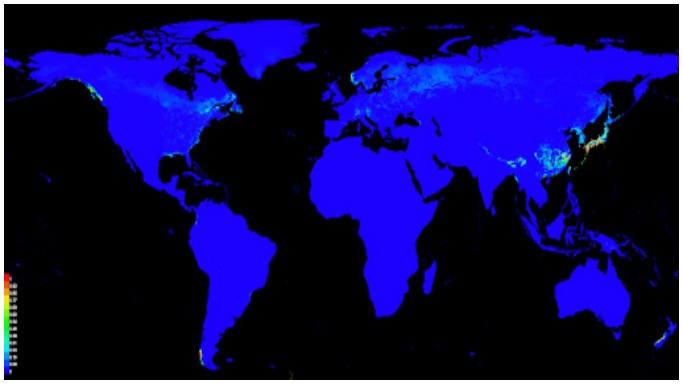
The fundamental niche of the Rhadinopsylla genus. This map displays the areas that contain the proper set of conditions in which these fleas can survive and reproduce. The warmth of color increasingly correlates with the most likely areas in which Rhadinopsylla can occur. These areas include the northwest quadrant of N. America, the Middle East, Japan and East Asia, southern South America, and southern Australia.

Figure 4. Predicted Niche Breadth of *Rhadinopsylla* syriaca Via MaxEnt.



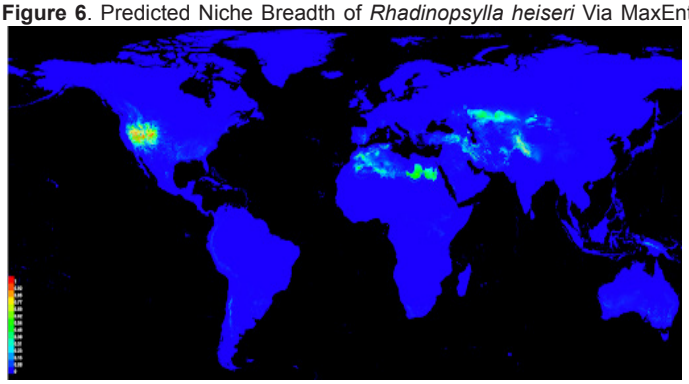
The fundamental niche of the Rhadinopsylla syriaca subspecies. This map displays the areas containing the proper set of conditions in which this subspecies can survive and reproduce. These areas include the Middle East, the western coast N. America, and a small fraction of western S. America.

Figure 5. Predicted Niche Breadth of *Rhadinopsylla rectofrontia* Via MaxEnt.



The fundamental niche of the Rhadinopsylla rectofrontia subspecies. This map displays the areas containing the proper set of conditions in which this subspecies can survive and reproduce. These areas include East Asia, Japan, and a portion of northwestern N. America.

Figure 6. Predicted Niche Breadth of *Rhadinopsylla heiseri* Via MaxEnt.



The fundamental niche of the Rhadinopsylla heiseri subspecies. This map displays the areas containing the proper set of conditions in which this subspecies can survive and reproduce. These areas include western N. America, northern Africa, and western Asia.

The predicted geographic range of the *Rhadinopsylla* genus (Fig. 3) matches the known distribution of these fleas (Fig. 1). The known distribution of *Rhadinopsylla* subspecies occurs in Utah & California U.S.A. (*R. heiseri*), Lebanon & Syria (*R. syriaca*), and Japan (*R. rectofrontia*) (Fig. 2). The possible geographic ranges of the subspecies overlap when these localities are compared to predicted niche breadth. The predicted niche of *R. syriaca* allows it to occur in areas of the Middle East and the western coastline of North America (Fig. 4). The predicted niche of *R. rectofrontia* allows it to occur in eastern Asia and Japan, the far western portion of North America, and northern Europe (Fig. 5). The predicted niche of *R. heiseri* allows it to occur in western North America, northern Africa, and parts of the Middle East and western Asia (Fig. 6). Comparing the MaxEnt predictions with known distributions (Fig. 2), the known localities of these *Rhadinopsylla* subspecies occur in the areas of the predicted fundamental niches. The results reveal the geographic ranges of *Rhadinopsylla* subspecies when considering their predicted fundamental niches (Fig. 4-6). The common areas in which the fundamental niches of *R. syriaca*, *R. rectofrontia*, and *R. heiseri* overlapped were western North America, the Middle East, and eastern Asia. These areas were also found to be occupied by the realized niches of the subspecies in (Fig. 2).

Discussion:

The areas in which the *Rhadinopsylla* subspecies occurred have similar climates and niche conditions year-round. The *Rhadinopsylla* subspecies samples were collected in Kyoto, Japan, Beirut, Lebanon, and Toole, Utah, U.S.A. The yearly climate of these areas has similar humidity, median temperature, and precipitation. Specifically, *R. rectofrontia* subspecies occur in western Japan, where the yearly temperature ranges from 33°F-88°F (*Climate-Data.org* 2021). This area of Asia is very humid and has an annual precipitation of 66.0 inches. *R. syriaca* subspecies occur in western Lebanon, where the temperature ranges from 50°F-81°F, and

the annual precipitation is 28.6 inches (*Climate-Data.org* 2021). Finally, *R. heiseri* subspecies occur in the western United States, where the yearly temperature ranges from 30°F-80°F, and the annual precipitation is 20.9 inches (*Climate-Data.org* 2021). Although the yearly precipitation is higher in Japan, these areas have similar temperature ranges in which the fleas can tolerate. Since temperature is a factor that influences where survival is permissible, the temperature range of species affects their niche breadth (Monahan 2009). Considering the similar temperature ranges in the regions where the subspecies are found, and the effect temperature has on niche breadth, we can conclude that the data support that *R. syriaca*, *R. rectofrontia*, and *R. heiseri* have equivalent niches. As *Rhadinsopsylla* temperature tolerances are influential in predicting their niche and geographic range, we must consider how these tolerances and adaptations evolve. Earlier, we discussed how parasite-host relationships influence the niche breadth of parasites. As fleas rely on their small rodent hosts for food and shelter, their relationship will ultimately determine the flea's geographical distribution. Therefore, we must look further into the host-specificity of fleas and their small rodent hosts. Again, *Rhadinopsylla* fleas are "nesting fleas," which means they are associated with their host for their entire lifespan (Medvedev et al. 2020). According to the categories of host-specificity above, *Rhadinopsylla* is classified as host-specific because they rely on small rodents for shelter and food. Therefore, *Rhadinopsylla* subspecies are expected to have the same geographic ranges and tolerances as their rodent hosts, as this outcome correlates with highly specialized parasites (Krasnov et al. 2005). As this specialized relationship is crucial to the geographic range of *Rhadinopsylla*, we must look further into the small rodent with which the subspecies travel on and interact with. A typical small rodent host of *R. syriaca* is the gerbil, specifically Tristram's jird (*Meriones tristrami*). Common small rodent hosts of *R. rectofrontia* are flying squirrels and voles; the samples did not specify the species from which *R. rectofrontia* was collected on. The usual rodent hosts of *R. heiseri* are squirrels, specifically, the white-tailed antelope squirrel (*Citellus leucurus*). Since squirrels are hosts of two of the *Rhadinopsylla* subspecies, we will look further into their evolutionary dispersal. Today, tree and flying squirrels occur across the world with their peak species richness in Asia, central and north Africa, and North America. The *Squiride* likely spread to its current distribution from the Nearctic and Northern Hemisphere (Koprowski & Nandini 2008). As these small rodents are non-aquatic, non-flying mammals, they can't cross large bodies of water readily. We must consider another route of dispersal because of the physiological limitations of squirrel, voles, and gerbils. A recent study by Medvedev et al. discovered that the distribution of *Rhadinpsylla* in the Nearctic and Palearctic is likely due to land bridges (Medvedev et al. 2020). They found land bridges as likely modes of dispersal because the number of *Rhadinopsylla* fleas increased from south to north; this is a trend that reflects dispersal via a land bridge. The current distribution is thought to have been achieved in three ways: from the northeastern Palearctic via the north Asian route, over the Beringia land bridge, and via the East-Central-Turano-Iranian route (Medvedev et al. 2020). Not only would these specific land bridges allow the dispersal of *Rhadinopsylla* subspecies via small rodents, but these routes would disperse to the areas in which our study discovered these subspecies. The Beringia land bridge connected North America and eastern Asia, while the Iranian route connected Asia to the Middle East during the Pleistocene. These routes align with our findings as *R. heiseri* is found in western North America, and *R. rectofrontia* is found in eastern Asia and Japan, then *R. syriaca* is present in Lebanon and Syria. Our first hypothesis was that *R. rectofrontia*, *R. heiseri*, and *R. syriaca* have similar fundamental niches and geographical ranges. This hypothesis is supported by our findings within MaxEnt (Fig. 3-6), which provide evidence that the fundamental niche of these subspecies allows them to survive in the localities in which they occurred in the ArcGIS mapping system (Fig. 1,2). Our second hypothesis is also supported, as the *Rhadinopsylla* subspecies are found on small rodent hosts like squirrels, gerbils, and voles. The presence of land bridges between North America, Asia, and the Middle East allowed for the global distribution of these small mammals. These dispersal patterns offer a mechanism for the distribution of *R. rectofrontia*, *R. heiseri*, and *R. syriaca* and the formation of similar fundamental niches.

Reducing yeast concentration in diet of Drosophila melanogaster negatively impacts female fertility

Allison Akins, Jessica Day, Amya Quillin and Rebecca Delventhal
Lake Forest College
Lake Forest, Illinois 60045

Abstract
Developmental timing and female fertility are dependent on a wide variety of factors. Before female flies can allocate resources to reproduction, they must account for the risk-benefit ratio. It is known that changing protein levels in fly food, among other manipulations, affects female fertility and developmental timing. However, the influence due to only manipulating yeast levels is not fully characterized. We evaluated the impact of decreased yeast concentration on the fertility and developmental timing in *Drosophila melanogaster*. We also tested if there was a difference in the effect of yeast concentration on the fertility and developmental timing of differently aged flies. We found that the yeast concentration played a significant role in decreasing female fertility and increasing developmental timing regardless of age, though age had a negative effect on its own.

Introduction
The strength and survival of animals is dependent on their diet. Thus, altering the diet of an animal will lead to visible as well as cellular differences in animals (Grangeteau et al. 2018). There are multiple ways to test the effect of food on the health of an animal. One such measure is through female fertility and developmental timing. Fertility refers to the ability for the female to produce offspring. Developmental timing refers to the timeframe in which the offspring reach certain measurable changes during their development. In order to test for these parameters, it is helpful to choose an animal with a short lifespan and early maturity. One such model organism is *Drosophila melanogaster*. *Drosophila* is a common model organism and one that is relatively easy to take care of in the lab. *Drosophila* is one of the most used model organisms, not only because of the low cost, short lifespan, and well-documented genetics, but also because most of the genes present in *Drosophila* are homologous to those in humans (Tolwinski 2017). There are multiple possibilities that could be explored when experimenting on the effect of food on *Drosophila*, but for this experiment, we only chose to test the effect of decreased yeast concentrations on fertility and developmental timing. We chose to test yeast because it is the protein source for the flies. Proteins are known to be important for development, and previous studies have shown that decreasing the yeast concentration emulates overcrowding and has similar effects on the flies during larval development (Klepsatel et al. 2018). Overcrowding is when there are too many flies present and so the availability of nutrients is depleted. Larval crowding has been shown to affect multiple aspects of fly development, including decreased body size, an extended life span, lower fecundity, etc. (Klepsatel et al. 2018). Thus, decreasing yeast concentration will likely lead to smaller flies and other physical changes. Food and access to adequate nutrients are also important aspects for mating. Mating is not necessary for survival but is necessary for reproduction, therefore it is an action that can be put off in times when survival is not guaranteed or when nutrients are low. Mating has been shown to take a toll on female fly health, and so the female flies need high nutritional food in order for the benefits of mating to outweigh the risks (Gorter et al. 2016). This indicates that a decrease in the food quality will lead to a decrease in the amount of female mating. Decreased mating will directly influence the measured fertility of the female. Protein rich environments have been shown to lead to higher levels of mating which is associated with the levels of offspring produced (Gorter et al. 2016). In addition, the percentage of protein present in the environment affects the development of larva (Gorter et al. 2016; Grangeteau et al. 2018). Therefore, there are previous studies that support testing the effect of yeast concentration on female fertility and developmental timing (Good and Tatar 2001). For our experiment, we measured female fertility by the number of eggs laid over a certain timeframe and we measured developmental timing by counting the number of flies that eclose over a series of days. Eclosion is the process when an adult fly emerges from the pupal casing. This is a physical change that is easily seen. More information on how fertility and developmental timing were measured will be provided later in the paper. Since there are common recipes to make food for *Drosophila* kept in the lab, we decided that we would keep the control group with the usual concentration of 4% yeast. In order to change the yeast concentration enough to have an effect, we halved the concentration of yeast in the experimental group down to 2% yeast. Since the concentration of the control is already relatively low, we hoped that a change of 2% yeast would be large enough, especially since we did not want to remove all yeast from the food source. As mentioned before, previous literature shows a preexisting connection between yeast concentration and the health and fertility of *Drosophila*, however further characterization is always necessary. Therefore, we hypothesize that if the yeast concentration in the *Drosophila* food source is halved, then there will be a negative impact on the fertility and development of the flies. To test this hypothesis, we manipulated both the yeast concentration as well as the time frame in which the flies were mated, five-day and twenty-day old female virgins, our "young" and "old" flies respectively. We tested both young and old flies to see if the effect of yeast stayed the same throughout the lifespan or if the effect was confounded in older flies.

Methods
Production of Fly Food
To make food with lower concentrations of yeast, the food had to be made from scratch as follows: 1% agar, 7% dextrose, 1500 mL water, and 5% cornmeal were combined and heated until boiling, stirring occasionally. Once heated the mixture was divided into equal parts. In separate beakers, the control and experimental food was created by adding 2% yeast and 4% yeast, respectively, then filled with water to desired volume and boiled again. The mixtures were cooled and 1.6% tegosept was added into each. 5 mL of food was aliquoted into separate vials for the control and experimental food groups. The food for both the in-lab group and at-home experiments were made at the same time in lab weeks prior to starting the experiment and stored in the fridge until used.
Fly maintenance and handling.
Fly stocks of genotype Canton-special (CS) were passed to fresh commercial food about twice a week to prevent microbial contamination and maintain freshness. When passing flies, the old vial was tapped on a foam pad so that flies fell to the bottom of the vial. The old vial was uncapped, and the new vial quickly aligned on top not allowing any flies to escape. The connected vials were then inverted, and the flies were then tapped down into the new culture vial. The new culture vial was then labeled with the date, genotype, food condition, student initials, and number of times the culture had been passed. Flies in the in-person lab were stored in an incubator at 25°C and 60% humidity to shorten the generation time. Flies reared in the at-home lab were kept at room temperature, which fluctuated throughout the experiment. To account for these fluctuations, the temperature on the home thermostat of the storage room for the fires was recorded and averaged over a period of two weeks.
Fly anesthetization in-person & at-home
To best sort, move, and identify the sex of flies over longer durations of time the flies were first anesthetized. Due to the variability of the availability of resources, the methods of anesthetization for the in-person and at-home labs differed from each other but remained consistent within each location. The in-person lab used carbon dioxide tanks to anesthetize flies. The at-home lab immobilized flies by lowering their body temperature via refrigeration. When handling and moving anesthetized flies, small paint brushes were used, taking special note to handle all flies delicately as not to injure, squish, or unnecessarily jostle them.
Developmental timing vial set up
To test developmental timing, two males and two females were placed together in vials of either experimental or control food groups. These flies were left to mate and lay eggs for roughly two days (~48 hours). The same parent pairs were removed from the vials to be used to start the egg lay pates. The vials were checked every few days for visual changes and dryness. If the food in any of the vials looked dry or began lifting from the sides of the vials 2-3 drops of water were added to moisten the food. Roughly 8 days after the start of the developmental timing test, vials were checked every 24 hours for eclosed flies. Each day after eclosed flies were observed, counts were taken of the number of eclosed female and male flies in each vial. This was continued until all flies had eclosed and then the vial was discarded.
Egg Lay Plate set up to measure fertility
To measure fertility, egg lay plates were assembled to enable egg counting

data to be collected. Grape agar plates were kept at 4°C until ~5-10 min before the preparation of the egg lay plates, and any condensation was wiped away before use. Granulated yeast (~¼ to ½ tsp) was mixed in a weighing boat with a few drops of water and spread in the center of the plate to attract females to lay eggs. Parents from developmental timing vials were anesthetized and transferred onto the edge of the corresponding plate, avoiding the yeast paste. The egg lay plate was then topped with a mating chamber, secured with tape, and incubated for 24 hours at 25 °C for in-person lab. The at-home lab mating cages were kept in a box at room temperature. After a day (~24 hours) the parents were discarded in the fly morgue. Data collection of the egg lay plates consisted of counting the visible eggs on the plate. This was done under a light microscope for the in-lab groups and with a camera and flashlight setup for the at-home experiment. While counting embryos the egg lay plates were marked on the reverse side with a line to signify where counting began. Eggs were counted until returning to this marked line. Each plate was counted at least twice to retrieve accurate counts and the average of each count was used for statistical analysis.

Statistical analysis of fertility and developmental timing

To determine significance of the data, two programs were used. Initially, the data analysis add-on in Excel was implemented to perform a two-sample F-Test for variances, the corresponding two-sample t-test assuming equal or unequal variances, and Two-Factor ANOVAs with replication for data sets with equal n in each condition. For days to eclosion, Jamovi was used to generate an ANOVA with unequal sample sizes and an estimated marginal means graph.

COVID-19 safety precautions

To maintain safe working environments, in-person lab and at-home lab safety precautions were taken to protect the health and well-being of students and other various individuals in labs. Materials used for this experiment were discarded in biohazard bags, in-person lab, or separate trash bags, at-home lab. While in lab and handling flies and materials, students refrained from touching their faces and frequent and proper hand washing procedures were followed. In-person students wore face masks and remained socially distanced as to maintain COVID-19 safety practices.

Results

Lower yeast concentration in food decreases female fertility

In order to assess whether the concentration of yeast in the food affected female fertility, we first needed to define fertility. For this study, fertility was defined as the number of eggs laid in a 24-hour period. To measure the number of eggs, grape juice agar plates with a small circle of yeast paste in the center were assembled, then two young male and two virgin female flies were added, along with a mating cage. We predicted that the young virgin female flies (5 days old) in the 4% yeast condition would be the most fertile based on previous literature. Initially, we counted eggs from the 2% yeast experimental and 4% yeast control food types using young virgin females. The average number of eggs laid by young females on 4% yeast was 23.65 eggs (n = 10), while the average for 2% yeast was significantly lower at 5.1 eggs (n = 10), $t(11) = 3.76$, $p = 0.003$ (Figure 1). It was also important to examine fertility in aged virgin female flies (20 days old). We used the same apparatus and counted the eggs 24 hours after set-up. The average number of eggs in the 4% yeast condition (n = 10) was 13.25 eggs, while the 2% yeast (n = 10) was similar to the young condition with 5.8 eggs (Figure 1). These results indicate significantly lower fertility in the aged females on 2% yeast food, $t(18) = 2.77$, $p = 0.01$. Because the averages for eggs laid in the 4% yeast condition by young and aged females was qualitatively different by about 10 eggs, we did a two-factor ANOVA with replication to examine whether age and food condition are jointly influencing fertility (Table 1). We found that the ANOVA supported the previous result that there were significantly less eggs laid in the 2% yeast condition ($F(1, 36) = 21.38$, $p < 0.0001$). However, any effects due to age were not significant ($F(1, 36) = 2.98$, $p = 0.09$), and there was not a strong significant interaction between age and food type ($F(1, 36) = 3.90$, $p = 0.06$). Thus, these results suggest a relationship between the concentration of yeast in diet and female fertility.

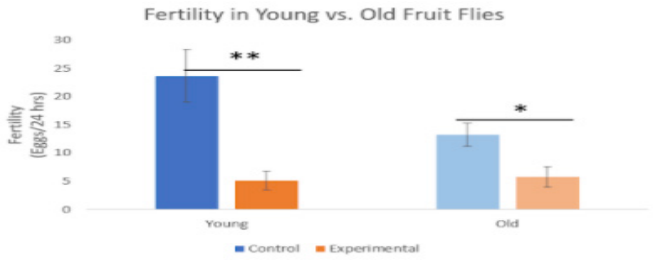


Figure 1. Fertility of young and old females decreases significantly in the 2% yeast condition (orange) compared to the 4% yeast control (blue). Fertility was measured by the number of eggs laid over 24 hours by virgin females of two ages, 5 day and 20 day. N = 10 for each diet and age. (**p < 0.01; *p < 0.05)

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample (Age)	235.225	1	235.225	2.976	0.0931	4.113
Columns (Food)	1690	1	1690	21.380	0.0000471	4.113
Interaction	308.025	1	308.025	3.897	0.0561	4.113
Within	2845.65	36	79.046			
Total	5078.9	39				

Table 1. Type of food significantly decreases fertility of young and old female flies. A two-factor ANOVA with replication was run to examine whether age and food have any interaction. The 2% yeast condition resulted in significantly lower fertility ($p < 0.0001$), while fertility based on age was not significantly impacted ($p = 0.09$). There is a slight interaction between age and food type, but this was not significant ($p = 0.056$).

Developmental timing differs with decreased yeast concentration

After determining that reducing the concentration of yeast in female diet affects fertility, we investigated whether there were any developmental timing differences caused by the different diets. To begin, we placed two young male and two young virgin female flies together in a developmental timing (DT) vial for 48 hours, after which they were removed. Once flies began to eclose in the DT vials, we recorded the number of flies that eclosed every 24 hours and the number of days to eclosion. For young female flies in 4% yeast, the average time to eclosion for their progeny was 10.67 days (n = 10), while the 2% yeast progeny was 11.4 days (n = 7) (Figure 2A). This was repeated in 20-day old females with similar results of the 4% yeast condition averaging 10.73 days (n = 10) and the 2% yeast averaging 11.71 days (n = 3) (Figure 2B). Qualitatively examining this data, the progeny from females on 2% yeast food had delayed developmental timing by about one day. This was determined to be true for the aged condition by a two-sample t-test assuming equal variances that indicated a significant increase in time to eclosion in the 2% yeast condition ($t(11) = -5.37$, $p < 0.001$). For the young female condition, we implemented a t-test assuming unequal variances due to significance in an F-test two-sample for variance ($F(9, 6) = 0.23$, $p = 0.02$). The t-test revealed that there was no significant difference in time to eclosion between diets for young females ($t(8) = -2.17$, $p = 0.06$), despite the qualitative difference. We again tested for any interaction between age and diet that might affect time to eclosion using an ANOVA. For this test, it was necessary to use a different program, Jamovi, because we had an unequal n for each condition. In the DT experiments, there were multiple vials in the 2% food condition that had no flies eclose and thus needed to be excluded from this specific data set. The ANOVA revealed similar results to the individual t-tests, in that the lower concentration of yeast significantly increased the time to eclosion of flies, ($F(1, 26) = 17.40$, $p < 0.001$), as seen by the higher orange line in Figure 3. However, there were no significant differences as a result of age ($F(1, 26) = 0.75$, $p = 0.393$) or due to the interaction between the two variables ($F(1, 26) = 0.32$, $p = 0.578$).

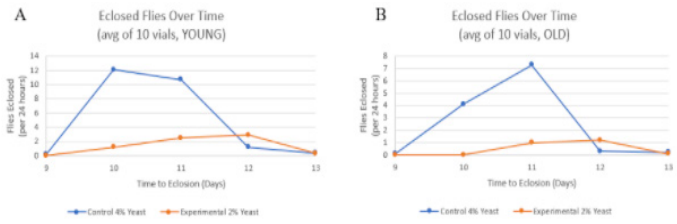


Figure 2. Flies on the 2% yeast food took longer to develop than those on 4% yeast food. The number of newly eclosed flies in each vial were counted every 24 hours until flies stopped eclosing. **A**) Flies eclosing from the young, 4% yeast condition (blue) averaged 10.7 days to eclosion while the young, 2% yeast condition (orange) took longer to eclose, averaging 11.4 days, but this was not significant (p = 0.06) Young 4% N =10, Young 2% N = 7. **B**) In the old, 4% yeast condition (blue), flies eclosed after 10.73 days, while the old, 2% yeast condition (orange) they averaged 11.7 days to eclosion, significantly longer (p < 0.001) Old 4% N= 10, Old 2% N = 3.

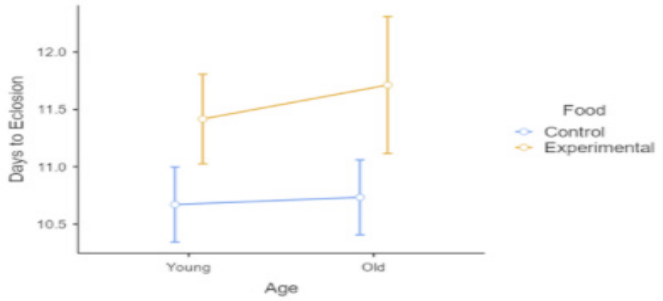


Figure 3. Flies took significantly longer to eclose on the 2% yeast food than the 4% yeast. A two-factor ANOVA was run in Jamovi due to different N in conditions (Young 4% N =10, Young 2% N = 7, Old 4% N= 10, Old 2% N = 3) and an estimated marginal means was generated. The experimental 2% yeast condition is in orange while the control 4% yeast is in blue. The food significantly influenced the time to eclosion (p < 0.001), but age did not have a significant effect (p = 0.393) and there was no significant interaction between food and age (p = 0.578).

Reduced fertility results in a smaller total number of eclosed flies

The previous experiment not only provided information about differential developmental timing, but also gave the total number of flies eclosed in each condition. This data can also be used as a qualitative measure of fertility, as it can be assumed that when more eggs are laid, more flies will eclose. Therefore, we expected to see the most flies eclosed in the vials where young females on 4% yeast laid eggs, and the least in the aged females on 2% yeast vials. In the young condition, the average flies eclosed across the ten DT vials was 25.7 flies in the 4% yeast and 6.9 flies in the 2% yeast condition. The average number of flies eclosed in the aged females were 12 flies for 4% yeast and 2.3 flies for 2% yeast (Figure 4). We next ran two t-tests to evaluate whether lower yeast concentration significantly decreased the total number of eclosed flies, and we found this to be supported. In both the young and aged females, there was a significant decrease in the total number of eclosed flies (t(18) = 4.46, p < 0.001 and t(18) = 3.98, p < 0.001 respectively). We next asked the question of whether the age of the female flies had any impact on the total number of eclosed flies. Through a two-factor ANOVA with replication (Table 2), we determined that both age and yeast concentration significantly decreased the number of eclosed flies across the DT vials (F(1, 36) = 14.11, p < 0.001 and F(1, 36) = 34.24, p < 0.00001 respectively). While both variables resulted in significant changes to the number of flies, there was no significant interaction between the two (F(1, 36) = 3.49, p = 0.07). Thus, these data suggest that both age and yeast concentration have an independent effect on female fertility.

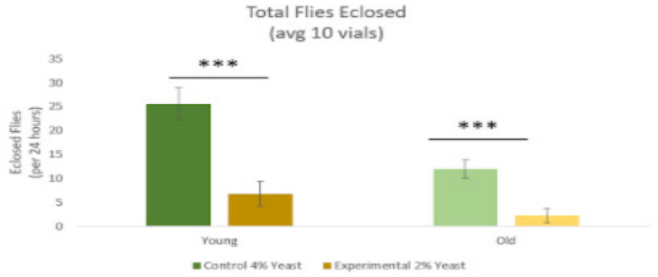


Figure 4. Female flies on 4% yeast food produced greater number of flies than the 2% yeast flies. Two male and two virgin female flies mated for 48 hours, then the number of eclosed flies were counted each day. Significantly less flies eclosed in the 2% yeast condition in both the young and old conditions (***p < 0.001) N = 10 for each age and food condition.

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample (Age)	837.225	1	837.225	14.116	0.000608	4.113
Columns (Food)	2030.625	1	2030.625	34.238	1.10E-06	4.113
Interaction	207.025	1	207.025	3.491	0.0699	4.113
Within	2135.1	36	59.308			
Total	5209.975	39				

Table 2. Total number of eclosed flies was decreased significantly by old age and 2% yeast food type. A two-factor ANOVA with replication was generated with the number of eclosed flies data (N =10 for all conditions). This test showed significant reduction in total flies across the young and old variable (p < 0.001), as well as across the 4% yeast and 2% yeast food conditions (p < 0.00001). The interaction between variables was not significant (p = 0.07).

Results from at-home lab experiments

No correlation between decreased yeast concentration and female fertility

The same experiments described above were performed outside of the laboratory. Figure 5 shows the measured fertility of young and intermediate aged flies cultured outside of the lab. Five-day old flies on 2% food conditions averaged 0.6 eggs while flies on the control 4% yeast conditions averaged 1.33 eggs. An F-test performed to account for variances (F(2) = 4.0, p = 0.20) prompted the use of a t-test assuming equal variances that showed no significant difference between food conditions in young flies (t(4) = -0.89, p = 0.422). Intermediate flies cultured on 2% yeast food conditions averaged production of 8.5 eggs. The control 4% yeast conditions of intermediate 12-day old flies produced an average of 17 eggs. To calculate variances, an F-test was employed (F(1) = 0.25, p = 0.295), from which a t-test assuming equal variances indicated no significant difference of the number of eggs laid on either food conditions (t(2) = -2.53, p = 0.127). There was a qualitative difference between the young and intermediate female fertility on different food conditions, so a two-factor ANOVA was performed on the data to test significance (Table 3). The results of this analysis suggested there was no significant difference in the number of eggs laid by females on the experimental food (F(1,8) = 0.562, p = 0.46). There was also no significant difference due to age (F(1, 8) = 5.06, p = 0.054) or due to an interaction between age and food (F(1, 8) = 0.90, p = 0.370).

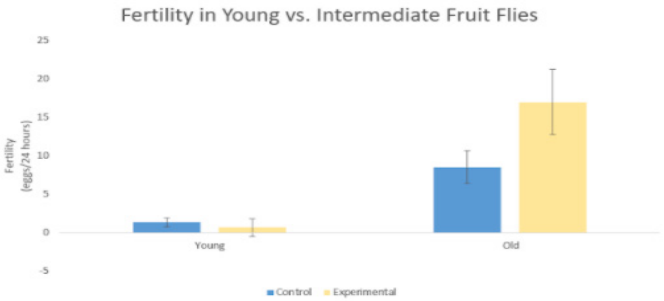


Figure 5. Fertility of young, aged 5 days, and intermediate aged flies, 12 days, measured by the number of eggs laid over a 24-hour period. Fertility of intermediate females shows an increase in fertility in the 2% yeast concentration (yellow) compared to the 4% yeast control (blue). An overall increase in fertility of intermediate females compared to young flies exists. However, due to error these differences are statistically insignificant (p=0.42, p=0.13) No observed differences yeast conditions in young flies.

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample (Age)	168.75	1	168.75	5.0625	0.05456	5.3176
Columns (Food)	18.75	1	18.75	0.5625	0.47473	5.3176
Interaction	30.08333	1	30.08333	0.9025	0.36992	5.3176
Within	266.6667	8	33.33333			
Total	484.25	11				

Table 3. Food type and age showed no statistical difference in fertility of young nor intermediate flies. A two factor ANOVA with replication revealed no significant change in fertility due to age (p = 0.055), food type (p = 0.475), or interaction between age and food (p = 0.370).

No differential developmental timing of altered yeast concentration

To further analyze the data for the developmental timing, the timing of when most flies eclosed needed to be addressed. Figure 6A and 6B displays the number of eclosed flies from parents cultured on control 4% yeast and experimental 2% yeast, as well as the time to eclosion. Young flies on both food conditions averaged 15.21 days to eclosion while intermediate aged flies averaged 11.67 days to eclosion. There was no significant difference in times to eclosion between either food conditions as all p-values were above the standard 5% threshold ($p > 0.05$) of significant difference.

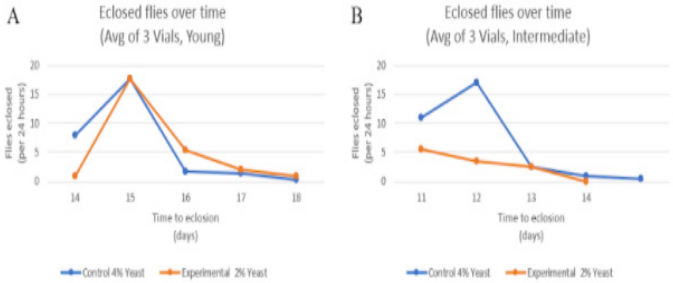


Figure 6. Mapped is the number of newly eclosed flies counted in each vial every 24 hours until flies stopped eclosing. A) Developmental timing of young five-day old flies. Flies on both food conditions had similar times to eclosion. The number of eclosed flies each day was not significantly different between the food conditions. B) Intermediately aged flies (12 day old) on 4% yeast conditions had a higher average of overall flies eclose. There is no significant difference in the number of flies eclosed between food conditions.

Female fertility by total number of eclosed flies at home

In extending the results shown in figure 6, the total number of eclosed flies was also used as a measure of fertility (Figure 7). The average number of eclosed young flies was 29 in controlled 4% yeast food condition. The average eclosed young flies on experimental 2% yeast was 27. As for the intermediately aged flies, the average number of eclosed flies was 16.6 under the controlled 4% food condition; while the average of eclosed flies for the experimental 2% yeast was 13. Any possible interaction of these results had no statistical difference on account of error and variance. Neither age ($F(1, 8) = 4.17$ $p = 0.075$) nor food type ($F(1, 8) = 0.09$, $p = 0.77$) were statistically different, and there was no significant interaction between age and food as well ($F(1, 8) = 0.005$, $p = 0.94$).

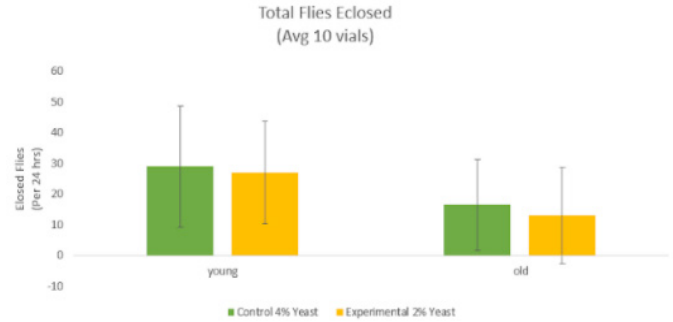


Figure 7. Comparison of the total number of eclosed flies of young and intermediate aged flies. Values of eclosed flies comparing young and intermediate flies implies a decrease in fertility with aging. However, the large error indicates there is not real significant difference. No significant difference between the control and experimental yeast condictiones within the young nor the intermediate groups.

Discussion

Based on our results, it is evident that the concentration of yeast in a female fly's diet directly influences the measured fertility. We found that with lower concentrations of yeast, young female flies laid fewer eggs and produced less progeny than those in the higher control concentration. The same result was observed in the old female flies. This indicates that flies that were placed on the 2% yeast food had reduced fertility compared to those on the control 4% yeast food. The type of food also negatively impacted the developmental timing of progeny, as eggs laid on 2% yeast took close to one day longer than the 4% yeast condition on average to eclose. Our results are supported by numerous previous studies that found decreasing the protein source in fly food also decreases female fertility, among other effects that we did results. Analysis of the at-home lab suggested there was no significant change in fertility or developmental timing due to reduction of yeast concentration in food conditions. Additionally, these results suggest that fertility increased as the flies aged when looking at female fertility but had reverse results when looking at the total eclosed flies in each food condition. The at-home lab did not have access to an incubator, resulting in all experiments being conducted at a fluctuating room temperature. The at-home experiments were subjected to large open spaces for longer periods of time. The lower temperatures alone gave reason to understand the inconsistencies of the at-home replication of these experiments. Additionally, the at-home lab also had slightly varied procedures and was subject to trial and error when conducting experimental methods. Lastly, the at-home lab had a much smaller sample size and fewer replications than that of the in-person lab. A comparison of in-person and at-home lab results would suggest conflicting analysis of both the female fertility and developmental timing. Notably, an understating of reviewed literature would also support that these are highly unsupported conclusions. Furthermore, with consideration of the multitude of external factors such as lower temperature, humidity and smaller sample size, it was determined best to refrain from statistically comparing the results from the two different lab environments. There were multiple limitations of this study that might have impacted our results. To begin, the food we made had the tendency to dry out after larvae were present in the vials and moving through the tube, even with the addition of water. However, the food dried out evenly across the experimental and control conditions, and between in-person and at-home labs. So, while the dryness of food was not ideal, it equally influenced the results from each condition and, therefore, is not the most important factor to consider. Another surprising effect that we did not anticipate was that many of the 2% yeast developmental timing vials did not show any pupae and had no eclosed flies. We initially had ten DT vials in each condition and the 4% yeast DT vials maintained an $n = 10$, but over the course of experimentation, the 2% vials in the young condition were reduced to $n = 7$, while the vials in the old condition were reduced even more to only three vials eclosing flies ($n = 3$). There could be multiple explanations for why the lower yeast concentration reduced mating. One possibility is that it could be more difficult for the developing eggs to survive with lower protein levels. It is also important to note that there may have been slight differences between how the 2% and 4% yeast DT vials were handled, as the conditions were not equally distributed, instead each author in-person recorded data for all the flies in one food condition. So, despite similar timing of data collection and similar techniques, there might have been some differences that impacted the 2% yeast flies. Another more likely explanation is that flies are less likely to mate on food with lower nutrition (Gorter et al. 2016). This could be supported by the fact that the same flies that mated in a DT vial that yielded no new flies were recorded to lay eggs on an egg lay plate that contained an unknown, but higher concentration of yeast paste. Unfortunately, we did not investigate whether the eggs laid on plates were fertilized, so it is difficult to predict whether the females were not fertile, or if they were not willing to mate on 2% yeast food to increase their survival. In terms of how this impacted our results, the smaller n in the old female condition could influence the significance of our results or whether age and food type interact to change time to eclosion. In the future, it would be interesting to explore whether the reduced fertility observed in low protein conditions is seen in multiple generations of flies. To begin the experiment described in this paper, we collected virgin female flies from mass produced commercial food, not our specific 2% and 4% yeast food. So, the female flies used in the fertility experiments developed on nutrient rich food rather than the nutrient poor 2% yeast. In following experiments, we would begin with a culture of flies on the control 4% and experimental 2% yeast foods conditions we made to investigate whether having lower levels of protein during development negatively impacts the adult fertility in a comparable way to the results of this study. It is possible that there could be an additive effect on the fertility of female flies and successive generations would lay fewer and fewer eggs and have fewer total flies eclose. On the other hand, the flies might have some adaptive mechanism that enables them to mate and survive on the low yeast food just as well as they do on the control food. Another study of interest would be to examine whether there is a relationship between measured fertility and lifespan of female flies on low-protein food. Some previous literature has noted that flies reared on low-yeast food show longer lifespans (Tatar 2007), while others have shown a more

complex relationship between yeast level, lifespan, and sex-specific responses (Duxbury and Chapman 2020). It is reasonable, based on results from Gorter et al. (2016), that the flies on low-yeast food reduce the energy intensive process of reproduction and mating to extend their lifespans. However, it would be interesting to see the difference of fly lifespans on 2% and 4% yeast and whether increased measured fertility is associated with decreased lifespan of female flies in one or both conditions. These results, and any future experiments, could be applicable to the importance of diet both before and during pregnancy for many organisms, including humans. If female flies who did not consume an adequate amount of protein are less fertile, then it may be reasonable to hypothesize humans who do not get the proper nutrition will have more difficulty in becoming pregnant. This interaction has been observed in human females, but the ideal amount of protein to consume remains unknown, although it is suggested that plant-based proteins are more likely to increase fertility than animal-based proteins (Silvestris et al. 2019). Overall, diet is extremely important to the survival and propagation of all living organisms, so a thorough understanding of nutrition is necessary to optimize survival.

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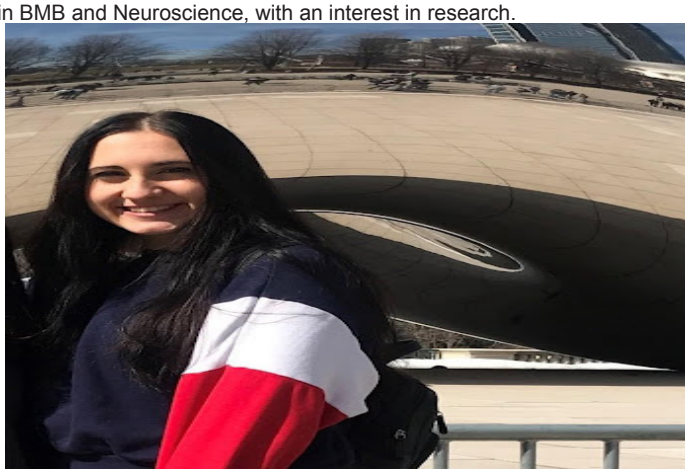
We would like to thank our professor, Dr. Rebecca Delventhal, for her guidance and assistance in both experimentation and interpreting results. We would also like to thank Lake Forest College and the Biology Department for providing resources that made this research possible. We acknowledge the programs Excel and Jamovi used for statistical analysis.

About the Authors

Fede Bertolotti ‘24 is from Montevideo, Uruguay. She is double majoring in BMB and Neuroscience, with an interest in research.

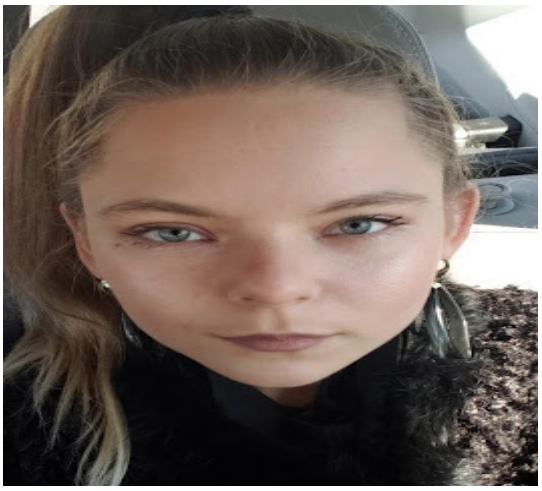


Brooke Carlock would like to thank her advisor Professor Anna Jones for helping her become the best writer she can be.

Naijila Dilosa is a junior majoring in psychology. She intends to become a pediatric occupational therapist and open her own therapy clinic in an underserved community. She hopes to also do research someday on therapeutical treatments for chronic pain associated with sickle cell disease.

Barry Khim ’22 from Morton Grove, Illinois is a Neuroscience major and a Chinese minor. He is a member of Nu Rho Psi with an interest in research.

Erica Knizhnik ‘22 from Grayslake, Illinois is a Computer Science and Finance student with a minor in Digital Media and Design. Her wide scope of interests helps her connect various educational communities, particularly art and technology, to prove that everything is art. After college, she hopes to take time off before pursuing a career in Computer Science with an emphasis on art and creativity.



Hannah Kobienia is originally from Willmar, MN. She transferred to Lake Forest College during her sophomore year and continued to pursue a major in biology and minor in Spanish. As a senior at Lake Forest College, she has taken many classes and had the privilege to learn and work with many talented professors and students. Virtually attending the 62nd Annual Drosophila Research Conference was an incredible experience for her. She met people from all over the world and learned about cutting-edge research that covered a wide range of developmental topics. After graduation, she plans to work as an EMT during her gap year before attending medical school in the Fall of 2023.

Jasmin Montes is from Des Plaines, Illinois. She is currently a sophomore at Lake Forest College, and a Biology and Neuroscience double Major with a Psychology Minor. She became a part of Eukaryon's publishing board this semester, and she looks forward to working with her professors and peers these upcoming years!

Kerry Mullen '22 from Sterling, Illinois is a biology major and an environmental studies minor. She is a captain of the Lake Forest Swimming and Diving team, a member of Tri-Beta, and a former member of Schwalbe's research lab. After graduation, Kerry plans on taking a gap year before pursuing a career in healthcare.

Mathieu Norcross, Senior at Lake Forest College majoring in Biology with a minor in Chemistry. All publishing done with the gracious support of the Lake Forest College Biology Department and the brilliant faculty that provided the basis for scientific experimentation and discovery.

Majo Orozco '22 is from Mexico City, Mexico is a neuroscience and psychology major. She is part of the Synapse and Alpha Phi Omega executive board, a member of Nu Rho Psi, and a research assistant at Delventhal's research lab. After graduation, Majo plans on pursuing a Ph.D. in Developmental Neuroscience with a focus on Autism Spectrum Disorder and Down Syndrome.

Ryan Osselborn is a junior at Lake Forest College double majoring in Neuroscience and Biochemistry & Molecular Biology. Currently, he serves as a part of the executive board of Nu Rho Psi, was recently nominated to the Student Academic Advisory Committee (SAAC) and is the Associate Chair of the Copy-Edit Board of Eukaryon. Ryan is extensively involved in Dr. DebBurman's lab where he researches the biomolecular nature of Parkinson's Disease in a yeast model. He hopes to one day research treatments and biomarkers of neurodegenerative disorders as an independent biomedical researcher.

Becca Ray is a sophomore at the college. She is majoring in neuroscience and psychology, and she runs cross country and track here at Lake Forest. She is also a part of Nu Rho Psi, Athletic Council, and Eukaryon's Copy-Edit Board. She is interested in studying sleep science and brain function.

Frances Rice recently graduated from LFC in the spring of 2021 with a degree in biology. She has interests in ecology and evolutionary biology, and is working towards attending graduate school in the future.

Raneem Samman is a sophomore at Lake Forest College from Jordan and Palestine. Raneem is part of the class of 2024; double majoring in Neuroscience and Data Science Computer Science track with a minor in Gender Studies. Currently, Raneem is on the executive board of Nu Rho

Psi and the journal of Eukaryon and plans on working in research with professor Zelenberg on analyzing data of mild brain traumatic injuries' (mTBI) fMRI scans and with Professor Barbosa on sexual selection and the alteration of traits and trade-offs.

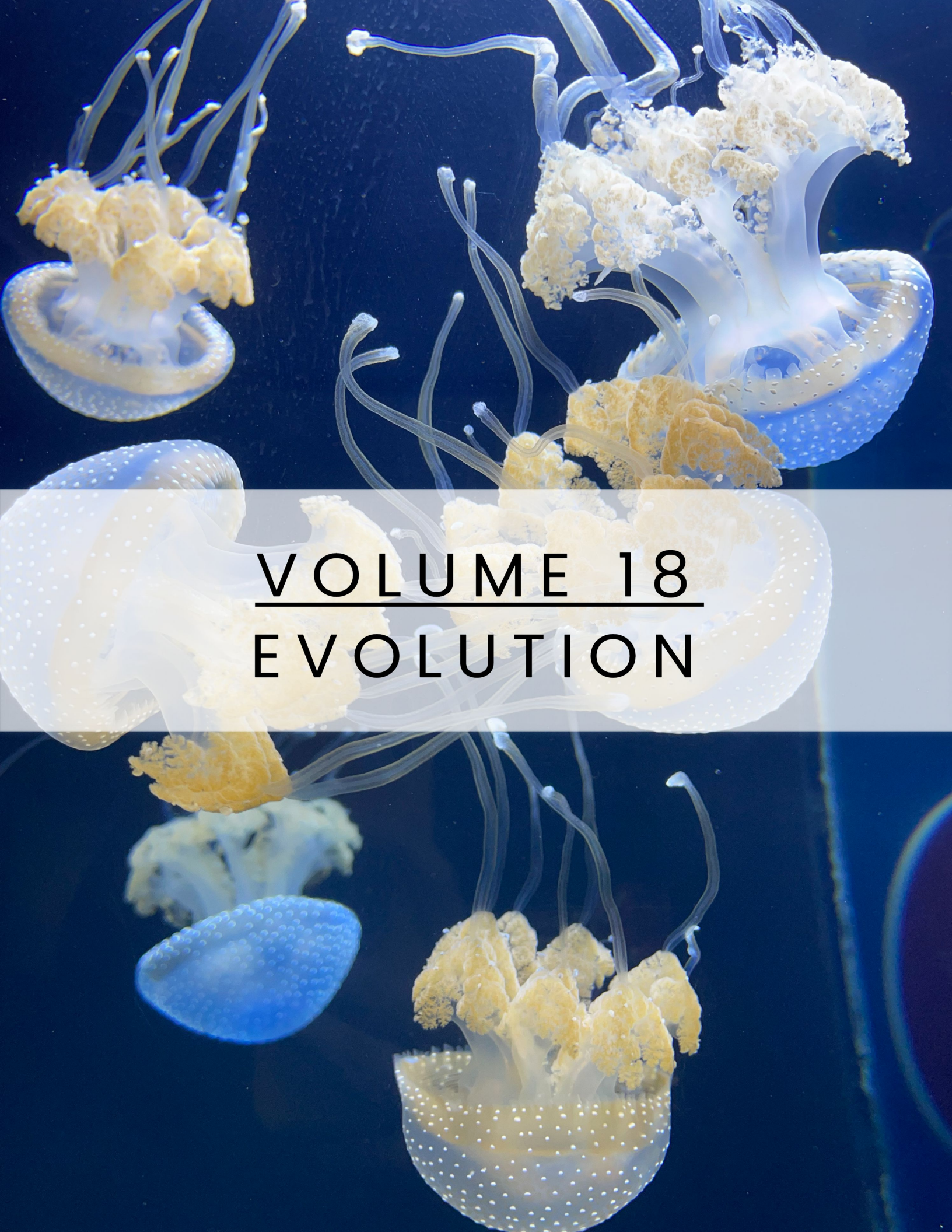
Ilona Sergeeva '24, is from Buffalo Grove, Illinois. She is majoring in BMB and minoring in Neuroscience. She is also interested in chemistry and molecular biology. After college, she is planning on going to medical school and pursuing a career in oncology.

Joy Shelton is a senior at Lake Forest College majoring in Biology with a minor in Spanish and Chemistry. She hopes to one day have a career in healthcare that assists individuals living with various health-related diseases.

Iman Shepard's film review caters to both the scientific and non-scientific community. She chose a scientific documentary she has enjoyed in many of her biology classes and with her friends and family. She is passionate about invasion ecology awareness, and also appreciates the eccentricity within the documentary's humor, which is unconventional for the genre. Ultimately, Iman is excited to share this review, and she hope others get the chance to watch the film!

Alyssa Silva is currently a junior at the college. She has a double-major in Neuroscience and Biology and minor in Religion and Chemistry. Some of her favorite subjects to learn about are human and animal physiology. In her free time, she likes to venture to Chicago and take photos of things that make her happy! The adorable critters she photographed were from the Shedd Aquarium. Enjoy! :)

Lea Wong is a senior at Lake Forest College. She is majoring in bio-chemistry and molecular biology. After graduation, her goal is to work in a lab where the research revolves around cancer treatments, and then one day attend graduate school to get her doctorates.



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