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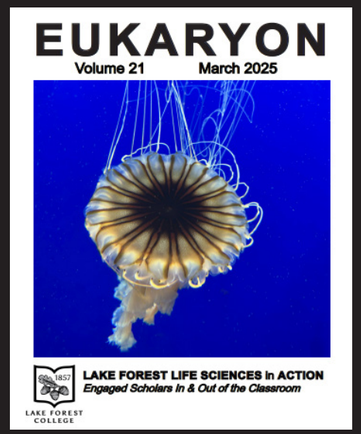


Snapshots (2024-2025)

Volume 22 | March 2026 EUKARYON JOURNAL www.lakeforest.edu/eukaryon

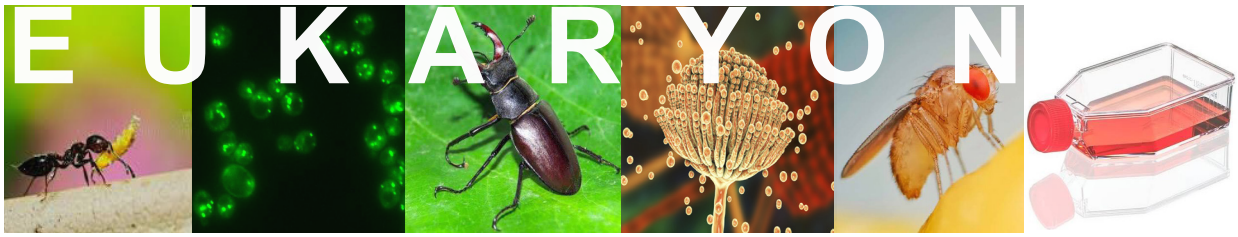
EUKARYON

Volume 22 March 2026

LAKE FOREST COLLEGE

LAKE FOREST LIFE SCIENCES 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 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 journal in 2004. The inaugural issue was published in January 2005 and featured seventeen articles selected by 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 the 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 the 2006 issue forth. In Spring 2005, the biology faculty selected student members of this Board. This Board not only reviewed articles, 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. As we, the 2025-26 Editorial Board have reflected on the journal, we have continued to evaluate our presence within the life sciences (Biology, Biochemistry and Molecular Biology, Neuroscience, etc). We hope to continue relating Eukaryon to the growing body of students, classes, and majors at Lake Forest and feel that this organization represents a unique and special facet of the life sciences at the College.

Front Cover: Photographed by Mieng Chandavimol. Read more about the inspiration behind the artist's work after the table of contents.

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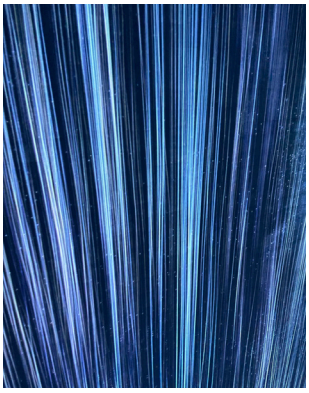

ABOUT THE COVER ARTWORK

"The Way of Water" by Shanamon (Mieng) Chandavimol

To me, the theme Beyond the Unobservable Universe is such a simple yet profound concept. Especially in Life-science, this theme refers to more than just what lies beyond the Earth. Our unobservable universe includes microscopic things, sometimes so small that exist beyond our eyes. However they will play a crucial role in shaping our world. At the first glance, many described this image to be space-like - a comet tail or a shooting star. This was actually a picture of the waterfall at the **TeamLab Borderless Museum, Azabidia Hill, Tokyo**. This specific piece is called 'The Rock where People Gather'. Each line represents the movement of water that created by using 3D technology called the Ultrasubjective Space. This allows that audience to influence the waterfall, hence, each picture taken at a specific moment, it will never be seen or replicated again. Linking back to our theme, water, a small molecule formed by two hydrogens and oxygen, gives rise to all living organisms. For us, without water, we will simply not exist. Beyond something we drink, water operates our body in an invisible level. From simple things like diffusion to homeostasis of our whole body. Water sustains every biological process in our life. In this sense, water mirrors the theme of Beyond the unobservable universe. The artwork reminds us that what seems to be unobservable doesn't mean insignificant. Sometimes things we cannot see play the greatest impact in our life.

Year 22: *Where Curiosity Leads Us*

About the Editor

Jeremy Levin '26 is a double major in Neuroscience and Biology with a minor in Chemistry. His academic and research experiences, includes his work in the Conrad Lab where he is conducting his senior project investigating potential drug targets for Tuberculosis (TB). He also has previously worked in the Schwalbe lab, were he investigated the properties on the lateral line system using the silver hatchetfish as a model organism. In addition to his scientific pursuits, he is actively involved in the college's music program, serving as the first-chair clarinetist in both the symphony orchestra and the concert band. Jeremy Levin is from Deerfield, Illinois and intends to pursue a career as a physician, and is currently applying and interviewing for medical school.

Dear Readers,

I am beyond excited to present the 22nd edition of Eukaryon, celebrating the extraordinary scientific scholarship at Lake Forest College. This year, our theme is "Beyond the Observable Universe," representing the curiosity and imagination that drive scientific discovery. From the smallest particles that make up matter to the vast complexity of our universe, science pushes us to ask questions that challenge what we see and what we think we know. Eukaryon exists to showcase that process, highlighting the work of students whose research takes them and us beyond the familiar.

We are honored to welcome Dr. D. Blaine Moore, Professor and Chair of Neuroscience at Lake Forest College as our featured speaker. Dr. Moore has over 20+ years of experience in neuroscience teaching and research, focusing on how brain cells work and how neurodegenerative diseases like Alzheimer's develop. His lab studies the molecular mechanisms behind Alzheimer's disease, including beta-amyloid plaques and the proteins involved in their formation. Beyond his research, Dr. Moore is committed to mentoring students and giving them hands-on experience in the lab. In his courses, students design experiments, read primary research, and learn to think like scientists. At the inauguration, he will present "How Experience, Serendipity and Molecular Scissors Shaped my Scientific Journey," offering insights that we hope will inspire curiosity and deeper engagement with science.

I want to express my sincere gratitude to the many people who made this edition possible. Our faculty advisor, Dr. Shubhik DebBurman, provided helpful guidance and support when we needed it most. The dedication of our editorial board was remarkable. Most importantly, thank you to the students and faculty whose submissions bring Eukaryon to life. Your creativity and persistence are the reasons this journal exists. It is also essential to recognize that science is not always valued or understood in the wider world. Misrepresentation of scientific findings, and cuts to research funding threaten scientists' ability to explore and solve problems, especially in times when political agendas overshadow empirical knowledge. Student scholarship and journals like Eukaryon matter even more. They show what is possible when curiosity, rigor, and collaboration are supported and celebrated.

As you explore the 22nd edition of Eukaryon, I hope you feel inspired to ask questions, challenge assumptions, and venture beyond what is immediately observable both in science and in life.

Sincerely,

Jeremy Levin 26' (ירמיהו לוין)

Editor-in-Chief, Eukaryon, 2025-2026

How Art Reveals What's Beyond The Observable Universe

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As extraordinary as humans are, our vision only allows us to see a minute fraction of life. Some would even argue that our eyes are the worst evolved part of our body. Thankfully, our brain is big and beautiful, hence we did not allow our myopic perception to halt our innovation. Thus, we invented tools to extend our visual horizon. Telescopes reveal distant galaxies, microscopes uncover magnificent cellular machinery, and brain scanners illuminate intricate neural networks.

As wonderful as these technologies are, they cannot compare to the human experience because they cannot observe what we feel and imagine. Pain, resilience, identity, and memory do not submit to quantification. Where telescopes and scanners reach their limits, art continues to document our existent by recording emotion, social interactions, history and more. Hence, art functions as a parallel mode of inquiry to what life is, often when data alone cannot or is slow to. Without our imagination, skepticism and curiosity cannot conceived. Without art, our imagination cannot be justly depicted. Therefore, art is a transitional womb that births scientific hypotheses and experimentation. Hence, allow me to indulge you in an interesting conversation about the importance of art to not just the life sciences but to scientific discovery, as a whole.

Unfortunately, science can only progress as far as its tools allow. Telescopes can only see so far and microscopes so close but somehow scientists we stay motivated. How? By mapping out the hypotheses in our heads and I his goes beyond flow charts and schematics. Long before Newton, 15th century medieval paintings like Salvador Mundi by Leonardo Da Vinci depicted the earth as as a crystal orb. Robert Hooke drew insects, tissues and even cells in the 17th century based on what he had see on rudimentary microscopes. Science fiction writers, like Jules Verne, envisioned space before NASA was even an idea. Simply put, art inspires science. Therefore, it should not be held in lower esteem in comparison to science nor should it be disregarded as a valid and fruitful career path. Without art, who knows where scientific discovery would be.

As with everything, science and medicine are fields with flaws. Although quantification is important, it is restrictive in what it can tell us. Pain scales are not adequate patients in chronic pain, depression questionnaires cannot encompass the lived experience, and BMI does not account for ethnic differences. Humans are undoubtedly the most mentally complex creatures on the planet, and our layered lives cannot be reduced to numbers on a scale. Constant quantification strips us of our humanity. It is no surprise that art is used to ease experience, as well as communicate where words fail. Furthermore, art removes stigma sometimes created by science. Like most mental disorders, schizophrenia is poorly understood by the public. Yayoi Kusama, a Japanese artist living with schizophrenia, paints what the disorder feels like to her. Upon viewing, people can see what she goes through, humanizing her and other patients. Her art also serves as a means of education and tool for dismantling misconceptions. Art reminds us that we are more than statistics.

In my opinion though, I think the most valuable role art plays in science is shedding light on the biases it often denies having. Life sciences have neglected BIPOC, queer, and disabled folks forever. In the field of psychology, non-white people are often excluded from studies. Historically, Black people have been used as test dummies for vaccines and medical remedies. Even today, trans people are denied equitable access to healthcare. Science is not immune to existing systems of oppression. When science has left people behind, art brings them forward to ensure they are not forgotten. The same way art has functioned as an activist in political movements is the same way it advocates for marginalized persons in science. In 1987, the AIDS Memorial Quilt was created. Each square sewn represented a life lost to AIDS. Eventually, thousands of squares were sewn together showing that AIDS was not just a niche issue. Rather, it was (and in some countries still is) a humanitarian crisis that should not have been ignored for so long just because it initially mostly affected gay men. Art makes the invisible visible.

The arts are being undermined now more than ever. Science is being questioned and invalidated at every opportunity. It is important that the fields recognize their intertwined purpose in order to fight what appears to be declining literacy and critical thinking. To safeguard the sanctity of human revelation, academics must abandon arbitrary wars and division. Instead, using our shared passion for knowledge and love of learning, let us endorse and protect each other. That way, we ensure that future generations are given the same (and hopefully more) opportunities to unearth what's beyond the observable universe.

An astronomy image taken from Orbital Today and a related news article on the theories behind what is behind the observable universe. Source: https://orbitaltoday.com/

Dr. Kirk: Unlocking Student Potential in the Life Sciences

Sebastian Henson
Lake Forest College
Lake Forest, Illinois 60045

Dr. Karen Kirk, beloved professor of Biology and Biochemistry & Molecular Biology at Lake Forest College (LFC), recently received theFoster G. and Mary W. McGaw Professorship in the Life Sciences for her outstanding academic achievements and contributions to the sciences at LFC. This article traces her surprising and relatable academic journey (including what led her to the study of telomeres significant segments of DNA that tell us much about stress and aging), her passion for research, and most importantly, her passion for helping students unleash their creative potential, persevere, and tackle scientific discovery in their own way.

The first people to come to mind when you think of high school dropouts are not college professors. However, Dr. Kirk is an exception to this rule. Unempowered by her high school at age sixteen, Dr. Kirk instead chose to enroll in college to further her intellectual development and get a head start, setting the stage for a long and fruitful career in academia.

She stumbled upon her love for chemistry and biology by accident. Dr. Kirk began college with a major in music because she loved playing piano, but was soon influenced by her brother, who had majored in chemistry and landed a job just after graduating. At the time, “it seemed like a reasonable thing to do,” she explained, laughing. It wasn’t until she transferred to University of Delaware two years later that she became captivated by the biological component of chemistry.

“I realized that biology was really the fascinating area, because you could do so much with gene splicing, genetic engineering, and other molecular biology tools,” Dr. Kirk explained. At the time, discoveries in genetics--coupled with new and powerful research methods--created emerging fields with infinite potential for research. After receiving her undergraduate degree, Dr. Kirk worked in molecular parasitology at Merck Pharmaceutical Company, studying how parasites interact with their livestock hosts at the cellular level. She then received her PhD in Microbiology and Molecular Biology from Rutgers University, and completed her postdoctoral fellowship at UC San Francisco with Dr. Elizabeth Blackburn, who received a Nobel Prize in 2009 for her discovery of telomeres. This experience opened a new world of interest and would inform Dr. Kirk’s work over the coming decades. “I loved [studying] telomeres…It gets more interesting all the time, because there appears to be a need to have a long telomere, and it’s important for the cell to have the chromosomes capped by a lot of these sequences…I’ve spent my life being fascinated by these kinds of things. So many, many things about telomeres.”

For Dr. Kirk, working with Dr. Blackburn was not just amazing because she was a groundbreaking scientist, but also because she was a woman, which was a rarity in the field at the time. Dr. Kirk highlighted that “now, that’s changed, but even still, it hasn’t changed that much, because only 6% today of the Nobel Prize winners are women. So, we have a long way to go.”

At Lake Forest College, Dr. Kirk has continued the work she started with Dr. Blackburn, researching with LFC students how telomeres (and the enzyme that makes them, telomerase) function in model organisms like the fungus *Aspergillus nidulans*. Telomeres are critical stretches of DNA that protect the ends of chromosomes in most living organisms-including humans-and are indicators of cellular aging. Over the years, Dr. Kirk’s lab has received nearly one million dollars in funding from the National Science Foundation, which has granted her and her students access to cutting-edge equipment and research techniques. Impressively, she and her student researchers have published 7 papers on telomeres to date. Recently, in April 2025, the scientific journal PLOS One published Dr. Kirk’s research with eight student co-authors, in which they investigated whether the RNA portion of telomerase (TER) associates with the telomerase protein (TERT) in the cytoplasm or in the nucleus of A. nidulans. What they found was striking: TER stays in the nucleus, as it does in vertebrates, and does not travel to the cytoplasm first, as it does in other fungi. Currently, Dr. Kirk and her student researchers are investigating whether telomerase expression is upregulated during meiosis.

During her nearly thirty years at Lake Forest College, beyond research with students, Dr. Kirk has developed and taught many of the core courses in the Biology Department, including Molecules, Genes, and Cells (BIOL 221), From Genotype to Phenotype (BIOL 352), Microbiology (BIOL 323), and more recently, Bio Inquiry: Gene Editing (BIOL 140). Infused in her lectures and lab sections is a motivation to show students that they are capable scientists and to inspire their curiosity. “My favorite thing is to teach labs, and the reason I love to do labs is because I always like to take one problem and have students spend the semester approaching it.” In this way, students who are initially technically shy or lack conviction in their research abilities slowly gain motivation and confidence. “I really like for students to learn to gain independence in the lab… I want them to gain that knowledge.” For Dr. Kirk, the most rewarding part of teaching is watching students transform from hesitant learners into confident scientists who trust their own ideas.

When asked if the Foster G. and Mary W. McGaw Professorship in the Life Sciences award feels validating of all the work she has done at the college, she smiled widely, humbly answering “Yes, it really does. It’s awesome. It is truly awesome.” She’s eager to highlight the impact that her students have made on the journey. “I can’t say enough: this is what I love the most…Thanks to all of the talented Lake Forest College undergraduates who have worked with me.”

Dr. Karen Kirk, Professor of Biology and Biochemistry & Molecular Biology, Foster G. and Mary W. McGaw Professorship in the Life Sciences, Lake Forest College.

The NPP Takes Off!

Elena Yoshovska
Lake Forest College
Lake Forest, Illinois 60045

The Nursing Pathway Program is a recently launched academic program for any LFC student interested in exploring nursing as a future career. With this program, students may focus on their chosen liberal arts majors while also gaining experience, coursework, and guidance from nurses and other health care professionals. This program works in tandem with the Health Professionals Program (HPP) to prepare students for careers in healthcare-related fields. Some experiences include: visiting hospitals to gain first-hand experience in patient care, attending seminars and networking events, and taking specifically curated courses in preparation for nursing careers.

Programs such as these are important for the development and improvement of the healthcare system. An interview with Dr. Lisa Hopp, Dean of the College of Nursing at Rosalind Franklin University, sheds light on this subject. “The big benefit is you get such a solid education, education with a capital E, from a liberal arts program. You get a well-rounded education that really teaches you how to think and to analyze. Those are a couple of the two really fundamental skills that you need in nursing and many other healthcare fields, all other healthcare fields,” Hopp states.

Nursing and the healthcare field as a whole require more than healthcare professionals with stellar scientific knowledge and performance. Both Hopp and Dr. Ann Maine, Director of the NPP, agree that real-world skills and bedside manners are the most important lessons to be gained from programs like the NPP. The program prides itself on the balance it requires from students between knowing the science behind healthcare and the humanistic parts that go along with it. Allowing students to question the system as well as take an interest in real-life experiences from nurses and healthcare professionals through networking events, as well as seminars. While students are encouraged to ask questions about scientific/technical topics they don’t understand, the real emphasis is on asking questions and being curious about the scarier part of the job: caring for other human beings. Caring for a life is a heavy burden, and the NPP does well to teach its students how to carry it with grace. Upon asking both Hopp and Maine about their thoughts on the future of nursing, particularly their hopes for the upcoming nursing students, they replied:

“We hope them to be top-notch problem solvers. You have to be able to work your way all around a problem and not [only] see through a single lens. That’s just critical and will remain so important across any healthcare field. Patients are complex, but it isn’t just their health issues that make them complex; it’s the rest of their lives,” Hopp explains.

Maine adds, “Having a health care provider who engenders that confidence and has those listening skills, you’re going to get that [well-rounded nurse], and you need those other liberal arts courses to understand people, to develop those skills. Yes, you need to know your chemistry and your biology. You could get A’s in those, but if you don’t have those other skills, you’re not going to be able to be the provider that your patients need.”

Programs like these are not for everyone; however, they are always available to anyone who wishes to use/join them. According to reports from Maine, the NPP has not only many accomplishments to be proud of but also much to look forward to. There have been improvements not only in the number of interested students (around 45!), but also in the ever-expanding curriculum.

“Well, we did develop a new course,” Maine starts. “Last year it was called Math for Healthcare Professionals, not just for the nursing, but it kind of came out of that. […] So I thought, well, why don’t we have a math class? All the problems are related to, hey, you’ve got, you know, the patient. You know, you go to the doctor, and they say, well, you’re 5 2, and weigh 128 pounds, but all the drugs are calculated per kilogram, right? So you need to do conversions. You need to do all of these different things. So I was really excited when I thought about that, and that the CPC Committee approved it. That’s being taught for the first time.”

The NPP not only teaches students through courses but also through experience. Gaining experience and graduating prepared are strengths that Lake Forest College excels at. It strives to bolster and motivate its students as they prepare for real-life experiences, especially in the fields they are entering. A value that rings true to our motto: “*Et veritas liberabit vos*.” Or in other words: “The truth shall set you free.”

Maine details: “We’ve got an internship that we finally got worked out at Lake Forest Hospital. […] Another student came back from doing an internship that another pre-nursing student had done, and she’s like, Oh, my gosh, I love this. I felt so affirmed that I want to go into nursing, that I could work with sick people, and that I could do a 12-hour shift. So I think the more experiences that we can get for students to experience and go, wow, this is what I want to do, [the better].”

Liberal arts colleges like LFC are crucial for the development of confident, well-rounded students. Especially in healthcare, doctors/nurses/healthcare professionals with patience, understanding, and a wide range of interests are needed. At the end of the day, the job is all about people and their care. Programs like NPP foster development and nurture students into well-rounded nurses and healthcare professionals. They provide community and support for students entering a field that can seem daunting when faced alone. Centering healthcare education on values centered on patient/human care will foster not only a sense of community within the field but also greater confidence between patients and healthcare providers. The NPP is only beginning its long and exciting life of bringing new generations of nurses into the healthcare field. What an honor to be a part of it and/or watch it grow!

A student intern part of the Nursing Pathyways Program shadowing at Lake Forest Hospital.

AI, Ethics, and the New Science Classroom

Javier De La Cruz
Lake Forest College
Lake Forest, Illinois 60045

Artificial Intelligence (AI) is simultaneously one of the most promising tools for advancing science education and one of its greatest challenges, a tension highlighted by the fact that nearly 80% of undergraduates already use it for their studies (Chegg, 2025). The rapid integration of AI into the population's daily lives since late 2022 (Figure 1) has created a conflict for educators. AI offers tools that can personalize learning and make complex science accessible; however, it also poses a significant threat to academic integrity by challenging traditional methods of student assessment.

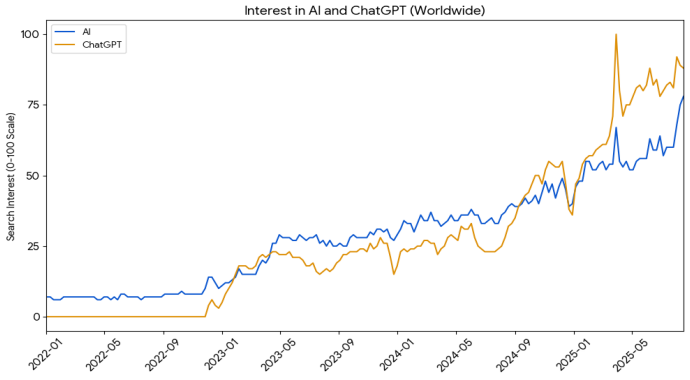

Figure 1. The rise in worldwide search interest for the terms "AI" and "ChatGPT" since 2022. The rapid spike beginning in late 2022 visually represents the rapid integration of AI into the public consciousness.

In science courses, AI is already proving to be a powerful pedagogical tool. For example, intelligent tutoring systems (ITS) can provide students with personalized support, offering real-time guidance on difficult concepts at their own pace, much like a dedicated personal tutor. If concepts such as stoichiometry in chemistry or the cell cycle in biology aren't making sense, an ITS can explain its process, adjust the difficulty for the next problem, and keep the student engaged. Platforms such as Carnegie Learning and Squirrel AI use this adaptive technology to identify a student's area of weakness and personalize their learning, thereby ensuring a solid grasp of the concept. Additionally, AI is making science courses more accessible to students with learning disabilities by offering tools that provide real-time transcription of lectures, summaries of large texts, and audio descriptions of visual information. This ensures that all students have an equal opportunity to engage with and master the material

Beyond personalized tools, AI is also transforming hands-on education through virtual labs. These platforms allow students to conduct experiments that would otherwise be too dangerous, expensive, or time-consuming for a traditional classroom. Platforms such as Labster allow students to explore AI-guided experiments virtually, from chemical reactions to CRISPR gene editing. These experiments create opportunities for fun, safe,and engaging learning experiences.

The same generative power these learning tools use also powers a challenge to academic integrity. It has become increasingly difficult to distinguish between human and AI-generated work, as students are turning to AI to write papers, generate code, or solve complex math problems (Figure 2). This challenge is heightened by the unreliability of AI detection software. As illustrated in Figure 3, AI detection software can incorrectly flag authentic student work. This could create a climate of suspicion in which students with unique writing styles or non-native English speakers are unfairly penalized. An over-reliance on such software is therefore flawed. This reality renders traditional assessments, such as take-home essays and problem sets, increasingly vulnerable.

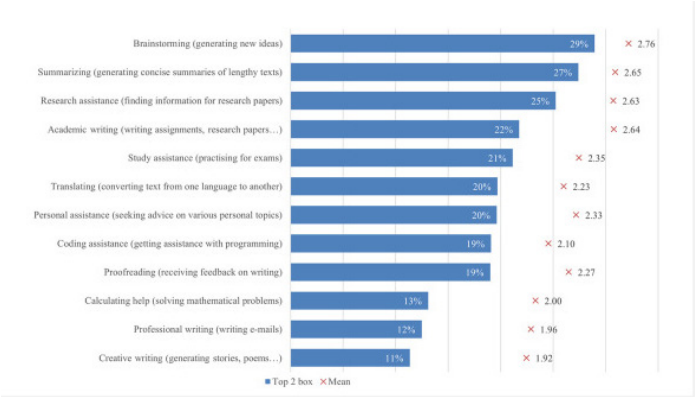

Figure 2. A survey of common academic uses for AI reported by undergraduate students. While tasks such as brainstorming and summarizing are prevalent, the use of AI to complete assignments and generate texts raises significant academic integrity concerns (Ravšelj et al. 2025).

This contrast places educators in a challenging position between innovation and maintaining academic honesty. The central conflict is no longer merely a matter of preventing plagiarism; it represents a fundamental pedagogical challenge. To outright ban AI and these tools appears counterproductive, as it would deny students access to a technology that is changing the scientific landscape. However, if we ignore how these tools are misused, we risk neglecting fundamental aspects of education, such as developing critical thinking skills and achieving true understanding of material.

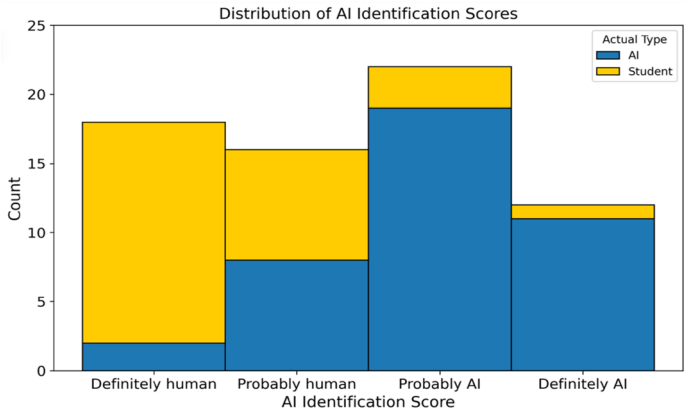

Figure 3. High Rate of Misclassification of Student Work by AI Detection Software. Graph shows how a detector scored known student and AI-generated texts. A significant portion of authentic work (yellow) was incorrectly flagged as "Probably AI" or "Definitely AI", highlighting the unreliability of these tools (Revel et al. 2024).

In response to the challenges created by AI, institutions are actively developing and implementing a range of strategies. These proposed solutions move beyond simple detection, focusing instead on adapting educational frameworks through new policies, emphasizing AI literacy, and fundamentally redesigning student assessment.

A primary institutional response has been to establish clear policies beyond requiring AI citations and to deepen ethical approaches. These approaches are designed to address more nuanced challenges, encouraging discussion on the limitations and biases of AI models. These models are usually trained on public datasets that can perpetuate societal biases, which are a major concern for scientific education. Furthermore, these policies confront the growing inequities in access to premium AI tools, creating a new divide and giving those with greater financial resources a distinct advantage. Therefore, effective institutional policies must not

only call for proper citation but also guide faculty in developing equitable assessments that account for these complex challenges (Chan 2023).

Recognizing that outright bans are often ineffective, many educators are integrating critical AI literacy into their curricula. The goal of this approach is to transform students from passive consumers of AI-generated content into critical users. One such strategy is the Peer and AI-Review and Reflection (PAIRR) model, in which students receive feedback from both AI and peers. In this model, students critique the AI's feedback, a process that improves their writing while simultaneously teaching them to recognize the strengths and limitations of AI-generated suggestions (Sperber et al. 2025). Additionally, institutions like Princeton University and UCLA are encouraging faculty to design assignments in which students are explicitly tasked with using an AI tool and then fact-checking, analyzing, and critiquing its response (Mangan, 2024).

Lastly, traditional essays and exams are becoming increasingly vulnerable to AI; educators are now using other forms of assessments that are more resilient to AI misuse because they target skills that AI cannot easily replicate. For instance, Davey et al. (2025) piloted an interactive oral assessment in an undergraduate bioscience course. Their study found that this method not only improved student performance from an average final grade of 68% to 75%, but was also effective at reducing academic misconduct from 27 formally investigated cases to 1. This interest in oral examinations is a direct response to the need for assessments that promote deeper understanding and real-time reasoning while protecting academic integrity.

The rise of AI presents a transformative moment in science education, requiring a new approach to teaching and learning. Rather than banning a tool that is becoming vital to scientific discovery, the scientific community should embrace the challenge it offers. A combination of clear ethical policies, AI literacy training, and assessment redesign provides a strong framework for this new approach. However, these strategies should not be viewed as a final solution, but as evolving responses. Ultimately, the future of science education will not teach students to avoid AI detection; it is about creating a new generation of scientists who know how to use these tools ethically, critically, and wisely. Successful integration of these tools will ensure that future scientists are equipped not only to use AI, but will also challenge it.

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Gene Editing: *A CRISPR Take on the Future*

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In November 2018, two twin girls were born in China. What makes this birth rather extraordinary is not the birth itself, but rather how it thrust the world into awareness of the possibility of modifying genes. The twins were the first to have their genes edited, namely the *CCR5* gene, which is believed to confer resistance to HIV in humans (Raposo, 2019). Society was already aware of gene editing, but it favored modifying vegetables, meat, and other organisms. This discovery sparked society's interest in gene-editing possibilities for humanity, made possible by the new CRISPR-Cas9. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) itself is relatively new. However, the rate of its growth requires scientists to assess its capacity and whether it should be used at all. It is this notion that has led faculty member Dr. Karen Kirk to create a new biological inquiry course at Lake Forest College. The new gene editing course, BIOL 140, explores not only the basis of CRISPR and its molecular foundations, but also the ethical dilemma regarding when and why the tool should be used.

Taught as one of the many Biological Inquiry Seminars, Gene Editing does not just explain the mechanisms of the technology but also its real-world applications and considerations. Dr. Kirk has "always been fascinated with molecular biology," she said, "and we did not have any courses like that here." Her passion for genes and the prospects that follow CRISPR's potential motivated her to create a course here for students who share similar interests. These problems can be better handled by students who receive early exposure to them, so they can brainstorm solutions. The course teaches students about genetic modifications we are more accustomed to, such as GMO foods and vaccinations. However, it also opens the door to new and experimental uses of CRISPR. This takes the shape of "designer babies," the curing of decade-long diseases such as malaria, and the side effects of free editing. With a combination of discussions and readings, the course places special emphasis on presentations, making it Speech-Intensive.

While all students are free to take this course, it has a prerequisite of introductory biology, BIOL 120. Most students who take this course are science majors fulfilling a biological inquiry credit or have a passion for gene editing. "It was actually interesting to see how people took different technologies from different times into one," says Diana Frankiv, a Biology Major who took the course her first year. The course focused on a set of readings and engaged students through debates and group presentations. "I found myself asking," stated Yasna Qureshi, "who sets the rules? Should it be each country individually, but then what about the dark market, and no one would know the consequences of that?" The introduction of moral dilemmas in this subject gives students a head start on how to approach similar conflicts in their future careers, making the class both educational and applicable. The use of CRISPR has ushered in a new era for science, but one we must approach only when we are ready.

The origins of CRISPR are relatively recent, with the Nobel Prize in Chemistry awarded only in 2020, making it crucial to have a coherent understanding of its consequences before using it on people. CRISPR was initially discovered during an immune response in Escherichia coli. While the scientists at the time were unaware of what this discovery would lead to, they proposed that it could be used in medical research. The reason it was so successful was that CRISPR loci have a "high degree of polymorphism in different strains of the same species of pathogenic bacteria" (Gostimskaya, 2022). Research continued to grow as scientists dove into the potential of these findings, and CRISPR repeats are now seen in most genomes of archaea and bacteria. Currently, scientists are highly motivated to use this device to cure diseases and save lives. Students learn about these positive uses and how they came to be throughout this course. Unfortunately, even if these edits can be inherited, there is a wall of technical and ethical difficulties facing them before this can be accomplished.

Although it can be easy to focus on the good that CRISPR can do, its application can be a slippery slope if not handled carefully. Malaria-resistant mosquitoes, cancer treatment, and food modifications are all positive outcomes of CRISPR taught in the class. However, this ideal would assume there is no safety risk associated with it and that the decision to use CRISPR is ethical. While most scientists who spoke at the International Summit on Human Gene Editing agree that germline genome editing should not be used for clinical reproductive purposes due to the risks, other instances are more grey. As we approach the modifications, it can be easy to shift from a therapeutic to an enhancement-focused approach (National Human Genome Research Institute, 2017). On top of these risks, there are potential accessibility gaps and consent conflicts, where regulation and guidelines are key.

Throughout history, scientists have developed tools for the betterment of humanity—such as nuclear energy, medicine, and genetically modified foods. While the intentions were in the right place, technology has a way of being taken and used for alternative utilization by other groups or causing unanticipated incidents. These concerns, on top of the obligation to act and help others, lend themselves to numerous debates. Gene Editing ensures students ask the right questions. Why do we use this technology? How can we make it better? With this mindset and preparedness from Dr. Kirk's course, future scientists continue to nobly push the boundaries of science.

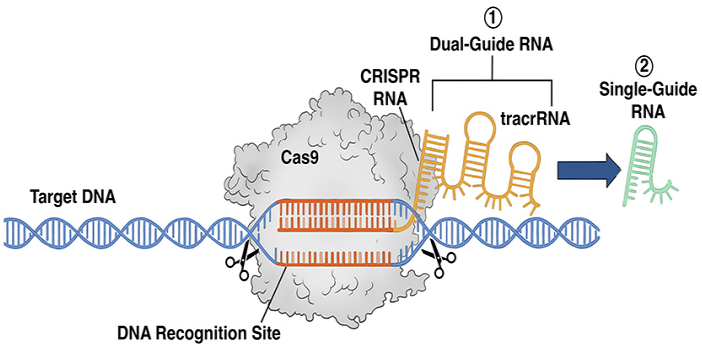

Figure 1. CRISPR-Cas9 system. *The following image above depicts the mechanism of the CRISPR-Cas9 system that has been used for current gene-modifications (Doudna 2024).*

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Artificial Intelligence: *Interdisciplinary Pathways Merging Innovation, Ethics, and Societal Impact*

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Lake Forest College has launched a new Artificial Intelligence (AI) minor, which reflects a growing recognition that AI is not just a technological phenomenon but also a cultural, ethical, and societal one. Two professors, Professor Davis Schneiderman and Professor Sara Jamshidi, play central roles as co-chair representatives of the new AI minor.

Faculty Inspiration

Professor Schneiderman, an English professor specializing in digital humanities, described his motivation for starting the minor: "If we don't want to wake up in a world designed solely by corporate interests, we need to think about AI as a humanistic subject of study." Schneiderman, who has directed three Mellon Foundation grants focused on technology and the humanities, explained that for him, AI must be studied alongside history, literature, and ethics as part of a broader liberal arts education. Similarly, Professor Jamshidi, whose Ph.D. in mathematics led them into applied AI research and governance, was influenced by their collaboration with TrustVector, a Chicago-based company that evaluates AI's role in healthcare. Through this collaboration, Jamshidi "came to understand that AI Governance is so large and complex that it needs to be taught in school." In her words, the course and later the minor were a "natural outcome." Together, their perspectives shaped a minor that emphasizes interdisciplinarity, ethical awareness, and responsible innovation.

Course Objective

The AI minor at Lake Forest College offers two tracks: AI Studies (humanities, ethics & big questions) and AI Governance (standards, oversight & real-world application). These tracks are designed to prepare students for a world increasingly shaped by automation, with an overarching goal not only to teach technical literacy but also to foster ethical reasoning, critical thinking, and collaborative problem-solving. As Jamshidi described, the governance pathway trains students to "review, assess, and draft governance frameworks for AI and data management systems, ensuring these technologies align with ethical standards and societal values." Similarly, Schneiderman emphasizes accessibility, highlighting that students without coding backgrounds should still become conversant in AI so they can understand how it is being deployed and confidently discuss its use in professional settings. The minor was designed to be intentionally flexible, inviting students across all disciplines to participate. For example, a Psychology major might examine how AI shapes mental health care delivery, whereas an English major could critique how generative tools reshape authorship. With this minor, the emphasis is on producing thoughtful leaders who understand the intricacies, risks, and potential of AI, as opposed to producing coders.

Teaching Strategy

Professors Jamshidi and Schneiderman emphasized applied, hands-on learning. Students will encounter case studies, policy drafts, debates, and collaborative projects that mirror real-world challenges. In the AI Governance course, Jamshidi explained that students will critique frameworks such as the EU AI Act, design governance proposals for different sectors (healthcare, finance, etc.), and participate in debates where they represent policymakers, developers, or affected communities. These projects and exercises highlight the ethical boundaries of utilizing AI.

Schneiderman's humanities-focused courses follow an "augment, not replace" teaching model, encouraging students to utilize AI tools while critically reflecting on their limitations. "I believe students benefit from multiple perspectives," Schneiderman explained, "and I want them to learn to use the technology ethically—but to use it." This approach shows a critical yet open approach to AI and its evolution, which students can expect to be reflected in the previously mentioned courses.

Innovation and Interdisciplinarity

A distinguishing feature of the program is its interdisciplinary scope. Specifically, AI Governance integrates ethics, law, policy, technology, and society. Jamshidi stressed that this interdisciplinary approach is crucial because "AI governance concerns so many stakeholders, like users, developers, policymakers, and the public at large." Schneiderman added that Lake Forest's program is the "first of its kind" in explicitly framing AI as a liberal arts subject. This allows students to merge humanistic inquiry with technical understanding as they navigate the complicated and varied landscape of AI.

Student Experience

The program is purposefully designed to be welcoming to students from diverse academic backgrounds, ensuring accessibility to all who are interested. Students will have the opportunity to connect their primary majors with AI as they engage in group projects, applied research, and ethical analysis. For example, a student studying education with an interest in systemic inequality might apply their knowledge to assess bias in AI grading software. Similarly, a business major could evaluate governance frameworks for AI within financial systems.By the end of their respective courses, students are expected to have developed technical literacy, analytical reasoning, and policy-writing skills, in addition to the ability to collaborate across disciplines with a wide range of students. As Jamshidi explained: "By the end of the course, students will develop technical literacy, analytical skills, ethical reasoning, policy and communication skills, and collaborative problem-solving."

Future Direction

Both faculty members acknowledged that AI evolves so rapidly that the curriculum must constantly adapt. Jamshidi rewrites significant portions of the AI Governance syllabus each semester to keep up with regulatory and technological shifts, a trend that will continue as global conversations around AI expand. Looking to the future, while the program has no plans to become a fully-fledged major, it is expected to incorporate more classes and strengthen collaborations with external organizations. Through these evolutions, the core mission will remain the same: to equip students with the insight and responsibility to guide AI's role in society. Schneiderman echoed the urgency of this mission, stating: "We are all living in the 'age of AI,' whether we like it or not, and we must engage with the way the technology is reshaping our world." Jamshidi offered a complementary reminder to students: "Grow and develop yourself because these are the things that make you distinct from AI. You are original; lean into it."

Conclusion

The AI minor at Lake Forest College is more than a new syllabus, it is a philosophy of education for the modern age. By blending innovation with humanism, ethics with policy, and technical literacy with creativity, the program embodies the multifaceted approach of liberal arts while addressing one of the most urgent issues of our time. For Professors Schneiderman and Jamshidi, the message to students is clear: AI will shape the world around us, but it is up to this generation to shape AI in return.

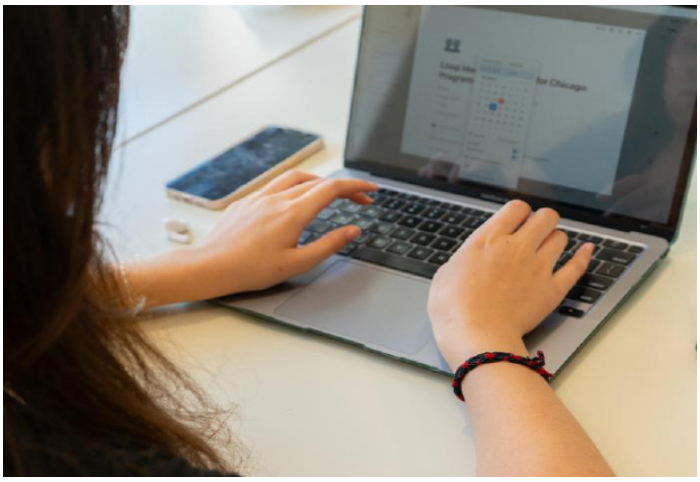

Figure 1. Increased prevalence and interest in Artificial Inteligence (AI) depicts need to learn and understand how to ethically use AI in and outside the classroom.

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Alumni in Focus: *Getting Into Top Graduate Schools*

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Just over 20% of Lake Forest College students pursue a postgraduate program after graduating, and many more begin graduate degrees after taking a gap year. Lots of anxiety comes from narrowing down interests and applying to programs, especially for those reaching upperclassmen status. Estella Tcaturian graduated from LFC in 2021 as the first-ever Neuroscience and Chemistry double major and is currently a PhD candidate in the Neuroscience department of Northwestern University. Majo Orozco Fuentes graduated from LFC in 2024 with majors in Neuroscience and Psychology and is a Genetics PhD student at Yale University. Both Estella and Majo seem to agree on two key things: Getting into top graduate schools involves not only what you accomplish on paper before applying, but who you become as a person.

What Does Being Prepared During College Mean?

Undergraduate preparation is key to success, but it is easy to misconceptualize what being prepared means. Estella came to college on the pre-med track and soon realized that other pre-meds seemed to enjoy chemistry a lot less than she did. This led her to decide to major in Neuroscience and Chemistry. However, she points out that what you pursue at LFC might be very different from what you pursue after. Though major choice and thesis completion are highly regarded accomplishments, preparing for graduate school includes extracurricular experience and community impact. Estella mentions that it was natural to help out and participate, eventually leading to leadership positions through executive boards and peer tutoring, which she considers a major factor in her admission to Northwestern. Not only is being a teaching assistant part of a typical PhD program, but the communication, management, and project management skills from extracurriculars are also considered translational skills by graduate programs.

Another unique aspect of LFC, as Majo explains, is the independence of the senior thesis. Majo notes that completing a senior thesis or master's thesis is extremely common in her current program, and for good reason. Not only is it extremely helpful to gain experience being in a lab, but Majo also explains that the long-term research projects "give you some agency. You start to decide which research you're going to do. Where are you taking those questions?" If possible, think about senior projects as early as possible. Additionally, Majo was an extremely active member of academic honor societies, organizations, and committees that served LFC faculty and students. She believes that communication is one of the top skills to gain from being involved on campus, and that "graduate school really pushes you to be independent…leadership positions helped me build those skills". Additionally, showing commitment to peers, faculty, and academics all play a role in both boosting a resume and growing as a person. Overall, preparing as an undergraduate student is not only about what is written on paper, but also about growing as a person and a scientist.

How To Plan Postgrad Life?

One similarity with science and graduate school is understanding that success is not always achieved on the first try. It is not always possible to control the timeline, but it is possible to make the most of the extra time provided. Estella took a gap year after being rejected from graduate programs. She emphasizes that "ego is your biggest enemy," connecting this back to her strong undergraduate background. However, she ended up connecting with a Northwestern lab and later got accepted to the school of her choice. Gap years can provide time for hands-on experience, access to campus information not available online, and stronger motivation letters.

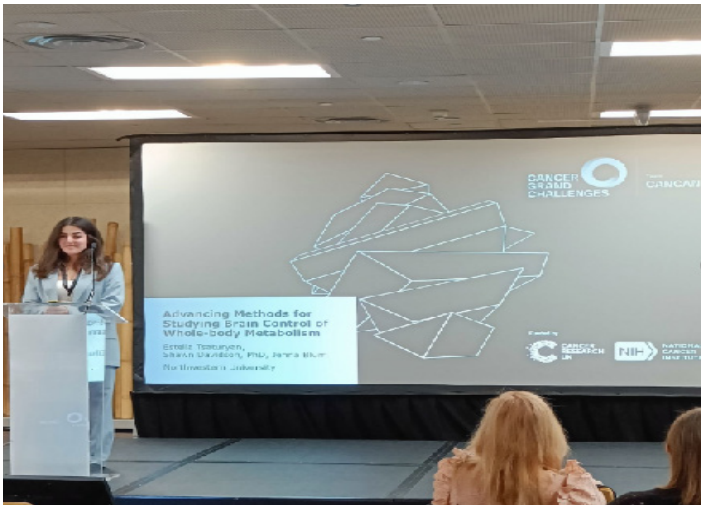
Majo Orozco Fuentes, LFC' 24, stands in front of Yale University School of Science, where her department resides.

Knowing when to apply is a decision unique to the individual. Majo applied and was accepted into Yale while she still attended LFC. Though there can be pressure and external stressors, Majo emphasizes that "it's you who's going to do the PhD, you who's going to move to a different place. So just do what feels right for you." When reflecting on personal experiences, utilizing resources from professors and alumni can help to provide more personalized feedback. Overall, planning life after graduation depends not only on life circumstances but also on a lengthy period of self-reflection.

After considering how to plan for post-graduation, the next challenge is standing out in a competitive applicant pool.Interviews are something that causes anxiety in many, and even the brightest acknowledge how normal it is to be nervous. Estella says that when practicing for interviews, "have examples in the back of your pocket for every skill you have on your resume." Drawing experiences provide an opportunity to elaborate on extracurriculars and research experience, which allows one to showcase a distinct set of skills and knowledge at every opportunity.

Another piece of advice from Majo is that it's important not to pretend to be somebody else. As cliché as it may sound, interviewers catch this facade. Instead, in a "pool of applicants where everybody's trying to impress the people that are interviewing you, you should just be yourself, because that will come off as genuine." The next tip is to be prepared to ask questions. Majo explains how crucial it is to read through the papers of the principal investigator (PI) you are being interviewed by. However, do not bring only technical questions, as this can come across as a critique. Instead, ask open-ended questions such as why a technique was used, why a gene was chosen for study, and what the implications of the work are. This allows for a natural conversation about science and is a way to connect personal research goals to the PI's research. It is okay to be nervous during interviews, but the true way to shine is to practice, be genuine, and be curious.

With interview strategies in mind, it is important to consider how to make the most of the resources available to you throughout your academic and professional journey. One of Estella's last pieces of advice is to not only create, but also to continue to tend to connections. She was connected to her post-baccalaureate position after speaking with Dr. Kelley, who knew of another LFC alumnus who had left their job. Estella explains that "the moment you know somebody inside, you skip that HR wall." In addition to asking professors for resources, befriending upperclassmen, lowerclassmen, and LFC alumni are great ways to build connections on a personal and professional level.



Estella Tcaturian, LFC'21, shares an experience at the Cancer Grand Challenges Future Leaders Conference 2024 in Barcelona, where she presented a talk on understanding the brain's role in systemic metabolic regulation and its application to cachexia.

On the other hand, though Majo did reach out to graduate school professors to chat before starting the application process, she does not owe her Yale acceptance to specific connections. She, however, credits professors like Dr. Delventhal for her personal, professional, and scientific development. She continues to invest in the relationships she built at LFC in a non-transactional manner. She "keeps [s] emailing them like, hey, guess what? Or here's a picture of my organoids. I'm very excited to share this with you." LFC has a unique community of professors and peers who want to invest in each other, and using these resources can support individual growth and help take steps toward success.

Majo Orozco Fuentes, LFC'24, shows off her workstation at Yale University, where she pursues neurogenetic study on how transcriptional regulation changes the brain.

Growing As a Person and Scientist

Overall, Estella and Majo have established that getting into their dream programs involved not just what was accomplished on paper, but also improving their extracurriculars and participating in long-term research projects. The crucial component is to continue growing as a person and scientist, and, of course, to continue questioning everything.

A More Ethical Science: *The Future of Animal Testing and Alternatives*

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In 2022, Congress passed the FDA Modernization Act 2.0, declaring that the FDA was no longer required to implement non-human animal testing as part of drug development. Due in part to this act, the FDA announced a plan in April of this year to replace its non-human animal testing requirements with non-animal alternatives. According to their roadmap, "In the long-term (3–5 years), FDA will aim to make non-human animal studies the exception rather than the norm for pre-clinical safety/toxicity testing" (Thomasy 2025). The stakes for this plan could not be higher. Non-human animal testing is based on the unjust principle that places perceived human benefit above non-human animal lives and welfare. Paradoxically, research and basic observation and experience have allowed us to recognize the feelings of intense pain, fear, distress, and depression that non-human animals undergo (Ferdowsian & Beck, 2011). Despite these facts, the status quo of anthropocentric tunnel-vision that has alienated and damaged humanity, the planet, and countless species remains dominant in society and scientific thought and practices. In 2015, an estimated 192 million non-human animals were abused in educational training, drug testing, and research (Taylor & Alvarez 2015). Unfortunately, many believe that animal testing is a necessity in the quest for further knowledge to improve human lives. Fortunately, as technology advances, it is becoming increasingly feasible to use "New Approach Methodologies" (NAMs) in lieu of unethical and barbaric non-human animal tests (Taylor 2019), the development of which is responsible for the announcements by Congress and the FDA (Thomasy 2025). Non-human animals must be given equal consideration with humans, and these NAMs are crucial tools towards this ideal due to their speed, efficiency, low cost, and promise to save more human and non-human animal lives than traditional methods.

Almost 70 years ago, when NAMs began to develop, replacement was difficult and slow. However, progress was still made in many key tests, which are now accepted standards (Taylor 2019). Once developed, standards are often accepted quickly due to their efficiency, as NAMs can be far faster and less expensive than conventional non-human animal testing methods. Due to differences between human and non-human animal anatomy, around 90% of all non-human animal-tested drugs never make it past human testing This results in a colossal waste of time, money, and human and non-human animal lives (Thomasy 2025). One of the NAMs demonstrating the most success is the Ames test, an in vitro test for mutagenicity, widely accepted as an industry standard in lieu of previous mouse-based tests. More in vitro cell cultures replaced horrific testing of polio, yellow fever vaccines, and even pregnancy tests (Taylor 2019). Advancing technology has led to five main NAM categories, all of which have demonstrated success, feasibility, and convenience, but the most excitement and promise lies with in silico and in vitro techniques.

In silico approaches make use of computational and AI/machine learning advances to model and predict the outcomes of a drug or treatment in the early stages of development. One such promising NAM is the Collaborative Acute Toxicity Modelling Suite (CATMoS). This uses computational modelling to predict the toxicity of compounds, and has so far shown extremely accurate results. In the future, models like this could replace notoriously painful acute toxicity tests. During its development, CATMoS published toxicity predictions for over 800,000 chemicals (Mansouri et al. 2021).

Thanks to these advances in NAMs and a shift in societal ethics, the end may be near for non-human animal testing, especially for toxicity testing where in vitro tests are simply better and more accurate than animal tests (Taylor 2019). However, some experts are skeptical. To many, the biggest challenges faced by new NAMs are that they are simply not complex enough and not as accurate as they need to be in predicting the complexity and chaos of a living body. However, simple human-based NAMs are much more relevant to humans than the chaos of a non-human animal system, and non-human animal tests are very poor predictors of human conditions (Taylor 2019; Ferdowsian & Beck 2011). Even so, researchers are building model complexity to address these criticisms by developing in vitro techniques reminiscent of science fiction: organoids and organs-on-chips (Singh et al. 2022). These models are layers of organ cells (induced pluripotent stem cells of human origin) supported by a vascular system and a 3D extracellular matrix. The models can effectively model the intricate intracellular interactions of human cells and organs, as well as the effects of drugs on these systems. These new technologies have been wildly successful. A liver-on-a-chip developed in 2022 was able to correctly identify the toxicity of drugs to the liver with near-perfect accuracy (Thomasy 2025; Singh et al. 2022). Most drug trials fail due to their liver toxicity, so such a simulacrum could vastly reduce the costs—in money, suffering, and time—of drug development. Other successful advancements include lung, brain, heart, kidney, skin, and gut-on chips, as well as combinations of multiple organs to mimic organ interactions. For example, an intestine-kidney chip was created to test both the absorption and nephrotoxicity of drug combination treatments (Singh et al. 2022). Such devices can even be utilized in personalized medicine to develop treatments tailored to each patient's needs (Skardal 2024).

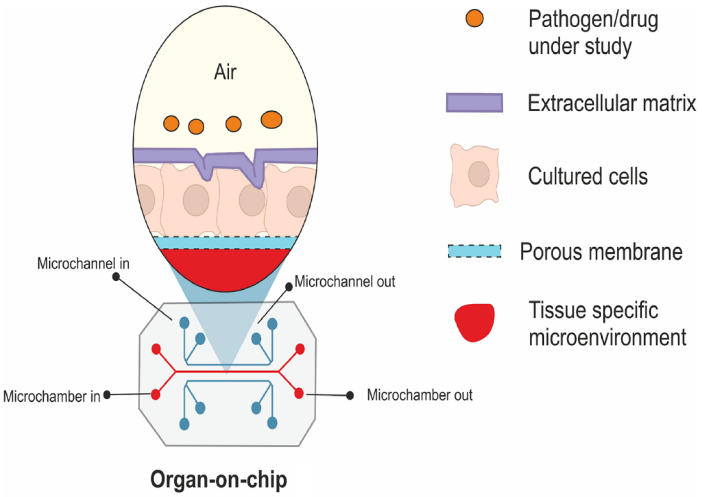

Figure 1. A diagram showing the typical arrangement of an organ-on-a-chip. Source:https://www.xiahepublishing.com/2572-5505/JERP-2023-00006S

Organs-on-chips have begun to attract attention from pharmaceutical companies and regulatory agencies, hence the FDA's announcement this April. Despite advances in biomedical and computational technology that seemed previously impossible, challenges remain. For example, the interactions between organs in vivo are still poorly understood (Thomasy 2025). Furthermore, the regulation of these new NAMs must be developed and standardized. Unfortunately, such advancements in new NAM technology and collaboration are at risk due to the Trump administration's funding cuts (Thomasy 2025). These cuts come at a crucial moment when funding is needed most to spur market demand, development, and innovation in these technologies and their fields (Singh et al. 2022).

In summary, NAMs have advanced greatly in the past three decades, allowing for the replacement of many unethical non-human animal tests with ethically and economically conscious NAMs; many entrenched benchmark tests have been completely replaced by NAMs. The importance of these advancements for both human and non-human animal welfare cannot be exaggerated. Researchers and politicians alike must do all they can to fight back against cuts and societal resistance while continuing to develop and refine NAMs (Taylor 2019). Going forward, regulatory agencies must implement more policies regarding and accepting the use of NAMs, such as organs-on-chips (Skardal 2024). Perhaps animal testing will remain a necessity in some fields, in which case society and science must be

willing to back down, especially if the research is knowledge for the sake of knowledge. There is most always a more ethical choice, if one cares to choose it (consenting humans in an experiment judged safe by predictive NAMs, for example). Non-human animals must be protected from unethical research to the same standards as humans are protected. The recent developments in NAMs and the announcements by Congress and the FDA are great strides towards a future where this ideal becomes reality, and science is truly used for what it should be—for the betterment of all.

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From Classroom to Lab: *Farhan Fuad*

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Undergraduate research allows students to extend classroom learning into real-world scientific practice. For Farhan Fuad, a Lake Forest College Graduate from the Class of 2025, this opportunity came through a summer research internship at the Shirley Ryan Ability Lab in Chicago. Formerly known as the Rehabilitation Institute of Chicago, the Ability Lab has been ranked the nation's top rehabilitation hospital for decades. Its unique model integrates patient care with active research, ensuring that discoveries in the lab directly inform treatment for individuals recovering from stroke, spinal cord injury, traumatic brain injury, and other conditions. For undergraduates, this setting offers not only technical training but also a chance to witness how science drives advances in patient care.

Farhan was drawn to the Ability Lab because of his interest in applying his background in neuroscience and psychology to real-world clinical problems. He was drawn to the institution's mission of advancing rehabilitation science through translational research and saw the internship as an opportunity to contribute to meaningful work while developing his research skills. The chance to move beyond theoretical learning and engage in scientific discovery within a leading research hospital was a compelling next step in his undergraduate journey.

His project focused on examining the genetic influences on post-stroke aphasia, a language disorder that affects communication ability following a stroke. Recovery outcomes for aphasia vary significantly across patients, with some regaining substantial language function while others experience lasting impairment. The research aimed to identify genetic markers that may explain these differences and provide insights that could eventually guide more personalized rehabilitation strategies. Farhan's responsibilities centered on molecular biology techniques, including DNA extraction, polymerase chain reactions (PCR), and allelic discrimination assays. Each procedure required precision and careful attention to detail, as errors in technique could easily compromise results. A typical day in the lab involved preparing samples, running assays, recording and analyzing data, and meeting with his supervisor to review progress. At times, he also joined discussions with the broader research team, giving him insight into how his work fit into the larger research effort.

Adapting to highly technical procedures under time constraints was one of the most demanding aspects of the internship. Like many researchers, Farhan encountered setbacks, including failed assays and inconclusive data. At first, these challenges were discouraging, but they became some of the most valuable lessons of the internship. He came to understand that unsuccessful experiments are not wasted efforts but opportunities to refine methods and strengthen problem-solving skills. This perspective shift improved his technical abilities and deepened his appreciation for the research process.

Through the experience, Farhan gained more than laboratory expertise. He developed patience, adaptability, and resilience, all qualities that are as essential as technical skills in scientific work. He also recognized that progress in research is rarely linear. Meaningful results often emerge gradually, after repeated trial and error. Accepting this reality not only made the work more rewarding but also shaped his outlook on science as a career.The internship also helped clarify his academic and professional trajectory. Working at the intersection of genetics, neuroscience, and psychology confirmed his interest in translational research and reinforced his plan to pursue graduate-level training, potentially through a PhD program. By contributing to a project with direct clinical implications, Farhan gained confidence that his studies could be applied to solving meaningful problems in medicine and rehabilitation.

Mentorship played an important role in his growth. Support from supervisors at the Ability Lab and from Professor Andrea Domenighetti at Lake Forest College challenged him to think critically, approach problems persistently, and view obstacles as integral to the scientific process. These experiences strengthened his identity as a researcher and underscored the value of thoughtful mentorship in undergraduate education.

Reflecting on his time at the Ability Lab, Farhan encourages future students to approach off-campus research with curiosity and flexibility. Asking questions, even those that seem basic, accelerates learning and fosters collaboration. He advises students to expect challenges and embrace them as part of the process, noting that research rarely proceeds smoothly. He also emphasized the importance of patience. Entering with the expectation of steady, linear progress had initially made setbacks more stressful. Recognizing that meaningful discoveries often come only after repeated trials would have made the experience less daunting from the start.

Farhan's summer at the Shirley Ryan Ability Lab illustrates the transformative impact of off-campus research. Immersed in an environment where clinical care and science are seamlessly integrated, he not only built technical skills but also developed the resilience and adaptability needed to thrive as a researcher. His experience shows how undergraduate research can clarify academic interests, prepare students for graduate study, and provide direction for professional goals. Lake Forest College continues to encourage student participation in research, both on and off campus. Experiences like Farhan's highlight the opportunities available to undergraduates who seek to apply their studies beyond the classroom. His work at the Shirley Ryan Ability Lab reflects not only his individual growth but also the broader benefits of undergraduate research: the ability to connect theory with practice, overcome challenges with persistence, and prepare for future contributions to science and medicine.

Farhan Faud, Class of 2025, after his graduation ceremony.

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Life AD-rift at Rosalind Franklin University

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During this past summer, I had the opportunity to join Dr. Robert Marr's lab at Rosalind Franklin University, and I would like to share more about him as a mentor. I will also discuss him as an associate professor, based on my interviews with him and his lab members. Dr. Marr started his educational journey at the University of Guelph in Ontario, Canada, earning his bachelor's degree in applied Biochemistry. He then completed a PhD in Molecular Biology, Genetics, and Cancer at McMaster University in Hamilton, Ontario. He had also joined the lab at McMaster, which is focused on gene therapy for cancer. After completing his education, Dr. Marr moved to California to the Salk Institute, where he began his postdoctoral research focused on gene transfer as a therapy for Alzheimer's Disease (AD). Today, he works as an associate professor at Rosalind Franklin University (RFU) in North Chicago, teaching medical/graduate school courses and leading a research lab that focuses on gene therapy approaches to Alzheimer's Disease.

This summer's primary focus of Dr. Marr's lab was to explore how, through viral delivery of a genetic construct, we could inhibit the NLRP3 inflammasome, which is a protein complex involved in inflammatory reactions in Alzheimer's disease. The NLRP3 inflammasome is an important protein complex in the innate immune system that, when activated, releases pro-inflammatory cytokines such as IL-1β and IL-18, which contribute to neuroinflammation in AD and worsen the condition. As a freshman with no prior laboratory experience, I found Dr.

Marr is to be incredibly mindful and supportive. He shared his perspective on mentoring by saying, "I think in one respect, it's a service that every mentor and laboratory should do. And two, it's a benefit because it attracts highly motivated people to work for us. And quite frankly, it causes things to be done that sometimes, when we take our time, we're dragging our feet on getting it done. So, it catalyzes things getting completed, and it's always a good learning exercise for the mentor, too." This philosophy was evident throughout my internship. Under Dr. Marr's mentorship, I became proficient in several laboratory techniques, including cell culture, lentivirus production, ELISA, and Western Blotting. He ensured that I understood the purpose behind each procedure, often giving mini-lectures to clarify complex topics or anything I was confused about.

His dedication to teaching was shared by members of his lab team. Mike, the lab manager, reflected on his experience: "I was a protein chemist in a previous lab. I did protein purification, cell surface expression, addiction models, and the like. This lab is mostly focused on molecular biology and gene therapy. After working with Bob for two and a half years, I can say he is a great teacher, and I quickly adapted and learned everything I needed to do my job here. It was a little challenging, but that's what made it interesting. I brought my knowledge about immunohistochemistry and various techniques that involve it, which other workers adopted. It's a great lab and place where you can easily fit in in a friendly environment. His depths of knowledge are very impressive; he is on a whole other level entirely. He designs the genetic makeup of the proteins. It is very impressive." Similarly, Sonia, a lab technician with a neuroscience bachelor's background from Loyola University, described the difference between Dr. Marr's lab and her previous experience: "In my past lab, we purchased every solution and virus. We didn't make anything ourselves. My previous lab was also more inclined towards behavioral techniques, whereas here it's less behavioral and much more cell culture, PCR, and genotyping. We didn't even do genotyping ourselves in my lab at Loyola; we just mailed them, and they were genotyped for us. This lab provides a unique experience in making everything yourself; everything is homemade."

The research project I was involved in aims to design a plasmid capable of inhibiting the NLRP3 inflammasome. The final goal was to make it enter the bloodstream via intravenous injection, cross the blood-brain barrier, and target cells in the CNS. We used the lentivirus gene delivery system to deliver those plasmids to the monocyte cells (THP-1 cell line), and later, by comparing the experimental and control groups, we assessed inhibition. We measured IL-1β levels (by ELISA) and Caspase-1 levels (by Western Blot) in the treated and control groups.

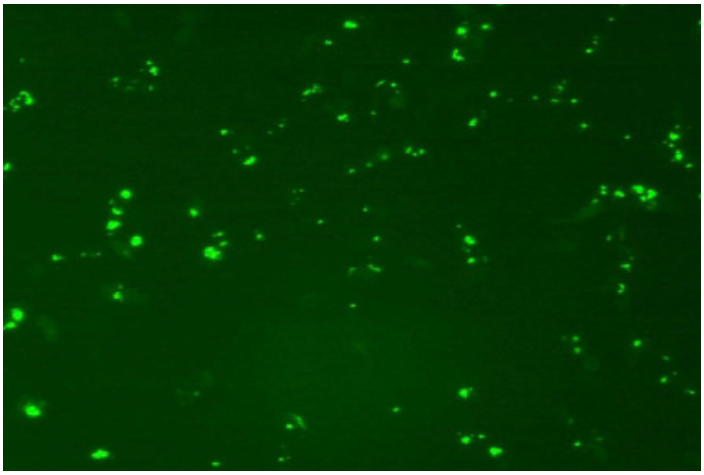

Figure 1. Visualization of virus transfection. GFP-Card (contains the self-made plasmid) medium magnification of lentivirus transfection in 293T cells (human kidney cells).

In the lab, students are welcome to work independently on procedures if they feel confident, with support from Dr. Marr or other lab members as needed. It was great to hear how many students got an opportunity to learn in such an environment. Since joining RFU, Dr. Marr has mentored a wide range of students, including three high schoolers, four INSPIRE students, ten summer interns from various colleges (including Lake Forest College and DePaul University), eight Summer Research Fellows, three student volunteers, five rotation students, twelve medical students, and numerous thesis students.

Dr. Marr's teaching extends beyond the lab. At RFU's medical school, he teaches courses on the limbic system, memory and emotional disorders, cerebellum, autonomic nervous system, and cranial nerves (7, 9, 10, 11, 12). At the graduate school, he leads courses on neurodegeneration and molecular biology and runs a journal club. Several students who took his courses were inspired to join his lab, including an MD/PhD student who eventually became a neurosurgeon. I asked Mike for his thoughts on whether this lab is suitable for students without prior research experience. He responded, "At least 10 students have been in his program. Everyone who was involved in his research and helped to make progress will get their name on the paper. He is very generous and happy to recognize people who contribute to the lab. Last summer, he also accepted a high schooler into his lab. Now that the student is applying to the University of Wisconsin-Madison. General interest in science is all that matters, so this is the place that would accept those students. It's a great opportunity for students to know if this is the area of research they would like to continue in the future, and just in general to find out what the actual research is like."

Beyond his professional role, Dr. Marr is known for being approachable and supportive, further enriching the lab community. Whenever there was free time, he would share stories about his students, give us (me and Sonia) advice if we ever planned to go to medical or graduate school, and answer any questions we might have had. When it came to explaining, his ability to break down complex concepts into understandable terms made a lasting impression on me. Even though I wasn't in his class as a formal graduate student, I still got a glimpse of his teaching style, though it was mainly focused on laboratory topics. Going through the experience of working with Dr. Marr might change one's perspective on the lab and, most likely, lead

to a realization of the lab community's importance. Dr. Marr's lab provides an environment where learning is constant, questions are welcomed, and every contribution is valued. One will learn early on that it's normal to make mistakes, and as Mike once told me, "Re in research stands for repeat, as it's the biggest part of the lab". Due to a lack of prior lab experience, I was nervous about making a mistake or doing anything without double-checking every step, making sure I didn't miss a word in the protocol. However, all the anxiety had faded away after the end of the first week.

Dr. Marr's lab community underscores the importance of being part of a lab that prioritizes both learning and strong mentorship. No matter if the participant is a high school student exploring research for the first time or a medical student preparing for their thesis, Dr. Marr's lab is a place where everyone will get an opportunity for growth and will be treated fairly. I am incredibly grateful for this opportunity and excited to continue building on these experiences in my academic and professional journey.

SICB 2025 in Review: *Bumblebee Gobies, Rubber Mallets, and Resilience*

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Overview of the Society and Its Mission

This year, the Society of Integrative and Comparative Biology conference was held in Atlanta, Georgia, from January 3rd to January 7th. According to Quinn (1979), the Society of Integrative and Comparative Biology began as the American Society of Zoologists in 1902. The change occurred as the organization expanded, increasingly taking on other aspects of biology. This integrative approach is a massive part of the beginnings of society as well. It was started with the merger of the Central Naturalists and the American Morphological Society and aims to include as many specialties within the field of Biology as possible. The Society of Integrative and Comparative Biology (SICB) aims to make organismal biology accessible to all and to further research in the field. One way it aims to do so is through its fully open-access journal, the Journal of Integrative Organismal Biology. In terms of inclusivity, SICB has devised an innovative plan to increase the number of underrepresented group members within the society (Wilga, Nishiguchi, & Tsukimura, 2017).

The Society of Integrative and Comparative Biology is not only interested in increasing the number of underrepresented group members but also in creating a space for them in a fun, welcoming way. Looking just at the conference held in Atlanta, Georgia, this January, the workshops ranged from an eDNA workshop to sessions on how to disrupt division and create spaces for specific underrepresented groups.

Poster Presentation: Bumblebee Gobies and Prey Detection

In addition to the workshops, the conference featured many interesting speakers and poster presentations. In a personal interview, Dr. Schwalbe (Schwalbe, M. Personal communication, August 28, 2025) described the talk and two posters presented by her lab at the 2025 SICB conference. The first poster, Bumblebee gobies using vision and their reduced lateral line canal system to find prey, was solo presented by Shrija Chhetri '24. Chhetri had graduated the semester before, having done her senior thesis on this project. The poster examined Bumblebee gobies' lateral line systems and their importance in prey detection (Chhetri, 2025). The lateral line system is a sensory system found in fish and some amphibians. Specifically, it is the hair cells within neuromasts that help mediate the fish's responses and detect disturbances.

The lateral line system is specifically helpful in prey detection and predator avoidance. The lab examined how prey-detection ability differed between light and dark conditions in Bumblebee gobies with their lateral systems intact versus those with a gentamicin-ablated lateral line system. To do so, the researchers used fluorescent microscopy to visualize the lateral line system and confirm that it had been successfully disabled. Then they recorded the feeding behavior of adult bumblebee gobies on live brine shrimp. The Schwalbe Lab found interesting results when examining the gobies' behavior more closely. For example, they had found that in the light, the gobies typically attacked the front of the shrimp's heads, but in the dark, they typically attacked the body of the brine shrimp. However, it is essential to note that there was variation in reaction distance and angle between individuals. Interestingly, the gobies with intact lateral line systems had similar striking distances in the light and the dark. However, in those with their lateral line systems disabled, the gobies had a greater striking distance in the light than in the dark. The Schwalbe Lab also found that the prey could detect two times its body length. As the primary finding, they found that feeding by the gobies was significantly reduced in fish with lateral line ablations, especially in the dark.

Poster Presentation: Silver Hatchetfish and Jumping Performance

The second poster, presented by Jeremy Levin and Hridey Kapoor (2025), was titled The Role of Vision and the Lateral Line System in the Jumping Behavior of Silver Hatchetfish. The poster looked again at the lateral line system, but in relation to Silver Hatchetfish. Similar to the Bumblebee goby poster, they sought to turn off the lateral line system in some of the fish and used fluorescent microscopy to confirm that it was successfully disabled. After turning off the lateral line systems in the fish, they conducted behavioral tests in light and dark conditions with both intact and impaired fish. The two studies differed in the behaviors they focused on. The Bumblebee gobies study focused on feeding behavior, while the Hatchetfish study looked at startle responses. To do so, Levin and Kapoor would use a rubber mallet to hit the tank counter, placing the fish precisely at a 1 cm height each time. The figure below shows the tank setup.

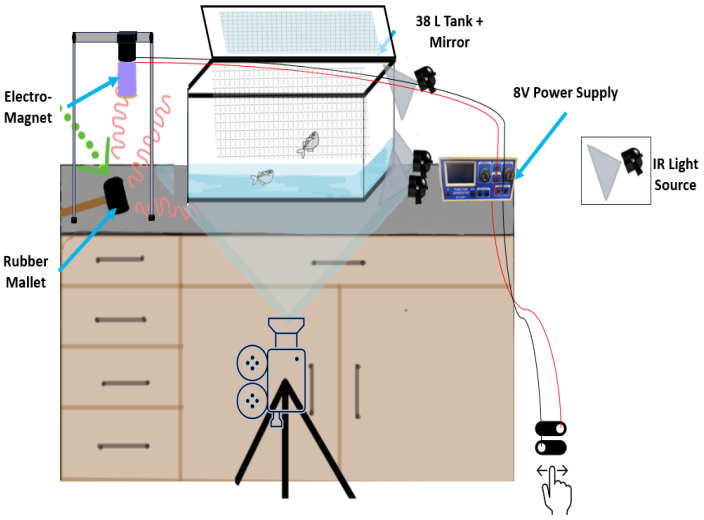

Figure 1. As shown in the image above, the following apparatus shows how the jumping performace of the silver hatchetfish is measured,

They observed the height of the jump, the number of jumps, the duration of jumps, as well as the number of c-starts performed by the fish. C-starts are a type of rapid escape response in which fish curve their bodies into a C shape to thrust forward and escape potential threats more quickly (Schwalbe, M. Personal communication, August 28, 2025). As for the results of this study, they found that the Hatchetfish that had their lateral line disabled made smaller jumps and performed them for a shorter amount of time in both the light and dark conditions. However, the Hatchetfish with disabled lateral lines performed more c-starts, and there were no significant differences in the number of jumps performed by the fish between the four conditions. The poster took first place at the conference for best student poster by an Undergraduate in the division of Neurobiology, Neuroethology, and Sensory Biology (Schwalbe, M. Personal communication, August 28, 2025).

Talk Presentation: Bluegill Sunfish and Flow Navigation

The talk presented by Dr. Schwalbe was titled Bluegills are stable in horizontal cross-flow vortices without their visual or lateral line systems (Schwalbe, M. Personal communication, August 28, 2025). In the interview with Dr. Schwalbe, she explained that the talk covered a study conducted in the lab examining how well Bluegill Sunfish navigate unexpected flow patterns when their vision and lateral line systems are disrupted (Schwalbe, M. Personal communication, August 28, 2025). To test this, the lab placed the fish in tanks with low and high flow speed tanks. As in the previous studies, they used light and dark to account for vision and intact and disabled lateral line systems. They continued using high-speed cameras to capture the movement of the fish across various conditions. They had found that the fish can recover from cross-flow vortices relatively well across conditions.

Implications, Technological Potential, and Future Research

The research presented and ongoing in the Schwalbe lab demonstrates the resilience of fish, as seen in their study with Bluegills. It also shows the systems at play that allow these fish to develop their skills in prey and predator detection, and how they enable the fish to operate at optimal levels. This research into lateral line systems can also aid the development of new technology. Namely, one being the ability of the fish to regenerate their lateral line system over the course of days after being disabled. The lateral line system, as stated above, consists of hair cells, which we also possess and use for hearing; however, our hair cells cannot regenerate. More research into this capability of the fish and their lateral line systems could yield many important findings. Moreover, the research being done by the Schwalbe Lab is focused on many different types of fish and lateral line systems, allowing the lab to understand the capabilities of the system not only in one model organism but across species.

The lab is expected to have a publication or two with SICB coming out in the following year (Schwalbe, M. Personal communication, August 28, 2025). Overall, the 2025 SICB conference was a highly productive and exciting endeavor for the lab. And as a perfect ending to another conference, the lab had gone out to the Georgia Aquarium to celebrate (Schwalbe, M. Personal communication, August 28, 2025).

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The Cicada Pulse: *Ants, Ecosystems, and a Rare Experiment*

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When periodical cicadas emerge from the ground by the trillions every seventeen years in northern Illinois, most people grab earplugs. For Dr. Sean Menke and his team, it was a once-in-a-generation scientific jackpot. The mass emergence of cicadas across Lake County gave Menke, a field ecologist at Lake Forest College, a rare natural experiment: a chance to test how sudden surges of resources—what scientists call "pulses"—reshape ecosystems. His project asks a simple question: when ants encounter a flood of free food, do they abandon their usual ecological roles or keep doing their normal work? The answer matters because it tells us how ecosystems respond when the rules suddenly change. Every 17 years, Lake County becomes an epicenter of ecological drama. Cicadas cover trees, lawns, and sidewalks. For homeowners and businesses, it's mostly a nuisance. For scientists, it's a pulse of energy big enough to alter an entire landscape. Cicadas are not small players in this story. Their collective biomass during emergence rivals that of many other organisms in the region. For about six weeks above ground, their shells cling to trees, their bodies pile in drifts, and their mating calls can reach 100 decibels, which is louder than a motorcycle. Then, just as suddenly as they arrive, they vanish, leaving behind nutrients in the soil and countless questions about what happened while they were here. Ants are often overlooked as pests, but in ecological terms, they're keystone workers. They disperse seeds of native plants, defend trees from herbivores, scavenge dead material, and regulate populations of other insects.

Their roles ripple through ecosystems, affecting plants, animals, and even humans. Because ants respond rapidly to environmental changes, they're also reliable indicators of ecosystem health. That makes them the perfect test case for Menke's question: do ants change their behavior when cicadas appear in overwhelming numbers? If so, what are the consequences for everything else tied to them? Most ecological studies are conducted in small, controlled settings such as plots, cages, or lab microcosms. Menke's project is different. "Most people who do experiments in ecology study small areas," he explained. "To study an entire county is unheard of." The cicada emergence effectively turned Lake County into a vast, real-world laboratory. Ecologists compare this kind of event to other "pulse experiments": whale carcasses sinking to the seafloor, cow carcasses decomposing in the desert, or fertilizer runoff flooding a river. In each case, the sudden arrival of nutrients changes how species interact. But almost none of those events happen on the scale of an entire county, making Menke's project one of the most ambitious of its kind. None of this work would be possible without support from the National Science Foundation (NSF). The grant funds three full years of study: the cicada year itself and two additional years to monitor lingering effects. Crucially, it also pays for people to do the work. Two undergraduates each summer, a full-time post-baccalaureate lab assistant, travel to conferences, and collaborate with partner labs on the East Coast. "All the money basically goes into student support," Menke said. That's not just a financial detail. It's a deliberate choice to make research accessible to students who might otherwise be shut out. Menke doesn't accept unpaid volunteers.

With this grant, ten students in just the first year and a half gained hands-on experience in the field and lab while being compensated for their time. For a liberal arts college, that kind of opportunity is transformative. Student research isn't about standing behind a microscope in climate-controlled labs. It's long drives to field sites, muddy boots, and hours of patient observation. Menke's team maintains five sites across Lake County, each requiring 2-4 hours of monitoring per week. When it rains, ants stay underground. When temperatures dip, activity stops. A week of bad weather can derail carefully laid plans. Students learn resilience the hard way: how to pivot when conditions change, how to keep collecting data even when the day doesn't go as planned. They also learn technical skills that carry far beyond college: field identification of ant species, statistical analysis of behavioral patterns, and the art of presenting results at national conferences. Many of these skills come from working alongside Menke in real time, troubleshooting problems as they arise. The immediate question is whether ants shift their activity during cicada pulses. But the broader implications reach far beyond Illinois. Ants disperse the seeds of endangered plants; if their foraging changes, plant reproduction could be affected. Ants also regulate insect populations; if they're distracted by cicadas, pests could temporarily spike. More broadly, the study speaks to how ecosystems respond to human-driven pulses. Fertilizer runoff, agricultural waste, and urban nutrient surges all create sudden shocks that ripple through environments. By comparing cicadas to these human-made pulses, Menke's team can generate insights that are widely applicable. "The results will be very generalizable because they cover such a large area," he explained. For Menke, cicadas and ants weren't always the plan. Originally trained as a herpetologist, his career shifted when his PhD advisor retired suddenly. He found a new direction through a lab focused on ants, and what began as a detour became a defining path. It's a story he shares with his students to remind them to stay open. "You don't know what you will like doing until you actually do it," he said. That philosophy fits neatly with the liberal arts ethos of Lake Forest College. Students may arrive with rigid expectations for careers in medicine, veterinary science, or molecular biology, but often find passion in unexpected places.

Dr. Sean Menke, Professor and Chair of Biology Department at Lake Forest College.

For Menke, an unwelcome surprise early in graduate school turned into a career in field ecology. For his students, it might be ants. Though the study doesn't directly tie into climate change, its relevance to local conservation is clear. Ants are key seed dispersers for native plants in Lake County, meaning that changes in their behavior could ripple through conservation efforts. The project also turns the county into a "living laboratory," connecting residents to science happening in their backyards. Neighbors notice the cicadas, but they may not realize their yards are part of an experiment that informs global ecological theory. Seventeen-year cicadas are more than a noisy curiosity. They're reminders that ecosystems are dynamic, unpredictable, and occasionally overwhelming. For Menke and his team, the 2024 emergence was not just a spectacle; it was an ecological experiment on a scale rarely seen. By tracking how ants respond to a pulse of free food, they're uncovering insights that apply from forests to rivers to deserts, and from Illinois backyards to global conservation. Although the cicadas won't return for another seventeen years, the lessons from this project may shape science and student careers for decades to come. In the end, the buzzing invasion left behind more than molted shells. It left a blueprint for studying, teaching, and learning from the sudden shocks that define life on a changing planet.

Fighting a Timeless Killer: *Dr. William Conrad's NIH-funded battle against Tuberculosis*

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Despite being preventable and curable, Tuberculosis (TB) was the world's leading cause of death from a single infectious disease. Even though the title of the world's leading cause of death has been held by the coronavirus disease (COVID-19) for the past 3 years, the World Health Organization (WHO) predicted that TB would reclaim its title. Causing twice as many deaths as HIV/AIDS, with up to 10 million falling ill increasingly every year, the United Nations calls for urgent action to end the global TB epidemic by 2030 (World Health Organization, 2024).

For scientists, the battle against the centuries-old disease is not just medical, but also scientific and social: how do we stop a disease that refuses to go away? At Lake Forest College, Assistant Professor of Chemistry William Conrad is taking up the challenge, and thanks to the $425,000 grant from the National Institute of Health (NIH), his lab is now ready to push the boundaries to discover new hope. For Dr. Conrad, this is not just a milestone. It is a turning point. It is a life-changing grant.

Dr. Conrad's fascination with TB grew from his fear of infectious disease after watching the movie Outbreak (1995). "It is something that scared me and became what I wanted to study and understand to find new therapies,". Prof. Conrad's journey with tuberculosis began in 2013 during his postdoctoral fellowship in microbiology and immunology at the University of Washington in Seattle. From there, he continued his passion with a second postdoctoral fellowship at the University of Cambridge in England. Here at Lake Forest College, he started a lab with 2 main goals in mind. First, to contribute to the TB research community, and second, to train the next generation of scientists and other professionals.

Since the 1930s, scientists have been developing some possible antibiotics against TB by studying bacteria in the lab using liquid culture or petri dishes. While it has been historically successful, Dr. Conrad believed it overlooks the realities of the disease. TB only causes problems when it is inside the human body. "I want to do something different to just see if there are other avenues where we could find antibiotics," Dr.Conrad mentioned. "In other words, the genes or the proteins that are needed for the bacteria when they are inside us". The prime suspect that could hold the key to unlocking the cure is a protein called BrkB (Bordetella resistance to complement killing gene B). In fact, BrkB is an ortholog (a descendant of a common ancestor) of *RV2707*, a disease-causing protein. "In simpler words, BrkB is the same protein but from the species Mycobacterium smegmatis (non-infectious to humans)," Alvaro Arroyo '26, a senior student from Conrad's Lab, explained. "Since they are so similar, conclusions on BrkB can inform us of the function of *RV2707*."

'When genetically deleting BrkB from the bacteria, it grows fine in a dish, grows fine in the flask, but does not grow in the host. ' Therefore, the NIH grant Prof. Conrad received is focusing on understanding BrkB with basic questions, including 'What is BrkB? What does it do and how does it work? In the long term, the Conrad lab would like these discoveries to serve as stepping stones toward a therapeutic approach for tuberculosis. The NIH grant not only supports his lab but also offers Lake Forest College students a lifetime opportunity to do real science. "The funding allows me to have a bigger lab," he said.

Dr. Conrad designed a lab where each student can take ownership of a project. With that lab ecosystem, "seniors can train the new students as they develop autonomy. I am hoping to build a web project". Jeremy Levin '26, a senior student majoring in Neuroscience and Biology, mentioned how the experience of training others is "rewarding, challenging, and makes me a better scientist". In addition, with a passion for working in the Medical field, "This trains me to communicate complicated topics with others, which in my future would be the patients." Further and detailed information about the lab is available on https://www.theconradlab.com.

Part of Dr. Conrad's grant also supports an in-class lab, CHEM 305: Advanced Biochemistry, which would be offered during the semester. The goal of this class is to continue where Dr. Conrad left off in his postdoc: the ESX secretion system. Specifically, the in-classroom lab will focus on the enzyme in the system, called the protease, which is highly "druggable". Many advanced protocols will be used in the lab, including purifying the protease, performing enzyme inhibition assays, and applying computational chemistry, such as modeling the protease's 3D structure. "It is so exciting about how and where we are living right now. Computational chemistry and biochemistry are growing so much, and it is democratizing science," Dr. Conrad commented.

Ultimately, what he hopes students will gain goes beyond technical knowledge. Dr. Conrad highlighted 3 important questions about science - questions he believes should always stay at the back of his students' minds as they pursue science. What is the problem? What do you want to solve? And what are some steps to solve the problems? "I don't want all of my students to end up being tuberculosis or even an infectious disease researcher. I want them to take the lessons of what we do in the lab and find the right tools to solve the problem elsewhere."

Despite the NIH grant's impact, Dr. Conrad is deeply aware of the fragile landscape of research funding in the US, which he argues is essential to global scientific progress. "Just like ATP is the powerhouse of the cell, the NIH is the powerhouse of scientific research around the world - Republican or Democrat, immigrant or non-immigrant, disease affects us all." His NIH grant currently supports more than a dozen students on campus; without it, his lab would not survive. "If my funding were taken away, I would have to shut down my lab. It would be devastating", he admitted. "My only wish and hope is that people keep speaking their truth out into the world, we benefit from fighting the disease. We need to stand up for science".

The fight against Tuberculosis is one of humanity's longest wars, but Dr.Corand sees a path forward. Because this disease survives only in humans, not in water, soil, or other animals, he strongly believes eradication is possible. "TB has been very effective at evolving against whatever new things that we give to it". For Dr.Conrad, victory is not just advancing TB research but preparing the next generation of scientists to solve the problems that matter.

Figure 1. The Conrad Lab.

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The Attack on DEI: *How Political Rhetoric Shapes Scientific Inquiry in America*

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The fight for diversity, equity, and inclusion (DEI) has always been central to American identity and discourse, even before DEI emerged as the socio-cultural paradigm we know today. DEI formally emerged from the Civil Rights Movement in the 1960s, and is best described as a framework that creates equitable opportunities, eliminates barriers to access, and fosters environments where everyone is valued and included. In the 21st century, violent acts of discrimination (such as the murder of George Floyd) catalyzed a new wave of DEI initiatives in the United States. This momentum, however, is threatened by the Trump administration's attempts to strategically shift the narrative surrounding DEI, turning a social justice paradigm into a political and cultural weapon. When detractors label DEI programs as reverse discrimination against majority groups, they equate equity with a zero-sum game and reduce the nuanced, on-the-ground work of social justice advocates to address structural and cultural violence. This misleading and emotionally charged rhetoric is dangerous, and critics who claim DEI impedes progress fail to recognize that it is, in fact, a prerequisite for the very societal and economic development they seek.

In October of 2024, Texas Senator Ted Cruz released a report titled "Division, Extremism, Ideology: How the Biden-Harris NSF Politicized Science." This report provides a case against government funding for research that implements principles of DEI, characterizing DEI as an intrusion of the far-left's political agenda into the objective, hard sciences: "...the Biden-Harris administration, through NSF, is deliberately and systematically inserting a divisive political ideology into 'scientific research.' Instead of identifying the best or most talented scientists, NSF funded researchers who prioritized filling out research teams and programs based on ethnicity, cultural background, or political perspectives." (U.S. Senate Committee on Commerce, Science, and Transportation, 2024)

The report claimed that supporting DEI in university research programs would lead to NSF's complacency in the "neo-Marxist indoctrination of students" and that DEI-based projects are one cause of anti-Semitic violence on college campuses (U.S. Senate Committee on Commerce, Science, and Transportation, 2024). Presenting DEI as a threat to meritocracy and a misuse of taxpayer dollars, Cruz's report effectively taps into potent conservative concerns and blames DEI as the root of these anxieties. However, the methodology used in the report—which categorizes grantsby their use of specific keywords such as "equity" and "social justice"—conflates the goal of broadening participation in science with the imposition of a political ideology. Ironically, the report itself serves as a case study, demonstrating through its own misrepresentations of DEI why public funding for propaganda and for bias research is necessary in the first place.

Through conservative educational and legislative crackdowns that vilify initiatives using DEI terminology, the acronym "DEI" becomes synonymous with the "radical woke left," and its true meaning is deliberately subverted to uphold structures of oppression and erase decades of scientific research. DEI has wide-reaching, measurable, and positive impacts, especially in the academic and corporate worlds. Research consistently demonstrates that DEI initiatives yield significant benefits across various sectors. In the corporate sector, a seminal study found a strong positive correlation between executive team diversity and financial performance, with companies in the top quartile for gender and ethnic diversity being 15% and 35% more likely to outperform their national industry medians, respectively (Hunt et al., 2015). Furthermore, in academic settings, the implementation of structured diversity interventions is empirically linked to improved outcomes; a longitudinal study concluded that campus diversity experiences significantly enhance students' academic development, intellectual engagement, and civic preparedness (Milem et al., 2005). DEI's positive impact extends to innovation, as further research reveals that companies with above-average diversity scores reported greater levels of innovation revenue—45% of total revenue—compared to 26% for companies with below-average leadership diversity, providing strong evidence that diverse teams are better equipped to drive novel solutions (Lorenzo et al., 2018).

As for STEM fields, a growing body of evidence demonstrates that enhanced diversity leads to more innovative and impactful research. Studies have shown that diverse teams are significantly more likely to produce novel and highly cited scientific work (Hofstra et al., 2020). This innovation is further linked to better financial outcomes and organizational performance (Gomez and Bernet, 2019). In medicine, racial concordance between provider and patient has been associated with an increased likelihood that patients adhere to prescribed treatments and receive preventative care (Marrast et al., 2014).

The literature reveals that DEI is a driver of economic growth and industry while fueling scientific discovery. However, DEI can and should be extended beyond the aim of "better science," explains Lauren Bauman, a Physics Research Coordinator and Consultant with a Physics Education group at the University of Washington (L. Bauman, personal communication, July 31, 2025). Beyond leveraging DEI for its tangible benefits within existing systems, scientists should aspire to a critical reimagination of the entire discipline– one that dismantles entrenched structures of exclusion and actively decenters the legacy of colonialism and white supremacy. We should transcend diversity quotas and box-checking requirements and question the very foundations of scientific practice by asking important questions: Whose knowledge is valued? What questions are deemed worthy of investigation? For whose benefit is research conducted? Bauman asserts that DEI initiatives grounded in interest convergence—the temporary alignment of diversity goals with institutional self-interest—are unstable and ineffective, and a systems-level approach must be taken instead (L. Bauman, personal communication, July 31, 2025).

By replacing methods built on historical inequities with frameworks that are inherently just and inclusive, we can create a scientific enterprise that is not only more equitable but also more rigorous and objective. Scholars argue that a lack of diverse perspectives can entrench methodological biases and blind spots, limiting the scope and validity of scientific inquiry (Intemann, 2009). The sciences therefore necessitate a "decolonial turn" and an active re-working to foster practices that are ethically engaged and socially accountable by critically examining how Western knowledge acquisition has historically been complicit in oppression (Bhambra et al., 2018). Ultimately, this necessary turn is not about lowering standards to increase diversity, but about raising standards by ensuring that science is built upon a foundation of plurality of thought realized through social justice and collective empowerment. It is precisely this transformative potential that makes DEI a target for political attacks, as it endangers the current balance of power in science.

Like all other scientific research groups in the United States, Bauman's team must submit grant proposals to the National Science Foundation (NSF) for funding. Whether or not a grant is allocated funding depends on the project's alignment with NSF priorities, which aim to "strengthen the U.S. economy, improve global competitiveness, and provide tangible societal benefits" (National Science Foundation, 2022). The Biden administration introduced DEI-focused priorities thatmany research groups used to guide their projects. However, in mid-April of 2025, the NSF, whose funding is provided by the federal government, updated their priorities to align with the Trump administration's interests, such as artificial intelligence, quantum science, and nuclear energy (University of Washington Office of Federal Relations, 2025). Consequently, funding was pulled from projects centering DEI and misinformation research (L. Bauman, personal communication, July 31, 2025).

Figure 1. Image above from a article by Urbina-Blanco et al.,2020 highlights how marginalized scientists need the required supporting systems to continue the process of change and destigmatization.

Bauman's team received an official letter of termination for their grant for "Spatial Justice in the Physics Classroom," which investigated the factors that make up a just classroom and involved a critical reimagining of mainstream approaches in Physics Education. The termination letter stated that their grant "no longer effectuated" the priorities of the NSF, and the resulting funding cuts permanently halted the project. Bauman's study was one of many projects liquidated as per the new NSF guidelines, and these sweeping reforms have had drastic consequences for STEM in the United States. "It was a complete 180," explained Bauman. Groundbreaking studies that had collected data for decades were shut down, and thousands of scientists now find themselves jobless due to the closures of entire research departments (L. Bauman, personal communication, July 31, 2025).

The ripple effect of shifting government priorities and the attack on DEI reveal themselves is an attack on the nature of STEM itself, which is to evolve and continuously self-correct. As Bauman puts it: "The critical work of interrogating the fundamental things we don't ever critique because they're 'just the way they are' is essential, even if it makes us feel shitty about the world in which we live." Questioning the world, however uncomfortable it may make us, is the engine of scientific progress. By stifling this process, we risk stagnation. The future of American science is at stake.

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STEM Education and Research: *Why Funding Matters*

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The current federal administration has proposed a budget for the 2026 fiscal year that would be utterly detrimental to scientific research. This proposal includes, but is not limited to, cutting the Centers for Disease Control and Prevention (CDC) budget by $4.3 billion, slashing the National Institute of Health (NIH) budget by about $18 billion, and cutting global health programs by about $6.2 billion (Cohen et al. 2025). Furthermore, as of May 7 of this year, the National Science Foundation (NSF) grants for STEM education have been cut by about $773 million (Miller 2025). Beyond the obvious ramifications for individuals in STEM fields, these budget cuts will likely create a significant ripple effect worldwide. STEM education and research are key components to a flourishing society, and therefore, any cuts in these areas will certainly hinder societal advancement and risk global health.

It is undeniable that society would not be where it is today without the work of STEM researchers. Every piece of technology we own and every medical advancement we have ever seen can be attributed to STEM research. As shown in Figure 1 (National Science Board & NCSES 2024), there is a wide distribution of occupations and specific concentrations within the STEM workforce. STEM careers are way more extensive than simply observing chemical reactions or looking at cells under a microscope. These jobs are consistently evolving to meet the demands of our growing society. For instance, the prevalence of artificial intelligence (AI) has hit an all time high. AI research is a rapidly growing field at this time, and has already begun to transform schools and workplaces. While it is important to be wary of the potential ramifications of AI technology, no doubt, continued AI research could transform society beyond our wildest imaginations.

Another example of crucial STEM research at this time is climate change research. 2024 was the warmest year on record so far, with Earth's temperature having risen about 2 degrees Farenheit since 1850 (Lindsey & Dahlman 2025). Undoubtedly, research in this field is crucial to the survival of our planet. Climate change and AI research, along with countless other fields, will determine what the future holds for humankind. To minimize the significance of this research would be downright foolish and detrimental to our planet.

Of course, there would be no scientists, technologists, engineers, or mathematicians without the process of being inspired, learning STEM fundamentals, and training to succeed in their work. STEM education as a whole offers students the opportunity to develop a unique set of skills, with each discipline offering its own benefits. For example, "The ideas of physics incorporate experiments, mathematics, logic, and philosophy," says Dr. Michael Kash, an esteemed physics professor here at Lake Forest College. In general, critical thinking skills needed in every aspect of our lives are primarily developed through STEM education. There is also an interdependency between STEM education and digital literacy, a crucial skill given that children interact with 21st-century technology every day (Govender 2025).

While all of these skills are valuable, the most important thing a STEM education can offer is a deeper understanding of the world around us. To understand things as simple as why little condensation droplets form on our cups, how connecting two wires can create electricity, or why we need food and water to survive allows us to connect and engage with the world around us. Without this knowledge, we would be walking around confused, with little understanding as to how we even exist. Our understanding of these concepts stems from math and science classes as children and allows us to grow into functioning, knowledgeable adults, whether we are in the STEM field or not.

For many individuals, however, this increased understanding of the world does more than that. It ignites a spark, and inspires them to pursue a career in STEM. This is the primary reason why societal development would come to a complete halt without the continuation of STEM education. Sure, no one would have the foundation of knowledge required to develop new technology or medication. However, more importantly, no one would have the passion to do so. "Teaching science early and helping young minds understand its fundamental and critical value is the responsibility of all cultures and nations since our future is in their hands," says Dr. Shubhik DebBurman, a well-respected professor of biology, neuroscience, and biochemistry and molecular biology here at Lake Forest College. "Disregarding, minimizing, or misusing it can cause serious harm to the progress of humankind and hamper our efforts to keep our fragile physical environment and our living world safe."

So, why aren't STEM research and education being prioritized? There are many misconceptions about the absolutely vital role of STEM in our everyday lives. We see headlines related to science in the news every day, such as that vaccines cause autism, or that federal funding is being spent on "making mice transgender." Clearly, there is a lack of understanding of scientific research. This lack of understanding, however, could put the world in immense danger. "We may be in an era where people who study a subject deeply enough to become experts ignore experts," says Kash. Efforts to combat climate change, mental health research, future medical developments such as a cure for cancer, and so much more are all being jeopardized by recent and future funding cuts. To combat this issue, it is crucial to continue advocating for STEM funding in every possible way. Furthermore, particularly in a democratic nation, we need to focus on electing leaders who will support, emphasize, and adequately fund scientific efforts. "Favoring shorter-term political advantage or economic greed over longer-term safety of human society and our fragile planet is something we all need to be wary of," says DebBurman. At the end of the day, without proper funding for STEM education and research, the world as we know it will change for the worse.

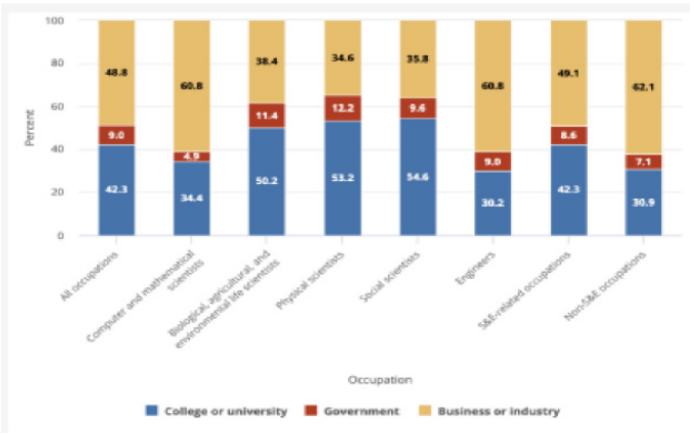

Figure 1. Fields of employment for doctoral recipients are distributed across various STEM fields—source: NCSES, Science and Engineering Indicators 2024.

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Beyond Punishment: The Work of Mental Health Court as a Model for Change

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If everyone who recognized your difficult days and knew when you were not your full self suddenly disappeared. It would leave anyone vulnerable. This reality, layered onto someone who is already struggling with their mental health, can create a space to be judged not based on the full story. That is not merely unfortunate; it is unfair and unjust. People can relate to the need for support, a community, and someone who truly sees them. The stigma against mental illness causes discrimination. And, our fear leads to the criminalization and ostracization of a person's differences. Thus, leading someone to incarceration.

Mass incarceration is a persistent and growing issue in the United States. Alongside this is the growing number of individuals living with untreated mental health conditions, visible and non-visible. These two crises are not parallel; they intersect. Mental Health Courts (MHC) provide a barrier to help filter out individuals who are entering incarceration and allocate potential resources that could prevent them from re-offending. Despite MHCs becoming a deterrent to imprisoned individuals with mental illnesses, many of those individuals are still not getting the help they need. Those with unaddressed psychological needs are often placed into the prison system not because of intrinsic criminality, but because of systemic neglect (Otto, 2020).

Background

In 1841, Dorothea Dix, while teaching in a Massachusetts jail, observed that a high number of inmates were not criminals, but people with mental illnesses (Dvoskin et al., 2020). Her observations led to her efforts to provide proper care to individuals who were struggling with mental illnesses and had been penalized for it. Most individuals at that time who were believed to have a mental illness were forced into psychiatric hospitalization or the jail system. About forty years later, Massachusetts enacted the first state law allowing voluntary admission (Dvoskin et al., 2020). Even more recent is the development of a Therapeutic Court: The Mental Health Court (MHC), first established in 1980 in Indiana.

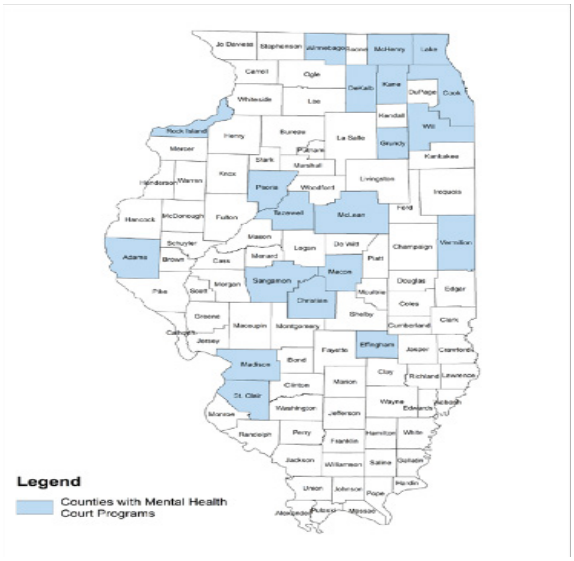

Figure 1. Illinois Cities with Mental Graph Programs as of 2020 (Otto, H. D.2020).

MHCs allow defendants to be seen in court as more than just a case, but as people struggling with mental illness. MHCs work specifically with defendants who have mental illnesses to balance specialized care and sentencing. MHCs serve as a useful tool to reduce recidivism, the likelihood of re-offence (Otto, 2020). When defendants are granted the court's permission to choose treatment over traditional sentencing, they gain access to support that can help disrupt the cycle of incarceration.

Mental Health Courts are one of several types of Therapeutic Courts. Currently, Lake County has a Drug Court, Veteran Treatment and Assistance Court (VTAC), and MHCs. By having MHCs, they are able to focus on a small group of individuals with severe mental illnesses, from schizophrenia to severe bipolar disorder. Depending on the severity of the violation, whether felonies or misdemeanors, this court offers tailored interventions. MHCs utilize the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) to define mental health disorders, symptoms, and criteria for the disorder (American Psychiatric Association, 2013).

When an individual is arrested, police are trained to recognize signs of mental health issues. They may not catch every individual; however, there is another filter, attorneys. Attorneys are informed by the Bar Association to assist in identifying clients who may benefit from MHC. Both police and attorneys play major roles in the first step: application.

Application

An individual with a pending case may apply to MHC with the understanding that they have a severe mental illness. The case is then screened by the MHC team, which includes two judges, two state attorneys, two probation officers, a case manager, and a psychologist/clinician. The psychologist conducts a screening to assess the severity of the illness and whether the individual is fit for programming. If their mental illness is deemed not to meet the criteria for the program, then they will be denied. This is because MHCs are small and need to be selective, as this process is long and intricate. However, this also highlights how individuals are excluded because of systems like the DSM-5, which may not capture the full experience of mental illness (American Psychiatric Association, 2013).

Furthermore, there is another choice to make. After assessment and confirmation, the case's legality must also be considered. Felony or misdemeanor charges are reviewed in accordance with federal law: "The court shall impose a sentence sufficient, but not greater than necessary, to reflect the seriousness of the offense, to promote respect for the law, and to provide just punishment for the offense" (18 U.S. Code § 3553, n.d.). Individuals must serve their sentences and will not get an "easy way out." Instead, they will have to work hard for their program and commit to treatment.

Processing/Entering of the Program

If the case is not approved, it may be dismissed or reconsidered. If approved, the individual enters the program under the supervision of a probation officer. Mental Health Courts (MHCs) then develop a tailored treatment plan, which may include group or individual therapy, prescribed medication, and other supportive programming.

Another form of treatment and programming offered through MHCs, as Frank Morelli, Manager of Probation in Lake County, explained in an interview, "Our adult probation department has several programs that are run by Probation Officers. Moral Reconation Therapy (MRT) for substance abuse and for domestic violence is part of that programming. It is equivalent to Intensive Outpatient Therapy for substance abuse"(F. Morelli, personal communication, August 24, 2025). MRT is a cognitive-behavioral treatment to enhance positive behavior, moral reasoning, and decision-making. Originally developed by Dr. Gregory Little and Dr. Kenneth Robinson to treat moral reasoning in offender populations and individuals with substance use issues (Correctional Counseling, Inc., n.d.).

For individuals with greater needs—such as lacking housing or

experiencing severe mental illness—Lake County provides access to housing with on-site services. Lake County is unique in its commitment to ensuring participants have everything they need—from rides to appointments to medication pickups. The biggest thing about the court and the team at Lake County is that they care. Comparatively, MHCs are small but thorough; they only take a limited number of participants at a time to ensure proper care. Yet many more individuals need help. That is why, as we move into the next stages of our lives, we must reflect on where we can make an impact.

The Impact

When an individual enters the Lake County Court, they join a family that wants them to succeed. They are required to attend court every Monday, where Judge Bishop or Judge Novak checks in with them, starting by asking how they are doing and genuinely trying to build connections. Manager of Probation Frank Morelli makes it clear that this leaves the individual vulnerable during treatment and in court (F. Morelli, personal communication, August 24, 2025). Then, the judge reviews their treatment progress by holding them accountable or offering incentives. Incentives include $10 gift cards, increased privileges such as an extended curfew, or having their case called first in court (Judge Bishop, personal communication, August 24, 2025).

Once probation is completed, individuals are encouraged to stay in contact with the team. Major outcomes I observed during my time at MHC included individuals gaining employment and achieving mental health stability. Beyond those outcomes, I witnessed people reconnecting with family, continuing their education, and most importantly, finding a support system. This system allowed them to make their own choices. Like Dix, I saw individuals who were struggling with their mental health finally begin to move forward (Dvoskin et al., 2020).

Thus, Mental Health Courts create a foundation of care and respect; they remind us that there is room for both justice and compassion. By challenging the idea that punishment is the only response to unwanted behavior, they offer something more that allows an individual to reflect, grow, and adapt to new and uncomfortable challenges. As we move forward in our academic and professional lives, it is imperative to ask ourselves whether the systems we want to build and grow within criminalize individuals for being different or recognize their humanity. The work being done in Lake County is not just legal work; it is rooted in emotional and scientific understanding. It shows what can be the outcome when an individual has support, a community, and someone who truly sees them. And that is the kind of justice worth working towards.

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Long-term Effects of Cancer-Associated POT1 Mutations on Telomerase-Telomere Interactions and TPP1 Function

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Telomere maintenance plays a crucial role in cellular aging and cancer development. This research proposal aims to elucidate the intricate dynamics of telomere maintenance mechanisms in cancer-associated mutations, with a specific focus on the interactions between telomerase, POT1, and TPP1. By leveraging the advanced capabilities of the CoPixie algorithm for high-resolution single-molecule imaging analysis, I seek to address two critical gaps in our current understanding: The long-term effects of cancer-associated POT1 mutations on telomerase-telomere interactions across multiple cell cycles and the indirect effects of these mutations on TPP1 function and its interaction with telomerase. I will employ live-cell imaging techniques and the CoPixie algorithm to analyze telomerase-telomere interactions in HeLa cells expressing cancer-associated POT1 mutants. My experiments will extend beyond the current 300-second observation window to multiple cell cycles, allowing me to assess cumulative effects over time. Additionally, I will investigate how POT1 mutations impact TPP1's interactions with telomerase by overexpressing wild-type and mutant hTERT. I hypothesize that the effects of POT1 mutations on telomerase-telomere interactions will become more pronounced over multiple cell generations and that POT1 mutations will indirectly affect TPP1's ability to regulate telomerase activity at telomeres. This research has the potential to significantly advance our understanding of telomere biology in cancer, potentially identifying novel targets for therapeutic interventions. By elucidating the mechanisms through which cancer-associated mutations disrupt telomere maintenance, we may contribute to the development of more effective strategies for cancer treatment and prevention.

Telomeres and Telomerase

Imagine a world where our cells contain an internal clock that ticks away with each cell division. Special structures called telomeres compose this internal clock. These telomeres act like the plastic tips that protect the ends of our shoelaces, protecting the vital information stored in our DNA. The telomeres are tandem genetic repeat sequences at the end of our chromosomes, but what happens when these protective caps wear down? Telomerase is a remarkable enzyme that can turn back this clock, extending the lifespan of the cells in our bodies. This crucial interaction between telomeres and telomerase plays a vital role in aging, cancer, and various diseases (Chan & Blackburn, 2003).

Telomeres, located at the ends of our chromosomes, are like biological countdown timers. Every time the cell divides, these protective caps get slightly shorter and shorter. When telomeres become too short, cells stop dividing and enter a state of senescence or even die. This is a natural process of aging, but it also serves as a safeguard against uncontrolled cell growth, a key feature of many cancers.

Telomerase, on the other hand, is nature's way of hitting the snooze button on this internal cellular clock. This enzyme can add DNA back to telomeres, effectively resetting telomere shortening caused by cell divisions. While most of our cells do not produce telomerase, specific cells, such as stem cells, can activate this enzyme (Cifuentes-Rojas & Shippen, 2011). This ability of telomerase to maintain or even lengthen telomeres gives these cells the potential for indefinite division. Unfortunately, cancer cells can also activate telomerase, allowing them to divide indefinitely.

Protecting our genetic material is not the job of telomeres alone. They work alongside a group of proteins called the shelterin complex. Returning to our imaginary world, the shelterin complex would be best described as a group of security guards, each with a specific role in guarding the ends of our chromosomes. The shelterin complex comprises six proteins, but the two key players are POT1 and TPP1.

POT1 binds to single-stranded DNA at telomere ends, where it acts as a lock. It prevents other cellular mechanisms in our body from mistaking the single-stranded telomeres as broken DNA and prevents telomere elongation (Rice et al., 2017). TPP1, on the other hand, serves as a bridge between POT1 and the rest of the shelterin complex. Together, they form a protective cap that shields telomeres from damage and regulates telomerase's access to and extension of telomeres.

The intricate interactions between telomeres, telomerase, and the shelterin complex maintain a balance in our cells. When this balance is disrupted in our cells, it can lead to various health problems, including cancer. In many types of cancer, cells have evolved a mechanism to reactivate telomerase, allowing them to divide endlessly (Jafri et al., 2016). It is as if these cancer cells have found a way to keep their internal clocks wound continuously, avoiding the natural processes that usually limit their growth.

Interestingly, some cancers are associated with mutations in genes encoding shelterin proteins, particularly POT1. These mutations can affect how POT1 binds to telomeres and interacts with the other shelterin proteins. This causes abnormal telomere lengthening and compromises the cell's ability to regulate telomerase activity (Prince et al. 2024a, 2024b). This telomere dysfunction contributes to the genomic instability often seen in cancer cells, allowing them to accumulate even more mutations and grow unchecked. Mutations in POT1 also disrupt the natural state of the POT1-TPP1 complex, leading to chromosomal abnormalities and cancer.

Understanding these complex interactions between telomeres, shelterin proteins, and telomerase is crucial for developing new strategies to combat cancer and age-related diseases. By unraveling the mechanisms by which cells maintain their telomeres, researchers hope to find ways to selectively target cancer cells or, potentially, slow the aging process in healthy cells. As we delve deeper into telomere biology, we hope to uncover new and exciting possibilities for future medical research.

Review at a more scientific level

While the overview of telomeres and telomerase provides a foundation for the understanding of cellular aging and cancer, a deeper dive into the molecular mechanisms and recent scientific advancements reveals a more complex and nuanced picture of telomere length regulation. The intricate system between telomerase and shelterin proteins in telomere length regulation maintains genomic stability and cellular longevity, with implications for aging and cancer development.

Telomerase is a ribonucleoprotein complex composed of a catalytic subunit (TERT) and an RNA component (TR or TERC). The telomerase RNA component provides the template for telomeric DNA synthesis and also contains elements important for the enzyme formation and regulation. The TERT subunit contains several conserved domains critical for its function: the N-terminal extension, the reverse transcriptase domain, and the C-terminal extension (Wyatt et al., 2010). The human telomerase (hTERT) adds TTAGGG repeats to chromosome ends, counteracting telomere shortening that occurs during cell division and DNA replication. Diverse transcriptional, post-transcriptional, and post-translational mechanisms tightly regulate the expression and activity of telomerase. At the transcriptional level, multiple transcription factors that bind to the TERT promoter regulate TERT expression (Robinson & Schiemann, 2022). These transcription factors integrate signals from

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numerous intracellular signaling pathways to either activate or repress TERT transcription. Accessory proteins such as dyskerin, TCAB1, and the shelterin complex regulate the assembly and localization of the full telomerase holoenzyme (Robinson & Schiemann, 2022).

The shelterin complex is a critical regulator of telomere structure and function. It is composed of six core subunits: TRF1, TRF2, RAP1, TIN2, TPP1, and POT1 (Amir et al. 2020). These proteins interact with each other to protect telomeres from DNA damage response pathways and regulate telomere length by controlling telomerase access. POT1 (Protection of Telomeres 1) is a key component of the shelterin complex and contains two N-terminal OB-fold domains that bind directly to single-stranded telomeric DNA (Amir et al., 2020). This binding of POT1 helps facilitate telomerase access. The C-terminal region of POT1 interacts with TPP1, another subunit of shelterin. TPP1 serves as an important bridge between POT1 and the rest of the shelterin complex. The N-terminal OB-fold domain binds telomerase and recruits it to telomeres (Amir et al., 2020). This TPP1-POT1 complex enhances POT1's DNA-binding affinity and regulates telomerase access and activity at the telomere. Disruption of this POT1-TPP1 interaction, through genetic mutations or other means, can lead to telomere dysfunction and the development of various diseases like cancer.

Mutations in the POT1 gene have been identified in several types of cancer, including chronic lymphocytic leukemia, familial melanoma, cutaneous malignant melanoma, Coats plus syndrome, cardiac angiosarcoma, and familial glioma (Prince et al. 2024a, 2024b). Many of these cancer-associated POT1 mutations cluster within the OB-fold domains of the protein, which are critical for binding to single-stranded telomeric DNA. Researchers have shown that these POT1 mutations disrupt POT1's ability to bind and protect the telomeric single-stranded DNA overhang, leading to telomere lengthening and fragility (Prince et al. 2024a, 2024b). Mutations in POT1 fail to inhibit telomerase access and activity at telomeres, thereby promoting telomere elongation that drives cancer progression.

The advent of single-molecule imaging at nanometer-scale resolution has revolutionized our understanding of molecular dynamics in living cells. However, quantifying colocalization events between single molecules in living cells remains a challenge. Current strategies for analyzing colocalization often rely on manual scoring, which limits data collection. To address this, researchers developed a new algorithm, CoPixie, that rapidly and automatically quantifies colocalization events across an unlimited number of imaging channels (Prince et al. 2024a, 2024b). Researchers have used CoPixie to study the dynamic interactions between telomerase (labeled with the hTR component) and telomeres (labeled with mCherry-TRF1) in living HeLa cells. HeLa hTR5MS2 cells, which stably express MS2-tagged hTR and mCherry-TRF1, were used as a model system to image single telomerase particles and telomeres simultaneously. This dual-color single-particle tracking data was then analyzed using the CoPixie pipeline to quantify colocalization events and binding dynamics between telomerase and telomeres. The high-throughput capabilities of CoPixie enabled the researchers to explore how cancer-associated mutations in the telomeric protein POT1 impact telomerase access and residence time at telomeres. The manual nature of previous analysis had limited the approaches to this question (Prince et al. 2024a, 2024b).

While researchers have made significant progress in understanding telomere length regulation and the role of POT1 mutations in cancer, several key questions remain: the long-term effects of POT1 mutations and the effects on telomerase-telomere interactions over multiple cell cycles. Current studies have focused only on short-term effects, measuring for only 300 seconds within a single cell cycle (Prince et al. 2024a, 2024b). There is a need to investigate whether the effects of these mutations would be more pronounced in subsequent cell generations. Another knowledge gap is the indirect effects of cancer-associated POT1 mutations on TPP1 function. Exploring how the mutations alter the formation and stability of the POT1-TPP1 complex.

Future Experiments

The proposed investigation seeks to elucidate the dynamics of telomere maintenance mechanisms (TMMs) in cancer-associated mutations, with a specific focus on the intricate interactions between telomerase, Protection of Telomeres 1 (POT1), and TPP1. This study will use CoPixie, a novel object-based colocalization algorithm that integrates pixel- and trajectory-based overlap analysis, to visualize and quantify these molecular interactions with high precision. The research proposal is structured around two primary objectives:

Aim 1: Longitudinal Analysis of Cancer-Associated POT1 Mutations on Telomerase-Telomere Dynamics

I first aim to investigate the effects of cancer-associated POT1 mutations on telomerase-telomere interactions over extended time periods. Previous studies using CoPixie to investigate the impact of POT1 mutations on telomerase-telomere interactions have shown that cancer-associated POT1 mutants increase telomere elongation by increasing telomere accessibility and enhancing telomerase retention at telomeres (Prince et al. 2024a, 2024b). However, these were performed in only one cell cycle, and long-term interactions were measured for only 300 seconds. Another study used single-molecule tracking with MS2 stem loops and CRISPR/Cas9 gene editing to tag and visualize hTR dynamics with POT1 and showed that POT1 plays a regulatory role in telomerase retention at telomere ends, and that mutations enhance this retention (Laprade et al., 2020). I want to explore the effects of cancer-associated POT1 mutations on telomerase-telomere interactions over a more extended period and across multiple cell cycles to determine whether POT1 mutation effects become stronger in later generations. I will explore this by using CoPixie to analyze hTR-telomere interactions using single-molecule tracking images and movies. I hypothesize that specific POT1 mutations will significantly alter the frequency and duration of telomerase-telomere associations across multiple cell cycles. I believe that the effects of the POT1 mutation will worsen over time, causing even more dysfunction in telomerase and telomere interactions, leading to increased telomere elongation in cancer-associated mutated cells.

Aim 2: Elucidation of TPP1 Dynamics in the Context of POT1 Mutations and Telomerase Activity

Secondly, I aim to apply the CoPixie algorithm to examine the dynamics of TPP1 in relation to POT1 mutations and telomerase activity. Previous studies have shown that the shelterin subunit TPP1 requires another shelterin subunit, POT1, to interact stably with DNA (Wang et al., 2007). Given that POT1 stabilizes TPP1, I believe mutations in POT1 will affect the interaction between the two shelterin subunits, further reducing telomerase-telomere maintenance mechanisms. I will explore this by applying the CoPixie algorithm to analyze telomerase interactions with TPP1 in the presence of various cancer-associated POT1 mutations, to observe any differences in TPP1 functionality. I hypothesize that POT1 mutations will indirectly affect TPP1's interactions with telomerase, altering telomere homeostasis.

Experiment 1:

Cell culture:

I followed the same methods as in Prince et al. (2024a, 2024b), using HeLa hTR^5'MS2 cells, Express mCherry-TRF1 to detect telomere foci & mCherry-CDT1 for filming and particle tracking. Cells are then cultured in DMEM, supplemented with 10% fetal bovine serum, two mM L-glutamine, and 100 U/ml penicillin-streptomycin. Induction of myc-tagged POT1 mutants will be performed using lentiviral vector infection. A positive control will include cells expressing wild-type POT1. A negative control will include cells with POT1 knockouts. I will also include cells transduced with an empty lentiviral vector to control for the effects of the viral infection process.

Western blot analysis:

The western blot analysis will be used to validate the expression of myctagged POT1 proteins in HeLa^MS2 cells, as in Prince et al.

(2024a, 2024b).

Immunofluorescence:

Immunofluorescence will be used to visualize the localization of myc-tagged proteins at telomeres in HeLa^MS2 cells, as in Prince et al. (2024a, 2024b).

Live-cell imaging of hTR and telomeres:

This will allow me to investigate the impact of cancer-associated POT1 mutations on the dynamics of telomerase-telomere interactions, following the methods by Prince et al. (2024a, 2024b). It will allow live-cell imaging and 300-second movies to observe longer interactions, which will be repeated in the same cells to observe changes over a longer period of time.

CoPixie analysis of hTR–telomere interactions:

This will be used to quantify colocalization events between single hTR particles and telomeres, as in Prince et al. (2024a, 2024b).

Mathematical and statistical analyses:

I will be analyzing survival probability, which measures the dwell times of hTR particles at telomeres, as in Prince et al. (2024a, 2024b). This will allow me to quantify the dynamics of individual hTR-telomere interactions and telomerase interactions at telomeres.

Predicted Outcome:

From this experiment, I predict that the effects of the POT1 mutations will be greater over a longer period and in subsequent generations. POT1 mutant cells may show a higher frequency of telomerase-telomere interactions compared to wild-type cells. This increase could become more pronounced over multiple cell cycles, indicating a cumulative effect of the mutation. Telomerase will spend more time at the telomeres of POT1 mutant cells. Because telomerase interacts more frequently and spends more time at telomeres in POT1 mutant cells, this leads to continuous elongation over multiple cell cycles, accelerating in subsequent generations. This will ultimately lead to tumor formation, with accelerating growth with each cell division. These potential outcomes would be analyzed using the CoPixie algorithm to quantify changes in telomerase-telomere interactions over extended time periods and multiple cell cycles. The results would provide insights into the long-term effects of cancer-associated POT1 mutations on telomere maintenance mechanisms, potentially revealing how these mutations contribute to cancer progression over time.

Experiment 2:

Cell Culture:

I followed the same methods in Prince et al. (2024a, 2024b) using HeLa hTR^5'MS2 cells. Express mCherry-TRF1 to detect telomere foci & mCherry-CDT1 for filming and particle tracking. Cells are then cultured in DMEM supplemented with 10% fetal bovine serum, two mM L-glutamine, and 100 U/ml penicillin-streptomycin, and myc-tagged POT1 mutants are induced by lentiviral vector infection. However, I will also induce lentiviral particles overexpressing hTERT-WT and hTERT-K78E, the mutant variant of the telomerase catalytic subunit hTERT, which is unable to interact with TPP1, and expression did not affect POT1 mutants' ability to enhance telomerase retention at telomeres (Laprade et al., 2020). The POT1 wild-type-expressing cells will act as a control, as will empty lentiviral vectors. There will also be hTERT-WT overexpressing cells without any POT1 mutations to isolate the effects of hTERT. Cells overexpressing the hTERT-K78E without POT1 mutations were used to isolate the effects of this specific hTERT mutation.

Dynamics of TPP1 in relation to cancer-associated POT1 mutations:

The same methods of live-cell imaging of hTR as described in the previous experiment, as described by Prince et al. (2024a, 2024b). However, the addition of overexpressing hTERT-WT and hTERT-K78E will allow me to assess telomerase-TPP1 interactions as in Laprade et al. (2020). I will compare the interaction between TPP1 and telomerase in cells expressing POT1 mutations with that in POT1-WT cells to determine whether POT1 mutations affect the dynamics of TPP1 and telomerase.

Predicted Outcome:

I believe that cancer-associated POT1 mutations will have both direct and indirect effects on TPP1 and telomere maintenance. With TPP1 acting as a bridge between POT1 and the rest of the shelterin complex, I believe a mutated version will amplify its influence. I believe that the mutated POT1 will indirectly cause increased telomerase retention and recruitment to telomeres via TPP1. I should see increased telomerase dwell time at TPP1 and increased telomerase abundance. Alternatively, it is also possible that mutated POT1 variants will no longer bind or interact with TPP1, disrupting the shelterin complex as a whole and potentially causing cancer.

Limitations

If undergoing multiple cell cycles, the cells may die or enter cellular senescence before they can enter new cycles with cancer-associated mutations. Especially telomere-altering mutations. There is also the possibility that additional mutations accumulate in cells, which could influence telomere maintenance, complicating the interpretation of results from POT1 mutations. These factors could introduce variability in long-term experiments, making it challenging to isolate the effects of POT1 mutations from other cellular changes over time.

While CoPixie is an advanced algorithm, the resolution of single-molecule tracking may still be limited, especially over extended time periods. Long-term imaging could lead to photobleaching or phototoxicity, potentially affecting cellular behavior or protein dynamics. These limitations could affect the accuracy of quantifying telomere-telomerase interactions, especially in long-term studies, potentially leading to underestimation or misinterpretation of subtle changes in these interactions.

Another limitation is that I am using only HeLa cells, which are the most common in research but may not fully represent the diversity of cancer cells; exploring other cell lines may be helpful. I am also using specific POT1 mutations, for which the effects may not be generalized to all POT1 mutations. The cells were cultured in vitro, which may not fully replicate the complex interactions and signaling that occur within a three-dimensional tissue environment, potentially limiting the translational value of the findings to clinical applications. Using controls that overexpress specific proteins, such as hTERT, may lead to protein levels that do not reflect natural cellular conditions, potentially altering the dynamics of telomere maintenance complexes. Protein tags, while necessary for visualization, may interfere with normal protein function or interactions. These factors could lead to observations that do not accurately represent the natural behavior of telomere maintenance proteins, potentially skewing the interpretation of how POT1 mutations affect telomere dynamics.

Conclusion

In conclusion, this research proposal aims to elucidate the intricate dynamics of telomere maintenance mechanisms in cancer-associated mutations, with a specific focus on the interactions between telomerase, POT1, and TPP1. By leveraging the advanced capabilities of the CoPixie algorithm for high-resolution single-molecule imaging analysis, we seek to address two critical gaps in our current understanding. First, the long-term effects of cancer-associated POT1 mutations on telomerase-telomere interactions across multiple cell cycles. Secondly, the indirect effects of these mutations on TPP1 function and its interaction with telomerase.

Our proposed experiments will provide unprecedented insights into the molecular mechanisms underlying telomere dysfunction in cancer. By extending the observation period and analyzing interactions

across multiple cell generations, we expect to uncover the cumulative effects of POT1 mutations, which may become more pronounced over time. This approach could reveal how these mutations contribute to the progressive genomic instability characteristic of cancer cells.

Furthermore, by examining the dynamics of TPP1 in relation to POT1 mutations and telomerase activity, we aim to unravel the complex interplay within the shelterin complex. This investigation may shed light on how disruptions in a single component of the telomere maintenance machinery can have far-reaching consequences for overall telomere homeostasis.

The results of this study have the potential to advance our understanding of telomere biology in cancer significantly. By elucidating the mechanisms by which cancer-associated mutations disrupt telomere maintenance, we may identify novel therapeutic targets. Ultimately, this research could contribute to the development of more effective strategies for cancer treatment and prevention, particularly for cancers associated with telomere dysfunction.

Use of AI

Throughout the grant proposal writing process, I utilized various AI tools to enhance my research and streamline my work. One significant tool was Undermind AI, which I used to conduct an in-depth search of research articles on single-molecule imaging techniques. This tool's iterative search capability mimics a human researcher's process, enabling a comprehensive "deep search" across titles, abstracts, and full texts when available. As a result, I uncovered a more thorough and relevant set of research articles compared to traditional search methods, potentially identifying 30-80% more relevant papers on my topic.

In addition to the literature review, I also used PerplexityAI to help interpret the results of various research articles. This AI-powered tool provided clear answers with clickable citations for easy verification, enabling me to ask follow-up questions for deeper understanding. By leveraging PerplexityAI, I gained a comprehensive grasp of complex research findings, which informed my experimental design and sparked ideas for future experiments.

The combination of these AI tools not only facilitated my understanding of existing literature but also played a crucial role in generating innovative ideas for future experiments. By synthesizing information from multiple sources, AI helped me identify gaps in current research that my proposal could address, ultimately increasing the relevance and potential impact of my proposed experiments.

References:

1. Amir, M., Khan, P., Queen, A., Dohare, R., Alajmi, M. F., Hussain, A., Islam, A., Ahmad, F., & Hassan, Md. I. (2020). Structural features of nucleoprotein CST/Shelterin complex involved in telomere maintenance and its association with disease mutations. Cells, 9(2), 359. https://doi.org/10.3390/cells9020359

This review article provides a comprehensive overview of the structural features and functions of the shelterin and CST complexes, which are critical regulators of telomere structure and maintenance. The authors discuss the key components of each complex, their domain organization, and how they interact to protect telomeres and regulate telomere length. Particular emphasis is placed on the role of POT1, a core subunit of the shelterin complex, and its interaction with TPP1. Disruption of these protein-protein interactions, often due to disease-associated mutations, can lead to telomere dysfunction and the development of various diseases, including cancer.

2. Chan, S. R., & Blackburn, E. H. (2004). Telomeres and telomerase. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences, 359(1441), 109–122. https://doi.org/10.1098/rstb.2003.1370

This review article provides a comprehensive overview of telomere structure and function, as well as the telomerase enzyme. The authors discuss how telomerase solves the "end-replication problem" by using an RNA template to synthesize telomeric DNA and maintain chromosome ends. They also describe how telomeres and associated protein complexes, such as shelterin, protect chromosome ends from being recognized as DNA damage. The authors highlight how mutations in the telomerase RNA template can disrupt telomere function and lead to cellular senescence. Additionally, they discuss the protective role of telomerase in preventing catastrophic telomere shortening and fusion events, even in the absence of telomere lengthening. This suggests telomerase has functions beyond just maintaining telomere length. The review emphasizes the complexity of telomere regulation and the limitations of simply using telomere length as a readout of cellular proliferative potential.

3. Cifuentes-Rojas, C., & Shippen, D. E. (2012). Telomerase regulation. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 730(1–2), 20–27. https://doi.org/10.1016/j.mrfmmm.2011.10.003

This review article provides a comprehensive overview of the regulatory mechanisms governing telomerase activity. The authors discuss the multiple layers of telomerase regulation, including transcriptional control of the TERT catalytic subunit, post-translational modifications of TERT, and regulation of the telomerase RNA component TR. They highlight how alterations in telomerase subunit gene dosage and alternative splicing isoforms can also impact enzyme activity. Additionally, the authors describe how telomerase recruitment to telomeres and its processivity at the chromosome terminus are tightly controlled, involving interactions with telomere-associated proteins and the long non-coding RNA TERRA. The review emphasizes the sophisticated networks governing telomerase function and the critical importance of these regulatory mechanisms for maintaining telomere homeostasis and preventing human diseases associated with telomerase dysfunction.

4. Jafri, M. A., Ansari, S. A., Alqahtani, M. H., & Shay, J. W. (2016). Roles of telomeres and telomerase in cancer, and advances in telomerase-targeted therapies. Genome Medicine, 8(1). https://doi.org/10.1186/s13073-016-0324-x

This comprehensive review article provides an overview of the critical roles of telomeres and the telomerase enzyme in cancer. The authors discuss how telomere dysfunction and telomerase reactivation contribute to the initiation and progression of the majority of human cancers. Key topics covered include: the organization and function of telomeres and the shelterin complex; the mechanisms of telomerase assembly, trafficking, and recruitment to telomeres; the significance of recurrent TERT promoter mutations in activating telomerase in cancer; and the development of various telomerase-targeted therapeutic strategies, including small molecule inhibitors, immunotherapies, and nucleoside analogs.

5. Laprade, H., Querido, E., Smith, M. J., Guérit, D., Crimmins, H., Conomos, D., Pourret, E., Chartrand, P., & Sfeir, A. (2020). Single-molecule imaging of telomerase RNA reveals a recruitment-retention model for telomere elongation. Molecular Cell,79(1). https://doi.org/10.1016/j.molcel.2020.05.005

This article uses advanced live-cell imaging techniques, including single-molecule tracking of the telomerase RNA component hTR, to provide unprecedented insights into telomerase dynamics in human cancer cells. The authors developed an MS2-tagging approach to visualize single hTR particles and track their movement through the nucleus, Cajal bodies, and at telomeres. The key findings include: 1st, hTERT controls the exit of hTR from Cajal bodies, where telomerase assembly and maturation occur. 2nd telomerase recruitment to telomeres involves a two-step "recruitment-retention" model. TPP1-mediated recruitment leads to short, highly diffusive telomere-telomerase interactions. Subsequent retention at telomeres is dependent on base pairing between the hTR template region and the telomeric single-stranded DNA overhang. 3rd the DNA damage response kinases ATM and ATR regulate the recruitment, but not retention, of telomerase at telomeres. Finally, cancer-associated POT1 mutations that disrupt the OB-fold DNA-binding domain enhance telomerase retention, providing a mechanistic explanation for how these mutations lead to abnormally long telomeres. Overall, this study provides unprecedented mechanistic insights into the spatiotemporal regulation of telomerase trafficking and its engagement with telomeres, which is critical for cancer cell immortalization. The single-molecule imaging approach provides a powerful tool for dissecting the multi-

step process of telomere elongation.

6. Prince, S., Maguemoun, K., Ferdebouh, M., Querido, E., Derumier, A., & Chartrand, P. (2024). Single-Particle Track Colocalization Using Copixie Reveals the Impact of Cancer-Associated POT1 Mutations on Telomerase-Telomere Interactions. https://doi.org/10.1101/2024.02.19.580537

This article introduces CoPixie, a new software tool for high-throughput quantification of colocalization events between single-particle tracks and other imaging objects in live cells. The authors demonstrate CoPixie's capabilities by applying it to study the impact of cancer-associated mutations in the telomeric protein POT1 on the dynamic interactions between telomerase and telomeres. Key findings: CoPixie accurately reproduced previous manual colocalization quantification between the telomerase RNA component hTR and telomeres, significantly increasing analysis throughput. Expression of cancer-associated POT1 mutants, including K90E, Y223C, and D224N, increased the percentage of telomeres that colocalized with hTR particles and the cumulative dwell time of hTR at telomeres, suggesting that these mutations enhance telomerase accessibility to telomeres. Interestingly, the POT1-K90E and POT1-Y223C mutants also increased the duration of long-lasting interactions between hTR and telomeres, unlike the POT1-ΔOB mutant. This indicates these mutations may impact an activity that limits telomerase elongation at telomeres. Overall, this work provides an important methodological advance in single-particle colocalization analysis, as well as novel insights into how cancer-associated POT1 mutations mechanistically drive telomere elongation by modulating both telomerase accessibility and retention at chromosome ends.

7. Prince, S., Maguemoun, K., Ferdebouh, M., Querido, E., Derumier, A., Tremblay, S., & Chartrand, P. (2024). Copixie, a novel algorithm for single-particle track colocalization, enables efficient quantification of telomerase dynamics at telomeres. Nucleic Acids Research, 52(16), 9417–9430. https://doi.org/10.1093/nar/gkae669

This article introduces CoPixie, a new software tool for high-throughput quantification of colocalization events between single-particle tracks and other imaging objects in live cells. The key features of CoPixie include the ability to identify colocalization events across an unlimited number of imaging channels, combining spatial and temporal information from particle trajectories. Flexibility to accommodate both diffraction-limited single particles and larger irregular objects, using either centroid-based or mask-based colocalization. Demonstration of CoPixie's robustness and accuracy in reproducing previous manual quantification of colocalization between the telomerase RNA component hTR and telomeres. The authors then applied CoPixie to study the impact of cancer-associated mutations in the telomere-binding protein POT1 on the dynamics of telomerase interactions with telomeres. Key findings include: POT1 mutants K90E, Y223C, and D224N increase telomerase binding to telomeres. Unexpectedly, the POT1-K90E and POT1-Y223C mutants also enhance the duration of long-lasting interactions between telomerase and telomeres, unlike the POT1-OB deletion mutant. The distinct effects of these POT1 mutants on both telomere accessibility and telomerase retention suggest a dual mechanism by which they promote telomere elongation in cancer cells. Overall, this work provides a valuable methodological advance in single-particle colocalization analysis and new insights into how cancer-associated POT1 mutations mechanistically drive telomere dysfunction.

8. Rice, C., Shastrula, P. K., Kossenkov, A. V., Hills, R., Baird, D. M., Showe, L. C., Doukov, T.,Janicki, S., & Skordalakes, E. (2017). Structural and functional analysis of the human POT1-TPP1 telomeric complex. Nature Communications, 8(1). https://doi.org/10.1038/ncomms14928

This article presents a structural and functional analysis of the human POT1-TPP1 telomeric complex. The authors determined the atomic structure of the interacting portion of the human telomeric POT1-TPP1 complex and investigated how naturally occurring POT1 mutations contribute to cancer development. The key findings include: POT1C consists of an OB-fold and a holiday junction resolvase domain, which make extensive interactions with TPP1, forming a tight heterodimer. Several cancer-associated POT1 mutations (P446Q, C591W, and Q623H) partially disrupt the POT1-TPP1 complex, reducing its ability to bind telomeric DNA efficiently. Partial disruption of the POT1-TPP1 complex results in longer, more fragile telomeres, contributing to genomic instability and cancer. The article provides insights into the molecular mechanisms by which dysfunction of the POT1-TPP1 complex can drive carcinogenesis. This study advances the understanding of the structural basis and functional consequences of POT1-TPP1 complex formation, as well as the role of cancer-associated mutations in telomere maintenance and genomic stability.

9. Robinson, N. J., & Schiemann, W. P. (2022). Telomerase in cancer: Function, regulation, and clinical translation. Cancers, 14(3), 808. https://doi.org/10.3390/cancers14030808

This comprehensive review explores the multifaceted roles of telomerase in cancer biology, emphasizing its dual functions in telomere maintenance and extratelomeric regulation of cellular processes. The authors detail the transcriptional and post-transcriptional mechanisms regulating telomerase components, particularly the TERT and TR proteins, and their implications for tumorigenesis. Additionally, the review highlights the extratelomeric contributions of telomerase to cancer progression through pathways like Wnt/β-catenin and NF-κB signaling. Clinical applications discussed include telomerase as a prognostic biomarker and as a potential oncology therapeutic target. This article is a valuable resource for researchers investigating telomere dynamics and therapeutic interventions in cancer.

10. Wang, F., Podell, E. R., Zaug, A. J., Yang, Y., Baciu, P., Cech, T. R., & Lei, M. (2007). The pot1–TPP1 telomere complex is a telomerase processivity factor. Nature, 445(7127), 506–510. https://doi.org/10.1038/nature05454

This study investigates the role of the POT1-TPP1 protein complex in telomere biology, providing significant insights into its dual functions. It reveals that the complex not only protects telomeres by capping chromosome ends but also enhances telomerase processivity, a novel finding contrasting its presumed inhibitory role on telomerase. The authors use structural biology and biochemical approaches to elucidate the molecular mechanisms by which POT1-TPP1 stabilizes single-stranded DNA and promotes efficient telomerase-mediated telomere elongation. This research advances our understanding of telomere maintenance and its implications for cancer biology, as telomerase activation is a hallmark of cancer. The article serves as a foundational reference for those studying telomere dynamics and telomerase-related therapeutic strategies.

11. Wyatt, H. D., West, S. C., & Beattie, T. L. (2010). InTERTpreting telomerase structure and function. Nucleic Acids Research, 38(17), 5609–5622. https://doi.org/10.1093/nar/gkq370

This comprehensive review article provides an in-depth analysis of telomerase structure and function, with a particular focus on the telomerase reverse transcriptase (TERT) subunit. The authors discuss: The importance of telomeres and telomerase in genome stability and human diseases. The composition of the telomerase holoenzyme includes the TERT and TR (telomerase RNA) subunits, as well as species-specific accessory proteins. The structural organization of TERT includes its N-terminal extension, central catalytic RT domain, and C-terminal extension. Recent structural studies on TERT, including the controversial crystal structure of Tribolium castaneum TERT, and the evolutionary conservation and variation of telomere-associated proteins across different species. The article provides valuable insights into current understanding of telomerase biology. It highlights areas for future research, making it a valuable resource for researchers in molecular biology, genetics, and cancer research.

KITLG Gene: *Why Am I Blonde?*

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Hair color differences are a clear example of phenotypic variation in humans. While many factors impact hair color, the human gene KITLG is associated with blonde hair. KITLG encodes a KIT ligand, a growth factor, for the KIT tyrosine kinase receptor. The KIT ligand binds to melanocytes, which play a fundamental role in pigmentation by producing melanin, a pigment that gives skin, eyes, and hair their color. Previous research has identified an upstream single-nucleotide polymorphism, a nucleotide A-to-G substitution, as associated with blonde hair using transgenic mice and comparing fur color between ANC-Kitl/+ (ancestral A allele) and BLD-Kitl/+ (blonde-associated G allele), which displayed lighter fur. However, the polygenic character of blonde hair is poorly understood. Therefore, the proposed experiment will investigate how multiple, blonde-associated genes interact, specifically KITLG and SLC24A4, under four experimental conditions: BLD-Kitl/+; BLD-Slc24a4/+; BLD-Kitl/+, BLD-Slc24a4/+; and +/+ (wild-type control).

THE PHENOTYPE

Why am I blonde? Hair color differences are a clear example of phenotypic variation in humans (Guenther et al., 2014). My family is an example of this variation: I am the only blonde, while my parents and siblings have dark hair. Growing up, I questioned this difference and was accused of dying my hair because of it. The familial difference inspired me to research blonde hair, searching for genetic factors that contribute to this variation. Research studies have demonstrated the association between the human gene *KITLG* and blonde hair.

Beyond my personal curiosity, blonde hair plays a culturally significant role worldwide. Contemporary popular culture has stereotyped blonde women as more attractive and having more fun, suggesting that blonde women, specifically, are happier and more popular (HandWiki, 2022; Dechter, 2015). This stereotype extends to ancient Greece, and ancient texts suggest that blonde hair was associated with youth and beauty. In both modern and ancient cultures, blonde hair has been imitated using bleaches, dyes, and wigs (Guenther et al., 2014). However, a 2011 study in Russia found that brunettes are considered more attractive, and a study in Brazil found that blonde women are looked down upon (Dechter, 2015). Therefore, geographical location contributes to how hair colors, specifically blonde hair, are perceived. In some cultures, blonde hair is associated with negative stereotypes: ghost-like abnormality, promiscuity, or unusual ancestry (Guenther et al., 2014). Furthermore, “blonde moment,” “dumb blonde,” and the “blonde myth” are stereotypes that portray blonde-haired women as attractive but scatterbrained and unintelligent (Dechter, 2015). Women face stereotypes due to their hair color, demonstrating societal pressure and expectations based on outward appearance. Therefore, the human gene *KITLG*, which is associated with blonde hair, plays a role for my family as well as women across the globe.

Fundamentally, hair color is determined by the amount of melanin, a type of pigment, in the hair. There are two types of melanin: eumelanin, associated with black and brown hair, and pheomelanin, associated with blonde and red hair. The type and amount of melanin in hair are determined by many genes (U.S. National Library of Medicine, n.d.). Melanin pigment is produced by melanocytes, specialized cells found in skin, eyes, hair, and other tissues that synthesize melanin. *KITLG* plays an essential role in the development, migration, and differentiation of many cell types, including melanocytes (Guenther et al., 2014). Specifically, *KITLG* encodes the *KIT* ligand, a crucial growth factor that binds to the *KIT* receptor tyrosine kinase located on melanocytes, the cells responsible for producing melanin (Guenther et al., 2014). Due to *KITLG’s* crucial role in melanocyte production and, consequently, melanin synthesis, it is associated with blonde hair color.

Not only does *KITLG* play a role in skin, eye, and hair pigmentation (melanogenesis), but it also has molecular roles in blood cell production (hematopoiesis) and gamete production (gametogenesis). (Allen et al., 2014; Guenther et al., 2014). Focusing specifically on melanogenesis, two associated disorders include Familial Progressive Hyper- and Hypopigmentation and Waardenburg syndrome type 2F.

Familial Progressive Hyper- and Hypopigmentation (FPHH) is a pigmentary disorder characterized by a mix of dark (hyperpigmentation) and light (hypopigmentation) spots on the skin (Wang et al., 2009; Xiao-Kai et al., 2017). FPHH is an autosomal dominant disorder associated with a heterozygous mutation in *KITLG*; therefore, only one copy of the gene is required to express the phenotype, meaning that only one parent needs to carry the gene. The skin spots are typically present at birth or develop during infancy but progress with age, with hyperpigmented patches increasing in size and number (Weizmann Institute of Science, n.d.-a). Research demonstrates that a gain-of-function mutation increases the melanin content by 109% compared to the wild-type *KIT* ligand. Mutations in *KITLG* can disrupt normal signaling pathways involved in melanocyte regulation, potentially leading to both overproduction of melanin in some areas (hyperpigmentation) and deficiency in others (hypopigmentation) (Johns Hopkins University, n.d.).

Waardenburg syndrome type 2F (WS2F) is an auditory-pigmentary disorder characterized by sensorineural hearing loss, hypopigmentation of the skin and hair, and heterochromia iridis. Sensorineural hearing loss refers to hearing loss of the inner ear or the auditory nerve, which connects the inner ear to the brain. WS2F is an autosomal recessive disorder associated with a homozygous mutation in *KITLG*; therefore, two copies of the gene are required to express the phenotype, meaning both parents must carry the gene. Symptoms present with congenital or neonatal onset (Weizmann Institute of Science, n.d.-b). Sensorineural hearing loss is often caused by damage to the hair cells in the inner ear, which convert vibrations into electrical signals that the brain can interpret. In addition to its role in melanogenesis, *KITLG* also regulates neural crest migration, during which embryonic cells derived from the neural tube move from their original location to various regions of the embryo to form diverse tissues (Vona et al., 2022). Therefore, mutations in *KITLG* can disrupt ear and hair formation, cause hypopigmentation of skin and hair, and result in heterochromatic eyes due to its role in melanocyte regulation.

Previous research has established an association between *KITLG* and blonde hair color, particularly in Northern European populations (Guenther et al., 2014). However, the interplay between *KITLG* and other genetic factors is poorly understood. Future experiments should address this limitation by examining how multiple genes influence blonde hair and its shades.

MOLECULAR FUNCTION OF THE GENE PRODUCT(S) AND MOUSE MODEL

The human gene *KITLG*, located on chromosome 12, encodes the *KIT* ligand, which binds the *KIT* tyrosine kinase receptor. The *KIT* ligand acts as a growth factor and binds to *KIT* receptors on cell surfaces, initiating signaling pathways involved in development and function (Guenther et al., 2014; Hoekstra, 2014).

The *KIT* ligand has been associated with melanocyte development, survival, proliferation, and migration, indicating its crucial role in pigmentation. As a growth factor expressed by various cell types, including those in the hair follicle, the *KIT* ligand interacts with the *KIT* receptor, which is primarily expressed on the surface of melanocytes (Hu et al., 2022). Melanocytes play a fundamental role in pigmentation by producing melanin. At the molecular level, ligand-receptor binding

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between the *KIT* ligand and receptor initiates intracellular signaling cascades that involve the phosphorylation of tyrosine residues on the *KIT* receptor and downstream effector molecules (Yarden & Ullrich, 1988). The signaling pathways regulate cellular processes in melanocytes, such as their differentiation and melanin synthesis, and are essential for maintaining a normal population of melanocytes and, consequently, pigmentation (D'Mello et al., 2016). Therefore, dysregulation of this signaling, often due to mutations in *KITLG* or *KIT*, can disrupt melanocyte homeostasis and lead to pigmentary disorders like Familial Progressive Hyper- and Hypopigmentation and Waardenburg syndrome type 2F.

KITLG, the human gene that encodes the *KIT* ligand, plays multiple roles in hematopoiesis (blood cell production), melanogenesis (melanocyte production), and gametogenesis (gamete production) (Allen et al., 2014; Guenther et al., 2014). Melanin plays a fundamental role in hair color and is produced by melanocytes; *KITLG* has a molecular role in this process (Hu et al., 2022). Specifically, *KITLG* is associated with blonde hair color due to a non-coding single-nucleotide polymorphism (SNP) (rs12821256), which substitutes nucleotide A to G, located over 350 kb upstream of the *KITLG* transcription start site, and is associated with blonde hair color (Guenther et al., 2014). The SNP affects *KITLG* expression in hair follicles, leading to reduced pigment production without altering expression in the rest of the body (Conger, 2014).

A transgenic mouse model was used to examine a regulatory region of *KITLG* that encodes the *KIT* ligand and is associated with blonde hair color in Northern Europeans. Specifically, the region contains a nucleotide A-to-G SNP (rs12821256) 350 kb upstream of the transcription start site. The results demonstrated that the blonde-associated *KITLG SNP, BLD-Kitl/+*, resulted in significantly lighter hair pigmentation than in control mice. Thus, a single-base change in the *KITLG* regulatory sequence is sufficient to significantly alter the activity of the functional hair follicle enhancer (Guenther et al., 2014).

To explore the functional impact of the regulatory variant, Guenter et al. (2014) used the Steel panda mutation (Slpan), an X-ray-induced Kitl (mouse gene homologous to human *KITLG*) allele caused by an upstream chromosome inversion, which reduces pigmentation. While mice homozygous for the allele were completely white, heterozygous mice had noticeably lighter hair color than the control mice, indicating that a single copy of the upstream displacement is sufficient to lighten hair color by reducing *Kitl* expression.

To determine the specific base-pair changes associated with blonde hair, three segments of human DNA spanning the 17.1 kb blonde-associated regions, as determined by a previous genome-wide association study, were separately cloned upstream of a minimal promoter and *lacZ* reporter gene: H1, H2, and H3. Only H2 drove consistent reporter expression in transgenic mouse embryos; thus, two subclones, H2b (kidney) and HFE (hair follicle enhancer), were examined. HFE drove consistent expression in developing hair follicles, and histological analysis confirmed that expression corresponded to a site of endogenous *Kitl* expression in the epithelial cells of developing hair and skin. Thus, the site of *Kitlg* expression attracts melanocytes to the developing epidermis and hair follicles.

Guenther et al. (2014) demonstrated the genetic basis of blonde hair color by examining a regulatory variant upstream of the *KITLG* transcription start site. Due to the lighter fur resulting from the heterozygous Slpan mutation, an upstream chromosome inversion, the study indicated that a single copy of the upstream displacement is sufficient to lighten hair color by reducing *Kitl* expression. Furthermore, using a *lacZ* reporter gene, the HFE region was determined to drive constant expression in hair growth follicles at a site of endogenous *Kitl* expression.

One specific aspect of the mouse model was the in vivo investigation of the effects of the rs12821256 SNP, a nucleotide A-to-G substitution. Guenther et al. (2014) generated matched lines of transgenic mice that expressed *Kitl* cDNA of either the ancestral (A; ANC-Kitl) or blonde-associated (G; BLD-*Kitl*) hair enhancer. To minimize differences due to transgene copy number, orientation, or integration site, the ϕC31 integrase system was used to generate single-copy integrants at the H11P3 locus on mouse chromosome 11. To prepare the mouse embryos, the HE-Kitl site-specific insertion plasmids were individually mixed with ϕC31 RNA and injected into the pronuclei of H11P3 mouse (FVB) embryos. Integration occurred at the same position in both transgenic lines, indicating that the phenotypic differences are due to the base pair present: A for the ancestral hair enhancer or G for the blonde-associated hair enhancer. Eight days postnatal, dorsal skin samples were analyzed using quantitative RT-PCR, and the *Kitl* mRNA expression revealed that the BLD-*Kitlg* enhancer drove a 21% lower expression of *Kitl* compared to the ANC-*Kitl* enhancer. Furthermore, the coats of the BLD-*Kitl*/+ mice appeared significantly lighter than the coats of the ANC-*Kitl*/+ mice and had lower pigmentation density in hair shafts. Thus, a single-base change in the *KITLG* regulatory sequence is sufficient to significantly alter the activity of a functional hair follicle enhancer.

Therefore, previous research has established a significant association between *KITLG* and blonde hair color, particularly in Northern European populations (Guenther et al., 2014). However, the polygenic character of blonde hair is poorly understood. Therefore, the proposed future experiment will investigate how multiple, blonde-associated genes interact, specifically focusing on *KITLG* and *SLC24A4*.

EXPERIMENT FOR THE FUTURE

Specific Aims

Previous research has established a significant association between rs12821256, a non-coding single-nucleotide polymorphism (SNP) that substitutes nucleotide A for G over 350 kb upstream of the *KITLG* transcription start site, and blonde hair color, particularly in Northern European populations (Guenther et al., 2014). However, the polygenic nature of blonde hair color and the interplay of additional regulatory elements remain poorly understood. To address this gap, the research study aims to investigate the polygenic contributions of *KITLG* and *SLC24A4* to the blonde hair color phenotype. The association of rs12896399, a SNP in the region that contains the first exons of *SLC24A4*, has recently been implicated in blonde hair (Han et al., 2008; Sulem et al., 2007). Specifically, the research study examines how variations in both genes, represented by the blonde-associated *KITLG* enhancer SNP and specific *SLC24A4* alleles, interact to influence hair pigmentation. The hypothesis of the research study is that mouse models carrying the blonde-associated alleles of both *KITLG* and *SLC24A4* will exhibit more pronounced blonde hair color than those with only one or neither of the alleles, indicating a polygenic effect on blonde hair color. To examine the hypothesis, hair pigmentation characteristics across four experimental groups will be compared: BLD-*Kitl*/+, BLD-*Slc24a4*/+, BLD-*Kitl*/+, BLD-*Slc24a4*/+, and +/+ (wild-type control).

Experimental Protocol

The experiment relies on transgenic mice generated using the protocol described by Guenther et al. (2014). The wild-type control mice are FVB/C57Bl/6J F1 hybrids that have not undergone genetic modification, as described by Guenther et al. (2014).

First, to begin the transgenic process, the allele lines are established. Both the *KITLG* and *SLC24A4* insertions are performed using the following protocol, adapted from Guenther et al. (2014), in which *Kitl* and *Slc24a4* are the mouse genes for *KITLG* and *SLC24A4*, respectively. First, the HE-*Kitl* site-specific insertion plasmid, containing the blonde-hair rs12821256 SNP, will be mixed with ϕC31 RNA and injected into the pronuclei of H11P3 FVB embryos. These are embryos from the FVB-H11P3 mouse strain, which align with wild-type control mice and provide a well-characterized genetic background that allows site-specific integration at the attP sites at the H11 locus. Therefore, this method allows for insertion at the H11P3 locus.

Following the insertion, genomic DNA from the ancestor and offspring mice will be analyzed with primer pairs PR387/PR425, PR522/Kg1576, and

Kg1580/Kg1581 to screen for site-specific and random integrations. HE-*Kitl* mice with confirmed site-specific insertions will be bred with wild-type controls to establish the BLD-*Kitl*/+ line. The process was then repeated using the HE-*Scl24a4* site-specific insertion plasmid (rs12896399) and *Scl24a4*-specific primers. HE-*Scl24a4* mice with confirmed site-specific insertions will be bred with wild-type controls to establish the BLD-*Scl24a4*/+ line. Lastly, HE-*Kitl* and HE-*Scl24a4* mice, with confirmed site-specific insertions, will be bred to establish the BLD-*Kitl/Slc24a4* line.

Breeding between the genetically modified mouse lines and wild-type control mice will lead to experimental groups. The BLD-*Kitl*/+ is heterozygous for the *Kitl* blonde allele. Therefore, they are the offspring of a homozygous wild-type control, +/+, and a homozygous *Kitl* blonde allele, BLD-*Kitl/Kitl*. Similarly, the BLD-*Slc24a4*/+ is heterozygous for the *Slc24a4* blonde allele, BLD-*Slc24a4/Slc24a4*. Therefore, they are the offspring of a homozygous wild-type control, +/+, and homozygous *Slc24a4* blonde allele, BLD-*Slc24a4/Slc24a4*. Lastly, the BLD-*Kitl*/+, BLD-*Slc24a4*/+ are heterozygous for the *Kitl* blonde allele and *Slc24a4* blonde allele. Therefore, they are the offspring of a homozygous *Kitl* blonde allele, BLD-*Kitl/Kitl*, and a homozygous *Slc24a4* blonde allele, BLD-*Slc24a4*/*Slc24a4*. These breeding pairs constitute the three experimental groups, which will be compared to the wild-type control FVB/C57Bl/6J F1 hybrids.

Phenotypic analysis will be performed by assessing the coat color by three methods: visual assessment, spectrophotometry, and histological analysis. First, the coat color of the mice in each group will be visually assessed at different developmental stages: postnatal at 3 weeks, adolescence at 2 months, and adulthood at 6 months. Representative mice exhibiting the hair-color phenotypes associated with the respective genes will be analyzed. Once visually assessed, the hair pigmentation will be quantified. Specifically, using reflective spectrophotometry, a technique that measures the amount of light reflected by a sample, the pigmentation of shaved fur will be quantified (Vaughn et al., 2009). Samples will be taken from each experimental group and from different body locations (head, ventral, and dorsal). Reflective spectrophotometry will provide quantitative data on lightness and darkness, and potentially on pheomelanin (light melanin) and eumelanin (dark melanin). Lastly, histological analysis will examine hair follicle morphology and melanin distribution (pheomelanin and eumelanin) in skin biopsies from each group. Tissue samples measuring 4 to 10 µm will be fixed, dehydrated, embedded, and sectioned over 2 days (Meng et al., 2022). This process will reveal potential differences in melanocyte number, size, and melanosome number, size, and pigment production.

Statistical analysis will determine whether the differences in hair pigmentation are statistically significant. Since there are four groups, ANOVA, which tests differences in the means of two or more groups, will be used. The coat color measurements will be compared using ANOVA for each method: visual scores, reflective spectrophotometry, and histology. Furthermore, histological data will be analyzed to identify any qualitative or quantitative differences in melanocyte characteristics. This will be conducted via visual scores. To verify visual accuracy, analysis will be conducted using machine learning techniques that process image data and pixel patterns to identify color (Komura et al., 2025).

The research study will provide insight into the interaction between *Kitl* and *Slc24a4* in influencing mouse coat color. The findings will promote a better understanding of the polygenic basis of blonde hair color, providing a model for human pigmentation.

Controls

The control for the research study is the wild-type FVB/C57BL6/J F1 hybrids, +/+. The control group possesses the wild-type alleles for both the *Kitl* and *Slc24a4* genes. The purpose of the wild-type control group is to establish the baseline coat color phenotype in the absence of the blonde-associated alleles, *Kitl* and *Slc24a4* genes. This will allow direct comparison with the experimental groups using the *Kitl*, *Slc42a4*, and both the *Kitl* and *Slc24a4* genes. Since the wild-type control group provides a baseline coat color, any deviations observed can be attributed to the presence and potential interaction of the gene variants.

Predicted Outcomes

It is expected that the wild-type control group (+/+) will exhibit the baseline coat color of FVB/C57BL6/J F1 hybrids. As demonstrated by Guenther et al. (2014), mice with the *Kitl* blonde allele, BLD-*Kitl*/+, exhibit a lighter coat color than wild-type control mice. Furthermore, it would be expected that the mice with the *Slc24a4* light-hair allele, BLD-*Slc24a4*/+, will also exhibit a lighter coat color compared to the wild-type control mice. These results would support previous research implicating *KITLG* and *SLC24A4* in blonde hair color.

However, the goal of the research study is to examine the polygenic influences on blonde hair color, focusing on the interplay between *KITLG* and *SLC24A4*. It is expected that mice with the *Kitl* and *Slc24a4* alleles will exhibit lighter hair color than those with either gene alone. If a lighter coat color is observed when both *Kitl* and *Slc24a4* alleles are present, it can be concluded that the alleles exhibit an additive effect in support of polygenic influence on blonde hair color, promoting lighter hair than a single-gene modification alone. However, if no difference is observed between the experimental group with both *Kitl* and *Slc24a4* genes and the groups with either *Kitl* or *Slc24a4* alone, then it can be concluded that there is no polygenic effect, and further research can be conducted to determine the most influential alleles.

Considerations

Regarding phenotypic variability, *KITLG* and *SLC24A4* are not comprehensive of the genes implicated in blonde hair color. Therefore, further research should be conducted, using a scientific basis from genome-wide association studies (GWAS) to examine other genes implicated. By generating and analyzing additional heterozygous combinations, the interaction could be examined more fully. Furthermore, epistatic interactions, where one gene masks the effect of another, should be considered. In addition, environmental factors may play a role in blonde hair color, which is not considered in the research study. Since the environmental factors were kept constant in the research study, a similar study could be conducted with various environmental factors, such as diet and housing conditions.

Lastly, homology to human blond alleles is a challenge of the research study. While robust research has established homology between *KITLG* and *Kitl*, less is known about *SLC24A4* and *Slc24a4*. However, when conducting studies in mouse models with potential implications for humans, it is important to ensure that the regions have the same functional impact in both mice and humans. Therefore, targeting conserved regulatory and coding regions between human and mouse genomes should be prioritized. In addition, in vitro functional assays could be conducted to assess the potential impact on gene expression.

CONCLUSION

KITLG and its gene product, *KIT* ligand, are associated with blonde hair color due to the *KIT* ligand's role in melanocyte development, the specialized cells that produce melanin, the pigment that gives color to skin, eyes, and hair. Previous research has demonstrated, using transgenic mice, that a nucleotide A-to-G SNP (rs12821256) 350 kb upstream of the *KITLG* transcription start site is associated with blonde hair color. However, there is a limited understanding of the polygenic character of blonde hair color.

The proposed future experiment examines the polygenic character of blonde hair, analyzing the relationships and, potentially, the compounding, inhibitory, or unchanged effects of *KITLG* and *SLC24A4*. To determine if the presence of both genes results in lighter fur color, the proposed experiment uses four experimental groups: BLD-*Kitl*/+; BLD-*Slc24a4*/+; BLD-*Kitl*/+, BLD-*Slc24a4*/+; and +/+ (wild-type control). While the experiment may not fully capture the complexity of polygenic inheritance, it provides an initial step toward understanding the interactions

among multiple genes contributing to hair color, an essential step toward moving beyond single-gene explanations. Polygenic effects are crucial for understanding how common traits are inherited when multiple genes are involved, and the proposed experiment can be extended to other polygenic traits and complex traits that involve environmental influences as well.

Artificial Intelligence Use Statement

I did not use artificial intelligence to augment my writing or my paper. Using the class discussion, both in general and specifically related to the paper "A molecular basis for classic blond hair color in Europeans" by Guenther et al. (2014), I became familiar with the paper and had an idea for a future experiment: a polygenic character. I appreciate Dr. Karen Kirk's assistance in better understanding the complex protocols presented in the primary literature.

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Investigations of Alzheimer's Disease: *Mechanisms of Assessing APOE 3 and 4*

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USE OF AI

AI, specifically ChatGPT and Perplexity, was used while writing this paper to assist me in the writing process. I used AI to generate initial ideas for the experimental design for future experiments, to help me organize the paper's structure according to the guidelines, to identify relevant scientific articles to use as references, and to proofread my paper for grammar mistakes and clarity.

Alzheimer's disease (AD) is a devastating neurodegenerative disease that affects millions worldwide, with no cure and limited treatment options. One of the most significant genetic risk factors for AD is the apolipoprotein E ε4 allele (APOE4). While we know that APOE4 increases risk for disease, the mechanisms by which it promotes neurodegeneration still remain unclear. Previous studies suggest that APOE4 can disrupt lipid metabolism, lead to synapse loss, and trigger inflammation in glial cells in the brain. These effects are often studied alongside other AD-related mutations, making it hard to determine which are caused directly by APOE4. The aim of this proposal is to isolate the effects of APOE4 by comparing cognitive and molecular changes in humanized APOE3 and APOE4 knock-in mice. Behavioral testing and gene expression analysis will be used to identify early-stage AD effects caused solely by APOE4. Understanding these mechanisms could offer valuable insight into AD progression and potentially help develop new strategies for early intervention in individuals who carry the APOE4 allele.

THE PHENOTYPE

Try to imagine losing track of a conversation, forgetting the faces and names of family and friends, or walking into your kitchen only to wonder why you're there. For many individuals with AD, these moments of forgetfulness are only the beginning of the disease. What might seem like ordinary brain fog can quickly evolve into the loss of independence and identity. AD is a progressive and degenerative brain disorder that primarily affects memory, thinking, and behavior. Over time, it destroys a person's ability to communicate, function, and recognize loved ones. In its final stages, AD often leaves individuals completely dependent on caregivers, as they are unable to carry out even basic tasks like dressing, eating, or speaking. For their families, the emotional burden can be overwhelming, and the sense of watching your loved one slowly fade away is devastating. While most people associate AD with the natural aging process, science has revealed that genetics can play a powerful role, especially in determining who is at risk of developing the disease and when. Among the many genes linked to AD, one stands out as possibly the most influential: *APOE*.

The *APOE* gene, short for apolipoprotein E, codes for a protein that helps transport cholesterol and other fats through the bloodstream. Its function is important throughout the body, but in the brain, *APOE* has additional important roles. It helps maintain neuron health, supports the repair of damaged brain tissue, helps regulate the blood-brain barrier, and can assist in clearing waste products, such as amyloid beta, the protein that clumps together to form plaques, a hallmark of AD.

However, not all *APOE* genes are created equal. There are three main alleles: ε2, ε3, and ε4. Every individual inherits two copies of the gene, one copy from each parent. The ε3 allele (*APOE3*) is the most common and is considered "neutral" for AD risk, as it doesn't significantly increase or decrease the risk of developing the disease. The ε2 allele (*APOE2*) is relatively rare but offers some protection against developing the disease. The ε4 allele (*APOE4*), however, is associated with an increased risk of AD and earlier onset of symptoms (Liu et al., 2013).

Previous research has shown that individuals with one copy of *APOE4* are about two to three times more likely to develop AD, and those who have two copies, homozygous carriers, are up to 12 times more likely to develop AD (Kloske & Wilcock, 2020). Research has found that more than half of all people with late-onset AD carry at least one *APOE4* allele (Mahley, 2016). Even more concerning is that research has shown that carriers of *APOE4* often experience brain changes decades before symptoms begin, such as reduced brain metabolism in memory-related areas like the hippocampus and increased inflammation and oxidative stress (Liao et al., 2017).

The symptoms of AD often begin subtly. An individual might start by misplacing items, forgetting names, or repeating questions. Over time, this progresses into deeper confusion, difficulty organizing thoughts, and eventually an inability to perform everyday tasks. Their language skills often decline, and emotional regulation can become impaired. In the late stages of AD, individuals may also lose awareness of the time, where they are, and even their own identity. It is important to note that not all people with AD progress at the same rate; carriers of *APOE4* tend to decline more rapidly, with earlier onset and more noticeable cognitive and behavioral symptoms (Neu et al., 2017).

Although *APOE4* can increase the risk of developing AD, it's important to understand that carrying *APOE4* is not a guarantee of developing AD. Some people with the allele never develop the disease, and some who do develop AD do not carry *APOE4* at all. This elevated risk provides a valuable opportunity for research. If scientists can understand what makes *APOE4* so damaging to the brain, they may be able to intervene before symptoms even appear. Catching the disease before widespread neuronal loss and its hallmarks are present could dramatically improve the chances of successful treatment or even prevention.

APOE4's impact is not limited to AD. It is also linked with worse outcomes after traumatic brain injuries (TBI), increased cardiovascular disease risk, and differences in response to some infections and medications (Tai et al., 2016). On the other hand, *APOE2* is protective against AD but is linked to a rare disorder called Type III hyperlipoproteinemia. These broader connections make *APOE* not just a gene of interest for AD, but also a central player in the health of the brain and body.

Because *APOE4* can affect so many processes in the body, from cholesterol transport to inflammation and neuronal repair, it provides a good target for studying how genetic risk can lead to biological damage. In my opinion, what makes this gene particularly interesting is its dual identity. *APOE* performs essential housekeeping functions in the brain but also contributes to its breakdown in the context of disease. My proposed experiment focuses on this, aiming to understand how *APOE4* influences brain cell function and gene activity before the onset of major disease symptoms. By doing so, the hope is to uncover molecular changes that could be reversed or slowed, potentially leading to new treatment strategies.

MOLECULAR FUNCTION OF THE GENE PRODUCT(S) AND MOUSE MODEL

The *APOE* gene, located on chromosome 19, encodes apolipoprotein E, a protein that plays an important role in the body's lipid metabolism, particularly cholesterol. In the brain, this protein has additional important functions. Unlike in the rest of the body, where *APOE* is primarily produced in the liver, the brain relies on local *APOE* production, especially by glial cells such as microglia and astrocytes. These cells produce *APOE* to help transport cholesterol and lipids to neurons, which need these fats for membrane repair, synapse formation,

*This author wrote this paper for Biology 352: From Genotype to Phenotype taught by Dr. Karen Kirk.

and normal signaling (Mahley, 2016). *APOE* also assists in the removal of waste products, including amyloid beta, a sticky protein that clumps into plaques in the brains of individuals with AD (Liu et al., 2013).

There are three major forms or alleles of the *APOE* protein: *APOE2, APOE3, and APOE4*. These alleles differ by just one nucleotide at two SNPs, rs7412 and rs429358, in exon 4, leading to a change of one or two amino acids. These small changes have a profound effect on how the protein folds and functions. *APOE3*, the most common allele, is considered "neutral," while *APOE2* offers protection against AD. *APOE4*, however, behaves quite differently. Its altered structure causes it to fold less efficiently, making it more prone to aggregation and misfolding. These changes disrupt its lipid transport and impair many of its normal functions. As a result, *APOE4* is less effective at clearing amyloid beta, contributes to synaptic loss, and triggers inflammatory responses, particularly by activating microglia (Mahley, 2016; Zhao et al., 2020).

The effects of *APOE4* on Alzheimer's disease are complex, as they vary depending on the context. In studies where *APOE4* is present alongside other AD-related mutations, such as those in the *APP*, amyloid precursor protein, or tau genes, it often amplifies the severity of the disease by increasing plaque accumulation and worsening cognitive decline (Shi et al., 2017). However, to truly understand what *APOE4* does on its own, researchers have created more refined models. One of these models includes humanized knock-in mice, in which the mouse *APOE* gene is replaced with the human *APOE3* or *APOE4* allele. These mice are generated using the CRISPR/Cas9 system or homologous recombination and are designed so that the human gene is expressed under the control of the mouse's regulatory elements (Foley et al., 2022). This avoids overexpression and allows for a more accurate representation of how *APOE* behaves in a real biological setting.

It's important to note that these knock-in models do not carry additional disease-causing mutations, unlike those found in *APP* or *PSEN1* transgenic mice. This makes them ideal for studying the allele-specific effects of *APOE*. Research using these mice has revealed that *APOE4* leads to early impairment of synaptic structure and plasticity, particularly in the hippocampus, which is important for learning and memory (Knoferle et al., 2014). Even in the absence of visible amyloid beta plaques, *APOE4* mice have shown deficits in behavior and cognition, as well as signs of microglial activation and oxidative stress. These findings suggest that *APOE4* may initiate the neurodegenerative process well before the pathological hallmarks of AD are detectable.

Previous studies have also used transcriptomics to examine how individual cells, specifically iPSCs, respond to *APOE4* at the level of gene expression. For example, using single-nucleus RNA sequencing (snRNA-seq), scientists have identified distinct gene expression patterns in iPSCs carrying *APOE4* compared to those carrying *APOE3*. These studies have shown altered expression of genes involved in actin filaments, regulation of epithelial-to-mesenchymal transition, axonal guidance, the endoplasmic reticulum stress pathway, and the innate inflammatory response (Zhao et al., 2020). This suggests that *APOE4* does not merely impair brain function; it can also alter gene expression in harmful ways.

Despite previous research on *APOE*, significant knowledge gaps remain. Many past studies focus on older animals or examine *APOE4* only in combination with other AD-related mutations. This makes it difficult to know whether observed effects are directly caused by *APOE4* or by a combination of various factors. Additionally, while inflammation and synaptic loss are frequently observed in older *APOE4* mice, little is known about the early changes that may occur in younger mice. Understanding these early changes is important, as it could provide opportunities for earlier therapeutic intervention.

This is what my proposed experiment will attempt to address. By using young humanized *APOE* knock-in mice without other AD-related mutations and pairing behavioral testing with cell-type-specific gene expression analysis, I aim to identify the earliest changes *APOE4* causes in the brain. Identifying these changes before major symptoms or irreversible damage occur could provide an opportunity for slowing or completely stopping AD progression.

EXPERIMENT FOR THE FUTURE

This research aims to determine how the *APOE* ε4 allele (*APOE4*) contributes to neurodegeneration by focusing on early synaptic dysfunction and neuroinflammation. Although *APOE4* is a well-established genetic risk factor for AD, the mechanisms by which it promotes disease progression remain unclear. To address this, I propose two experiments using mice that express human *APOE3* or *APOE4* alleles: one to assess behavioral and histological changes in memory-related brain regions, and another to examine cell-type-specific gene expression. I hypothesize that *APOE4* will disrupt synaptic signaling and promote inflammation prior to clinical symptoms, thereby identifying early changes that could be targeted for intervention.

The first experiment will evaluate whether *APOE4* expression alone is sufficient to affect cognitive performance and brain function in aging mice, independent of other AD-related mutations. Most previous studies have examined *APOE4* in combination with other genes linked to AD, such as the amyloid precursor protein (APP), making it harder to observe the individual contribution of *APOE4* to the early stages of the disease (Youmans et al., 2012; Shi et al., 2017). To better understand the impact of *APOE4*, I will use the CRISPR/Cas9 system to create genetically modified mice that express either the human *APOE3* or *APOE4* allele at the mouse *APOE* locus. These mice will still maintain regulation of *APOE* expression and are free of additional AD-related mutations, making it easier to isolate the effects of *APOE* gene alleles on cognitive health.

Behavioral testing in this experiment will be done on both male and female mice at two different ages: six months and twelve months. I chose these time points to capture the earlier stages of aging and any brain changes that *APOE4* might cause. The mice will undergo several behavioral tests, including the Morris water maze to assess spatial learning and memory, the novel object recognition test to assess recognition memory, and the open field test to assess exploratory behavior and anxiety (Knoferle et al., 2014). After behavioral testing, the mice's brains will be harvested for histological analysis. Immunofluorescent staining for synaptic markers such as PSD-95 (postsynaptic density protein 95) will be performed in regions of the brain critical for learning and memory, and particularly vulnerable in AD (Liao et al., 2017). The purpose of this first experiment is to determine whether APOE4 expression alone can cause measurable cognitive and synaptic problems before other features of AD, such as amyloid plaques or tau tangles, appear.

The second experiment will use single-nucleus RNA sequencing (snRNA-seq) to examine gene expression changes across multiple brain cell types in *APOE3* and *APOE4* mice. This will allow for examining how different cell types, such as neurons, glial cells, and immune cells, are affected by the presence of *APOE4*. This approach was chosen over single-cell RNA sequencing (scRNA-seq) because brain tissue can be difficult to dissociate into single cells, and isolating cells for scRNA-seq can trigger stress responses, altering gene expression. The nuclei for sequencing will be isolated from frozen hippocampal and cortical tissue collected from six-month-old *APOE3* and *APOE4* mice. These nuclei will then be analyzed to measure gene expression that may be altered by *APOE4*.

Previous studies have shown that *APOE4* affects microglial activation, reduces the expression of synaptic plasticity-related genes, and triggers inflammation in supporting brain cells, such as astrocytes (Shi et al., 2017; Zhao et al., 2020). However, these studies often focus on later stages of AD or use mouse models that are genetically modified in multiple ways. By focusing on middle-aged mice, this experiment aims to uncover the earliest molecular changes specifically linked to *APOE4* expression, before the onset of other disease markers. The results of this experiment will be analyzed to identify genes that are up- or downregulated in *APOE4* mice, as

well as any biological processes that may be affected by *APOE4*. I expect that *APOE4* mice will show upregulation of genes involved in immune activation alongside downregulation of genes involved in neuronal plasticity.

To ensure the results from both experiments are reliable, appropriate controls will be used throughout. *APOE3* mice will serve as the main comparison group to *APOE4*, as this allele is considered neutral with respect to AD risk (Liu et al., 2013). Regular lab mice (wild-type C57BL/6 mice) will also be included in molecular analyses to provide baseline data on *APOE* function. Due to some studies observing differences in *APOE*-related pathology between sexes, particularly with *APOE4* posing a higher risk to women in some human populations (Neu et al., 2017), both male and female mice will be analyzed in equal numbers. For the snRNA-seq, controls will include removing low-quality samples, filtering out data containing more than one nucleus, and normalizing gene counts to ensure consistency across all samples.

If my hypothesis is correct, I expect that *APOE4* mice will show behavioral impairments and reduced synaptic marker expression compared with *APOE3* mice. This would suggest that *APOE4* disrupts neuronal signaling even in the absence of amyloid pathology, aligning with the idea of a "preclinical" stage of AD driven by genetic risk factors. At the molecular level, I anticipate that snRNA-seq will reveal clear differences in gene expression between *APOE3* and *APOE4* mice. In *APOE4* mice, microglia will likely show a shift toward increased inflammation, while neurons may show decreased expression of genes critical for synaptic maintenance. These findings would provide strong evidence that *APOE4* causes early changes in the brain that could later lead to AD, highlighting potential targets for early intervention.

On the other hand, if *APOE4* mice do not show any significant behavioral or molecular differences from *APOE3* mice at the two examined ages, it would suggest that *APOE4's* effects occur later in life or that environmental and genetic stressors may be required to fully trigger phenotypes associated with *APOE4*. This wouldn't mean that *APOE* isn't important, but rather underscores its role as a risk amplifier whose effects may depend on age, stress, poor diet, or other variables to fully trigger disease processes.

There are several challenges to keep in mind when designing and interpreting these experiments. First, the effects of the *APOE4* gene may emerge slowly, requiring longer-term aging studies or the introduction of stressors, such as high-fat diets or low levels of inflammation, to reveal them (Tai et al., 2016). Second, although snRNA-seq provides insight into gene expression across different brain cells, it also generates a very large amount of data. Going through all the data will require a lot of time and computer programs to aid in analysis. This can be managed by working with researchers who specialize in bioinformatics and by using methods like DESeq2 to ensure results are accurate and meaningful. Finally, it's important to note that this research project focuses on the effects of *APOE* in a simplified genetic model. In the real world, AD risk involves complex interactions between many genes and environmental factors. Future studies could build on this approach to include other risk genes, such as *TREM2*, or examine how *APOE4* affects brain health when the body is dealing with other problems.

Together, both experiments provide a focused and realistic approach to uncover the early impact of *APOE4* on brain function. By combining behavioral testing, synaptic imaging, and single-nucleus RNA sequencing, this research project aims to determine how *APOE4* influences brain aging before visible signs of Alzheimer's disease appear. These findings have the potential to identify new therapeutic targets that could delay or prevent disease onset in genetically at-risk individuals, supporting the broader goal of developing precision medicine for individuals who carry genetic risk factors such as *APOE4*.

CONCLUSION

Understanding the molecular and cellular effects of the *APOE4* allele is essential for uncovering how *APOE* causes Alzheimer's disease. This grant proposal focuses on evaluating early behavioral, cognitive, and molecular changes in humanized *APOE3* and *APOE4* knock-in mice, specifically in the absence of additional AD-related mutations. By also analyzing brain tissue from mice and profiling gene expression in different brain cell types, I aim to pinpoint how *APOE4* influences neuronal health and immune responses in the brain, long before plaques or other hallmarks of the disease appear. This focus on early-stage effects of *APOE* fills an important gap in knowledge, as much of the existing research has focused on later stages of disease or used models that introduce multiple mutations at once.

If successful, this research proposal could reveal the earliest brain changes associated with *APOE4* and identify molecular pathways that could serve as ideal targets for early intervention. These findings could also help inform drug development aimed at inflammation, supporting synaptic function, or targeting *APOE*-related signaling before irreversible damage occurs. In conclusion, this research proposal aims to contribute to the growing field of knowledge on neurodegenerative disease by providing insight into how individual genetic differences can shape disease progression and potentially offer new treatment strategies.

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AAV-Mediated Gene Editing for the Late-Onset GJB2 p.V37I Mutation: *Potential for Restoring Hearing in Aging Mice*

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ARTIFICIAL INTELLIGENCE PREFACE:

I found Artificial Intelligence to be a very valuable tool while writing this research grant. First off, it helped me narrow down the research articles I wanted to choose by generating summaries of the main findings. These summaries helped me decide whether the papers aligned with my personal research goals and whether I should devote time to reading them in full. This helped me save a ton of time during the literature review process. After finding and fully reading all the research papers I wanted to integrate into my final, I used AI to help generate ideas for future experiments. Although I had lots of ideas in mind, I wanted to make sure that they were focused experiments, with a clear objective. AI helped me turn my ideas about the timing of gene therapy treatment into an interesting future experiment. This also ensured I addressed a knowledge gap, so I didn't repeat an experiment that had already been done.

Some of the lab procedures described in the papers were also quite complex, and I used AI to learn more about them and how they worked, so I could confidently write about them in my proposed experiment section. I haven't been able to personally complete these experiments in the Lake Forest Lab because they are expensive and complicated, so AI helped simplify the procedures in terms I would understand. For example, I was confused about how transgenic mice and AAV vectors are generated, and artificial intelligence guided me through a step-by-step process to accomplish this in the lab. In my proposal, I also used AI to help with my technical language, especially in the proposed experiment section. I wanted to make sure the structure of my sentences sounded like a standard research grant proposal, and AI helped me make better word choices in scientific terminology. Overall, I felt AI was a great resource for learning about complex lab procedures and the decision-making process researchers must go through when designing future experiments.

Hearing loss is a common condition and is linked to mutations in the GJB2 gene, which encodes the connexin-26 channel in the cochlea. Early-onset mutations have been widely studied, but late-onset mutations, such as p.V37I, are largely unexplored. While gene therapy treatments are proving useful for treating neonatal mice, this study aims to address the efficacy of gene therapy in middle-aged to geriatric patients with the p.V37I mutation. Building on the most recent GJB2 gene therapy approach (Ukaji, 2025), this study proposes using an AAV-mediated Cas9 base-editing system to target and correct the point mutation in transgenic mice across multiple age groups. This research provides crucial insight into the influence of age on gene therapy efficacy and provides hope for expanding the therapeutic window for older patients experiencing progressive hearing loss.

PHENOTYPE

My Grandpa loves to socialize - some would argue too much! He goes to the gym not for a workout, but to talk to the people around him. He will retell the same story to anyone who will listen because he's perfected it, and he knows it will get a good laugh. He lives for those mundane interactions with strangers that most people would find insignificant. When his hearing loss progressed, I could tell that he felt disconnected from his family and friends and isolated from one of the things that brought him the most joy in life. It was disheartening to watch him sit at the head of the table at Christmas dinner without talking - not because he didn't want to, but because he couldn't hear the conversation happening just 3 feet away from him. Although physically and mentally, he was healthy in his old age, his hearing loss made him feel as though he wasn't. Seeing firsthand the profound impact that hearing loss can have on a person's daily life is what drew me to study it, particularly the effects of a single-gene mutation.

The most common gene linked to age-associated hearing loss is *GJB2*, which stands for Gap Junction Beta-2 Protein. This gene encodes Connexin 26, an essential protein for cellular communication in the cochlea of the inner ear. When this Connexin 26 protein is dysfunctional, cellular signals cannot be transmitted in the cochlea. These disrupted sound signals are difficult for the brain to interpret, thus leading to partial or whole hearing loss. Additional details on the molecular function of this protein will be explained in the next section.

Genetic hearing loss is common and increasing; by 2050, it is estimated that 2.5 billion people will have some degree of hearing loss, and 700 million of those will have debilitating hearing loss (Ma, 2025). Important characteristics of *GJB2* mutations include the severity and timing of hearing loss, as well as vestibular symptoms (e.g., balance disturbances, visual disturbances, vertigo, nausea). These symptoms differ depending on the type of mutation in the gene, but are generally divided into two categories of Connexin 26 activity: partial functioning and nonfunctioning (Sakata 2023). Partial protein function leads to less severe symptoms, whereas nonfunctional proteins lead to more severe symptoms. Hearing loss severity is categorized as mild, moderate, severe, or profound. Patients with mild hearing loss can detect sounds ranging from 21 to 40 decibels, while those with moderate hearing loss perceive sounds between 41 and 70 decibels. Severe hearing loss corresponds to a range of 71 to 95 decibels, and profound deafness applies to any sound exceeding 95 decibels (Cryns, 2004).

A common mutation that leads to a partially functional protein is p.V37I, a missense mutation at position 37 that causes a valine-to-isoleucine amino acid substitution in the connexin 26 protein. 65% of patients with this mutation had congenital hearing loss, which means they were born with this mutation. The remaining 35% of patients had delayed-onset hearing loss, indicating they acquired the mutation after birth. Environmental stressors, such as loud noise, can cause non-hereditary mutations in the *GJB2* gene (Lin, 2019). The p.V37I mutation creates a partially functional protein with a broad spectrum of phenotypes. Some individuals with this mutation show no signs of hearing loss, whereas others have severe hearing loss. In most cases, though, hearing loss is strongly correlated with age, with symptoms worsening much more in later life (Lin, 2019).

A common mutation that leads to a nonfunctional protein is 35delG, a deletion of a guanine base at position 35. This creates a frameshift mutation, in which every amino acid encoded after this mutation is altered. This is because amino acids are coded in base pairs of three, and if one of these bases is deleted, then every following group of three will be altered by one pair. This mutation leads to the production of completely nonfunctional connexin 26 proteins, resulting in total hearing loss. This mutation is most common among Caucasian populations, and it is congenital in 75-80% of cases (Li, 2023). In congenital cases, affected individuals can be homozygous or heterozygous for this mutation, meaning that either only one or both copies of the gene are affected. Mutations in both copies of the gene cause significantly greater hearing loss, while a mutation in only one copy results in milder impairment. (Cryns, 2004).

Due to the wide range of phenotypes associated with *GJB2* mutations, future research is needed to determine which gene therapies and clinical treatments are appropriate for each phenotype. For instance, how does the effectiveness of *GJB2* gene therapy vary with the individual's age at the time of administration? Is there a difference in gene therapy effectiveness between individuals with early-onset hearing loss versus adult-onset hearing loss? While some studies have shown that older mice exhibit limited recovery following gene therapy, these studies used mice with early-onset *GJB2* mutations (Guo et al., 2021). What about late-onset mutations - could gene therapy still be effective in those cases?

*This author wrote this paper for Biology 352: From Genotype to Phenotype taught by Dr. Karen Kirk.

MOLECULAR FUNCTION OF CONNEXIN 26 AND MOUSE MODELS

The *GJB2* gene is located on chromosome 13q12.11 and encodes the connexin 26 protein. Connexin 26 is a member of the gap-junction protein family and is found in the epithelial cells of the cochlea in the inner ear. A gap junction acts as a direct channel between two cells and is composed of six subunits, which combine with another six-subunit protein on the other cell to form a pore between the cells (Ma, 2025). These channels differ from other ion channels because they form much larger pores that allow signaling molecules to pass through. If these subunits become dysfunctional due to mutations in their genetic code, they cannot form fully functional channels. In addition, if the channels are deformed, lysosomes in cells degrade them, even if they are only partially active (Xu, 2023). Degradation and loss of connexin 26 channels have catastrophic impacts on cellular communication and function in the inner ear.

Connexin 26 is essential for generating electrochemical gradients that facilitate potassium cycling and the transfer of signaling molecules (Ke, 2025). The scala media, one of the major fluid compartments in the cochlea, must maintain a high K+ concentration and provide a positive potential to allow passive flow of K+ into the hair cell mechanoreceptors. These hair cell mechanoreceptors become depolarized when K+ flows through them, initiating a chain of events that eventually transmits the electrical signal the brain interprets as sound. ATP also acts as an important signaling molecule in the cells of the inner ear, serving as an energy source to actively pump K+ ions into the scala media through connexin 26 channels and to regulate channel activity (Zdebik, 2009). The mechanism of hearing is complex, but the main takeaway is that cell signaling is essential for converting sound waves into signals the brain can interpret, and the connexin 26 protein is vital for this process. Mutations in the *GJB2* gene that encode this protein have been widely studied using mouse models to investigate its relationship to different severities of hearing loss and to develop potential gene therapy options.

A target ablation of the connexin26 protein in the epithelial cells of the inner ear was completed in CRISPR-generated knock-out mice to prove the overall importance of this gene function (Cohen-Salmon, 2002). After only 14 days, the supporting cells and cochlear hair cells died. Apoptosis occurred first in the supporting cells, which hold hair cells in place and maintain ion balance. Next, the inner hair cells (IHCs), which convert sound vibrations into electrical signals, underwent cell death. Shortly after, outer hair cells (OHCs), which amplify sound, underwent cell death. The death of these cells leads to altered epithelial cell shape in the cochlea and to eventual Corti collapse, a small organ in the cochlea. Despite this cell death, the mice maintained vestibular function, such as balance, but exhibited complete hearing loss. This proves that the connexin 26 channel in the inner ear is directly linked to hearing, but not vestibular function. The researchers had several predictions for why the loss of connexin-26 caused this cell death. The first is that without the connexin channels, a toxic buildup of potassium occurs around the hair and supporting cells. This overstimulates cells, leading to oxidative stress that triggers the extrinsic cell death pathway. In this pathway, external stimuli bind to death receptors on cells, triggering a cascade of events that culminates in apoptosis. When the supporting cells die first, the hair cells cannot survive (Cohen-Salmon). These results provide a strong foundation for future studies on the impact of gene-knockout timing and on how the phenotypes of specific frameshift or point mutations differ from those of an entire gene knockout.

After determining that the *GJB2* gene causes hearing loss, tamoxifen-induced knockout mouse models were generated to investigate how the timing of a *GJB2* mutation influences hearing loss. They first created a transgenic mouse with the *GJB2* gene surrounded by loxP sequences. When tamoxifen was administered, it activated the Cre recombinase enzyme, which cut the loxP sequences, thereby removing the *GJB2* gene. They found that when the *GJB2* gene was deleted before the inner ear was fully developed (at 1 day postnatal), the mice experienced more severe hearing loss than when the gene was deleted 14 days postnatal, after inner ear development (Guo, 2021). The severe structural changes in the cochlea that occurred during early gene deletion imply that the connexin 26 protein is an essential part of inner ear development. Another important finding was that hair cells, which cannot regenerate once lost, remained intact for 2 months post-*GJB2* deletion, which provides hope for future gene therapies, as gene therapy is not an option if the hair cells are already deteriorated. To begin investigating gene therapy, the researchers used an adeno-associated virus with a healthy copy of the *GJB2* gene and injected it directly into the cochlear nerve. Although connexin 26 expression increased, hair cells began expressing the protein, leading to their degeneration and worsening hearing loss (Guo, 2021). Although this research did not restore hearing, it lays a foundation for developing more precise and targeted gene therapies.

To develop precise gene therapies, it is important to understand the different types of mutations in the *GJB2* gene, particularly the optimal timing of gene therapy administration for each mutation type. Frameshift mutations lead to alterations of every amino acid coded after. The 35delG and 235delC mutations are common frameshift mutations that occur in the *GJB2* gene in humans. Knock-in mice were developed by constructing CRISPR-edited embryonic stem cells carrying these mutations, injecting the cells into blastocysts, and then implanting the blastocysts into female mice to develop. The mice with these frameshift mutations showed early-onset hearing loss that progressed into profound deafness (Li, 2023). Because this mutation leads to such early hair cell loss, it would be difficult to develop a gene therapy that could effectively restore hearing. Other types of mutations, however, lead to a much slower onset of hearing loss and offer greater potential for gene therapy to restore function.

Point mutations, which are the change of one base pair, can have less dramatic effects on the function of the connexin 26 protein. The p.V37I mutation is a missense mutation that substitutes a valine for an isoleucine at position 37 in the amino acid sequence and has been studied using knock-in mouse models. Transgenic mice carrying the mutation were generated from an embryo, and they found that this amino acid substitution led to no significant hair cell loss but did decrease the length of gap junction plaques. These gap junction plaques are clusters of Connexin 26 channels where much cellular communication occurs. As these plaques diminish, endocochlear potential decreases, leading to mild, progressive hearing loss as potassium and other signaling molecules accumulate. Because there was no loss in hair cells or the motor proteins in the inner ear, this change in endocochlear potential is the predicted result of missense mutation-induced hearing loss (Lin, 2019). Given that hair cells are not lost by this mutation, it offers hope for gene therapy interventions to restore connexin 26 function and thereby restore hearing.

Gene therapy is often delivered inside viral vectors. Adeno-associated virus (AAV) vectors are used because they do not target dividing cells, reducing the likelihood of adverse side effects. To study the efficacy of AAV-mediated gene therapy, researchers generated complete *GJB2* knockout mice and, shortly after birth, surgically injected the gene therapy vector into the cochlea. The DNA remained in the nuclei of cochlear epithelial cells, where it was transcribed into mRNA and eventually translated into functional Connexin 26 proteins. This gene therapy restored connexin 26 expression in the mice, despite not actually being integrated into the mice's genomes via a double-stranded break. (Iizuka, 2015). This study highlights the potential of gene therapy, but doesn't acknowledge that many people receiving the treatment would not receive it shortly after birth or would have the genotype for a complete knockout. In one of the most recent and groundbreaking studies regarding treating *GJB2* hearing loss caused by a point mutation, viral vectors were utilized to deliver gene therapy to edit the mutated DNA in the genome. Researchers developed a catalytically impaired Cas9 called Cas9 Nickase, which induces a single-strand break at the point mutation. A deaminase enzyme then edits the base pair to fix the mutation. This system targeted the R75W point mutation, which replaces arginine at position 75 with tryptophan. The goal was to target the single-base-pair mutation and correct it in all cochlear cells. They found that after administering the vector in vivo to mice on postnatal day 1, functional Connexin 26 was partially expressed,

indicating that the gene therapy partially reversed hearing loss (Ukaji, 2025). This exciting research opens up a whole new set of possibilities. Now that we know the inner ear can be treated with in vivo AAV-mediated gene therapy systems to edit and restore gene function in the context of point mutations, this approach can be applied to a plethora of more specific studies. For example, can the point mutation p.V37I, which is a common mutation linked with progressive adult-onset hearing loss, be treated with an AAV-mediated system at a variety of ages besides just postnatal day 1?

FUTURE EXPERIMENT

AAV-Mediated Gene Editing for the Late-Onset GJB2 p.V37I Mutation: Potential for Restoring Hearing in Aging Mice

SPECIFIC AIMS

Building on recent advancements in AAV-mediated gene editing in the inner ear (Ukaji, 2025), I plan to expand this research to investigate whether hearing loss associated with the *GJB2* gene p.V37I mutation can be prevented or restored in aging mice. The p.V37I mutation is a common cause of progressive adult-onset hearing loss, yet the applicability of gene therapy for this mutation has not been studied in older mice. I hypothesize that AAV-mediated gene editing will be effective at halting the progression of mild adult-onset hearing loss by restoring Connexin 26 expression in aging mice with the p.V37I mutation. By developing transgenic mice carrying this mutation, we can evaluate the efficacy of AAV-mediated CRISPR-based treatments across different age groups. This information will provide valuable insights into assessing the therapeutic window for treating late-onset hearing loss, particularly in determining whether older mice with established hearing loss can halt progression, restore Connexin 26, or even experience hearing recovery.

EXPERIMENTAL PROPOSAL

The type of gene mutation, the timing of gene therapy administration, and the age of patients receiving the treatment can affect its efficacy. We know that *GJB2* mutations that cause nonfunctional connexin 26 proteins are detrimental to inner ear development, resulting in severe hearing loss. In comparison, *GJB2* gene mutations that result in partially functional connexin 26 proteins are not detrimental to inner ear development and cause milder, progressive hearing loss. In cases of these less severe point mutations, there was a window for therapeutic intervention to restore hearing loss, as the inner ear's structural integrity remained intact (Guo, 2021). The p.V37I mutation would therefore be a good candidate for a future study, as mice with the mutation have normal cochlear development and hearing at birth but develop progressive hearing loss over time - very similar to how it presents in humans. It is also the most common mutation associated with adult-onset hearing loss in East Asian Populations, so this work could be applied to many people (Lin, 2019). Recent advancements in inner ear AAV-mediated gene editing demonstrate that in vivo editing of a point mutation is feasible, restoring Connexin 26 and partially rescuing hearing in young mice. This research, although exciting, has yet to show implications for older mice with late-onset mutations (Ukaji, 2025). These findings justify the immense promise of my proposed future study and make it an essential next step in hearing loss research. I will structure the gene-editing procedure very similarly to that in Ukaji's recent 2025 study, "AAV-mediated base editing restores cochlear gap junction in *GJB2* dominant-negative mutation-associated syndromic hearing loss model". The knock-in mouse model with the p.V37I mutation is the first and essential step in my experiment. A previous study by Xin Lin successfully generated a knock-in mouse with this mutation, and I will follow their procedure to ensure I also generate a successful animal model (Lin, 2019). To accomplish this, I would locate the precise base pair 109 in the mouse *GJB2* gene that corresponds to the human *GJB2* and induce a mutation in embryonic stem cells. Homologous recombination is the most common and precise method to induce these mutations. This process involves creating a DNA construct vector containing the mutation using site-directed mutagenesis. Oligonucleotide primers carrying the p.V37I mutation are designed, and PCR is used to amplify the *GJB2* gene. Because the primers include the mutation, the amplified *GJB2* gene will also contain the mutation. To ensure that only amplicons containing the mutation are inserted into embryonic stem cells, a process called restriction enzyme digestion is used to recognize and cut amplicons lacking the mutation. Gel electrophoresis can be used to extract the longer (uncut) amplicons with the mutation. Then I will use Topo-cloning to integrate the mutated gene into a plasmid, which is then taken up by E. *coli*. E. *coli* bacteria with the plasmid will grow colonies on an ampicillin plate, since the plasmid will also contain an ampicillin resistance gene. The process amplifies the mutated gene, which can then be isolated from E. coli cells and purified to high concentration. The sequence will be verified with Sanger sequencing before inserting into embryonic stem (ES) cells.

The plasmid vector will be introduced into the ES cells by electroporation. The ES cells will be placed in an electroporation buffer, to which the plasmid DNA will be added and mixed. An electroporation machine applies an electric field to the solution, temporarily making the ES cell membranes permeable and allowing the DNA plasmid to enter the cells. The ES cells are removed from the buffer mixture and incubated for at least 24-48 hours to allow DNA repair processes to incorporate the plasmid into their genome. Like Topo-cloning in E. coli cells, because the plasmid in the ES cells contains an ampicillin resistance gene, we can incubate the ES cells on ampicillin-containing plates. The surviving colonies should harbor the mutated *GJB2* gene, and these cells will undergo Sanger sequencing of *GJB2* to confirm the presence of the proper p.V37I mutation. Then, the modified ES cells will be injected into blastocysts, which are early mouse embryos. Fertilized blastocysts develop in the female mouse for about three to four days before euthanizing the mouse, dissecting out her uterus, and flushing the blastocysts onto a petri dish. These blastocysts are placed under a microscope, where a micropipette is used to inject between 10 and 20 of the edited ES cells directly into the inner cell mass of the blastocyst. The target ES cells inserted will integrate into the developing mouse fetus, creating a chimeric mouse with a mixture of normal and modified cells. The blastocysts are then implanted directly into the uterus of a surrogate mouse. In 19-21 days, the surrogate will deliver our transgenic mouse model. This mouse model can be tested for the presence of modified cells by taking blood or tissue samples and completing sequencing. These new chimeric mice can be bred together to produce offspring with the mutation, rather than going through the intensive process of creating them from scratch.

Now that the knock-in model has been generated, we must develop an adeno-associated virus (AAV)-mediated editing system to target the p.V37I mutation. Takeo Ukaji developed this system to target their *GJB2* point mutation of interest, R75W, and I plan to use a version of their editing technology for my experiment (Ukaji, 2025). In the p.V37I mutation, the base change that leads to the amino acid change is Guanine to Adenine at base position 109. Instead of using a standard Cas-9 enzyme to induce a double-stranded break, a catalytically impaired Cas9 paired with a base editing enzyme will be used to chemically change the Adenine back to a Guanine at that position. This catalytically impaired Cas9 is called Cas9 nickase, which induces a small cut in only one strand of the target DNA. Like standard Cas9, Cas9 nickase also uses a guide RNA to target a specific mutation in the *GJB2* gene. Once cut, the deaminase enzyme completes the base editing. The specific deaminase that converts A to G is called adenine deaminase. The Cas9 nickase and deaminase combination must be put into an AAV vector. This impaired Cas9 and adenine base editor is more compact than the standard CRISPR system, allowing the entire complex to fit into one vector. The smaller size of the editing system, along with an AAV vector specifically targeting cochlear supporting cells, offers greater therapeutic potential than past techniques. Once a genetic mouse model and the AAV vector have been developed, we can begin assessing the therapeutic window for treating late-onset hearing loss. Because my experiment focuses on efficacy across different age groups, I plan to test 6 age groups. Group 1 will be of neonatal mice at postnatal day 1 (P1). Group 2 will be juvenile mice at 2 months of age. Group 3 will be young adult mice at 5 months. Group 4 will be mature adult mice at 8 months. Group 5 will be middle-aged mice at 16 months, and Group 6 will be geriatric mice at 22 months.

To deliver the AAV-mediated gene therapy to cochlear cells, we will use a technique called round window membrane (RWM) injection. The round window membrane is a thin membrane at the base of the cochlea, and when treatment is injected here, it can reach cells inside the inner ear. Different surgical approaches must be used for each age group due to variations in the inner ear structure. This will ensure that the gene therapy is reaching the correct epithelial cells. For example, younger mice have delicate membranes, whereas geriatric mice have thick membranes that are harder to inject. Smaller needles should be used for the younger mice, and larger ones for the older ones. In all groups, the surgery is completed by making a small incision behind the ear and using a microscope to locate the RWM. The treatment is then injected, and the incision site is sutured closed. Therapy effects can vary, so I would like to assess auditory function in the mice pre-treatment and at weeks 2, 4, and 8 post-treatment. Auditory brainstem response (ABR) will be used to assess this function, which tests how well sound waves travel from the ear to the brainstem. We will gradually reduce the sound intensity delivered to the mice in a plastic box, and once no further waves are detected by the ABR machine, the machine will indicate the quietest sound the mouse can hear. In theory, after gene therapy is administered, hearing loss should halt or even be restored, and mice will be able to hear lower sound intensities than they would without the treatment. After the last auditory function assessment at 8 weeks, mice will be euthanized and have their cochlea removed. The connexin 26 proteins in cochlear cells will be stained with colored antibodies using immunohistochemistry to visualize the amount of connexin 26 in the cells. Brighter staining indicates greater levels of connexin 26, indicating higher expression.

CONTROLS

For each age group, there should be three control mice: two negative and one positive. Control 1 (negative) should have mutant mice untreated with the AVV vector. This control will act as the baseline for the disease phenotype. This mouse will confirm that any improvements in hearing are due to the treatment, and not random or spontaneous chance. Control 2 (negative) should be treated with an AAV vector lacking the Cas9 system. This will ensure that the vector's toxicity does not cause detrimental effects or otherwise influence the disease phenotype. Control 3 (positive) will be wild-type mice without the mutation. This mouse represents a normal healthy phenotype that shows what normal ABR and connexin levels should be in each age group. If we observe an increase in ABR and connexin 26 expression levels after gene therapy, we can conclude that high editing efficiency and hearing restoration are possible in that age group. If we observe no increase but also no decrease in ABR and connexin 26 expression level, we could conclude that further deterioration of connexin 26 was prevented, even if not reversed or fully restored. If there was a decrease in ABR and connexin 26 expression, we could conclude that the gene therapy was ineffective at preventing deterioration or restoring hearing loss. Overall, I think this research will confirm that early intervention is better and also show that late intervention prevents further deterioration of connexin 26 and cochlear hair cells.

CONSIDERATIONS

The most important consideration is the variability in mouse models due to age differences. Immune response increases with age, which could lead to the older mice having more tissue damage or higher rates of mortality due to AVV-related complications. In addition, cochlear vulnerability increases with age, and if hair cells are lost, *GJB2* gene therapy treatments would not prove useful. To prevent cochlear damage, surgical procedures must be done extremely carefully in older groups. We must also consider that older cochlear implants may take longer to respond to gene therapy due to slower healing rates. Gene therapy transduction efficiency also decreases with older age, so increasing the vector dose may help combat this issue. Sample size is important in this experiment, too, especially given its long-term nature. Ensuring that many transgenic mice are generated per group will ensure that, even if mortality rates are higher than expected within groups, there will still be enough participants to obtain statistical data. His study aims to determine whether an AAV-mediated gene-editing system can target the p.V37I mutation in the *GJB2* gene to restore connexin-26 function and prevent or even reverse hearing loss. Mild, adult-onset hearing loss is often not addressed clinically until patients are geriatric, which is why I propose using six different age groups to test the efficacy of the gene therapy, specifically focusing on whether older models respond well to treatment. Efficacy can be confirmed through auditory brainstem response tests and connexin-26 expression levels.

Studying the efficacy of gene therapy across multiple ages could provide insight into when it should be administered for optimal results. It would also be the first study done for this specific mutation, investigating whether geriatric individuals with late-onset hearing loss can experience restoration or prevention of further hearing loss with this gene therapy approach. The findings could offer hope to patients who have already missed the window for early intervention treatment. Cohen-Salmon, M., Ott, T., Michel, V., Hardelin, J. P., Perfettini, I., Eybalin, M., Wu, T., Marcus, D. C., Wangemann, P., Willecke, K., & Petit, C. (2002). Targeted ablation of connexin26 in the inner ear epithelial gap junction network causes hearing impairment and cell death. Current biology: CB, 12(13), 1106–1111. https://doi.org/10.1016/s0960-9822(02)00904-1

This study completes a full *GJB2* gene knockout and focuses particularly on the effects of cochlear cell death and its mechanisms. Oxidative stress, apoptosis, activation of caspase-activated pathways, and overall metabolic stress caused by a *GJB2* knockout result in loss of cochlear hair cells, leading to irreversible hearing loss.E., Murgia, A., Huygen, P. L., Moreno, F., del Castillo, I., Chamberlin, G. P., Azaiez, H., Prasad, S., Cucci, R. A., Leonardi, E., Snoeckx, R. L., Govaerts, P. J., Van de Heyning, P. H., Van de Heyning, C. M., Smith, R. J., & Van Camp, G. (2004). A genotype-phenotype correlation for GJB2 (connexin 26) deafness. Journal of Medical Genetics, 41(3), 147–154. https://doi.org/10.1136/jmg.2003.013896

This review examines the genotype-phenotype correlation in hearing loss caused by mutations in the *GJB2* gene. It classifies hearing loss severity by decibel measurements and considers homozygous mutations to be more severe than heterozygous mutations. Idmore, J. M., Cimerman, J., Prieskorn, D. M., Beyer, L. A., Swiderski, D. L., Dolan, D. F., Martin, D. M., & Raphael, Y. (2021). *GJB2* gene therapy and conditional deletion reveal developmental stage-dependent effects on inner ear structure and function. Molecular therapy. Methods & clinical development, 23, 319–333. https://doi.org/10.1016/j.omtm.2021.09.009

This study found that when the *GJB2* gene was deleted in mice during early stages of inner ear development, they exhibited severe hearing loss, whereas when the gene was deleted after initial inner ear development, they exhibited mild hearing loss. Losing connexin-26 function early in development leads to a loss of structural integrity in the cochlea, so when they used gene therapy with viral vectors to reintroduce a functional *GJB2* gene, they found it was most useful when administered in younger, less developed mice, with limited recovery in older mouse models. K., Gotoh, S., Sugitani, Y., Suzuki, M., Noda, T., Minowa, O., & Ikeda, K. (2015). Perinatal *GJB2* gene transfer rescues hearing in a mouse model of hereditary deafness. Human molecular genetics, 24(13), 3651–3661. https://doi.org/10.1093/hmg/ddv109

Perinatal gene transfer using viral vectors during the prenatal stage restored connexin 26 function in mice at birth or shortly before. Rather than using gene editing, a functional copy of the gene is introduced to compensate for the dysfunctional one in the mouse's DNA. Sun, Y. (2025). Regulatory mechanisms of connexin26. Neuroscience, 570, 9–15. https://doi.org/10.1016/j.neuroscience.2025.02.027

This is the most recent review of research on the regulatory mechanisms of the connexin 26 protein, both pre- and post-transcriptionally. Transcription factors, microRNAs, ubiquitination, and calcium ion concentration can all affect the protein's functionality. This paper exemplifies the importance of understanding the molecular biology aspect of disease, specifically, electrochemical gradients in the inner

ear. ao, R., Yin, X., Wang, D., Cheng, Y., Huang, B., Wang, L., Yan, M., Zhou, J., Zhao, J., Tang, W., Wang, Y., Wang, X., Lv, J., Li, J., Li, H., & Shu, Y. (2023). The pathogenesis of common Gjb2 mutations associated with human hereditary deafness in mice. Cellular and molecular life sciences: CMLS, 80(6), 148. https://doi.org/10.1007/s00018-023-04794-9

This paper examines a complete *GJB2* gene knockout, specifically the common mutation in Caucasian populations, *35delG*, which deletes a guanine base at position 35. This creates a frameshift mutation, in which every amino acid encoded after this mutation is altered. This mutation leads to a completely nonfunctional connexin 26 protein, resulting in early-onset complete hearing loss. This showcases that although there is a connexin 26 protein, it is completely dysfunctional due to this mutation.

Lin, X., Li, G., Zhang, Y., Zhao, J., Lu, J., Gao, Y., Liu, H., Li, G. L., Yang, T., Song, L., & Wu, H. (2019). Hearing consequences in Gjb2 knock-in mice: implications for human p.V37I mutation. Aging, 11(18), 7416–7441. https://doi.org/10.18632/aging.102246

This paper examines one particular mutation in the *GJB2* gene - a common missense mutation in East Asian populations found at location p.V37I that causes a valine to isoleucine substitution on the connexin 26 protein. This mutation is linked to mild, progressive hearing loss caused by loss of gap junction plaques, compared to severe, complete hearing loss in childhood that other mutations in the *GJB2* gene may cause. They are also linked to these mutations, leading to a non-congenital p.V37I mutation.

Ma, S., Chen, X., Wang, Y., & Guo, Y. (2025). Mechanisms of congenital hearing loss caused by *GJB2* gene mutations and current progress in gene therapy. Gene, 946, 149326. https://doi.org/10.1016/j.gene.2025.149326

This review discusses the actual mechanism behind *GJB2* gene mutation hearing loss, the most common types of inheritance patterns, and provides statistics to back it up. It also discusses the challenges in gene therapy and some future directions, including various delivery methods. A., Koyama, M., Urata, S., Koyama, H., & Yamasoba, T. (2023). Hearing and Hearing Loss Progression in Patients with GJB2 Gene Mutations: A Long-Term Follow-Up. International journal of molecular sciences, 24(23), 16763. https://doi.org/10.3390/ijms242316763

In this long-term follow-up study, they found that different genotypes are associated with varying severities of hearing loss. Their research helps predict how severe hearing loss will progress and aids clinical diagnosis and treatment, specifically by dividing hearing loss into two categories: partial-functioning versus non-functioning connexin 26. Tsutsumi, H., Nakagawa, R., Matsumoto, F., Ikeda, K., Nureki, O., & Kamiya, K. (2025). AAV-mediated base editing restores cochlear gap junction in GJB2 dominant-negative mutation-associated syndromic hearing loss model. JCI insight, 10(5), e185193. https://doi.org/10.1172/jci.insight.185193

This study focuses on gene therapy using adeno-associated virus-mediated base editing to correct a mutation in the GJB2 gene without inducing a double-stranded break or inserting new DNA, but rather inducing a single-stranded break and using a base editing enzyme to change a single nucleotide. This experiment showed that gene therapy can restore connexin-26 protein levels and hearing in mice treated on postnatal day 1.l, X., Xie, L., Qiu, Y., Liu, X. Z., Wang, X. H., Kong, W. J., & Sun, Y. (2023). Degradation of cochlear Connexin26 accelerates the development of age-related hearing loss. Aging cell, 22(11), e13973. https://doi.org/10.1111/acel.13973

This study emphasizes that as connexin 26 degrades in the cochlea, hearing loss progresses. The actual mechanism of connexin degradation involves lysozyme, and lysosomes were found to degrade the proteins even when they were partially functional.

Zdebik, A. A., Wangemann, P., & Jentsch, T. J. (2009). Potassium ion movement in the inner ear: insights from genetic disease and mouse models. Physiology (Bethesda, Md.),24,307–316.https://doi.org/10.1152/physiol.00018.2009

This review provides important insight into the normal functioning of the inner ear, specifically how ion movement is a necessary component of hearing. Different signaling molecules, such as K+, glucose, ATP, and Ca+2, are dependent on Connexin-26 channels, and without them, their buildup can become toxic to the cell.

Excessive Telomere Shortening and Accumulation of DNA Damage Signals May be to Blame for Latent Cancer Diagnoses in Pedriatric Survivors

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Disclaimer: I did use AI in my paper, but only to find scholarly articles that mentioned «The Trinity at the Heart of the DNA Damage Response.» I had previously heard about the «holy trinity» of DNA damage markers in several other papers discussing telomere damage, but no specific proteins were discussed. This led me to conduct a general Google search for «holy trinity of DNA damage to telomeres». The first results came from the AI overview, which listed ATM, ATR, and DNA-PK as the primary regulators of damage responses. This overview provided the sources from which the information was pulled, and the aforementioned article was included. After reading this article, I gained a general understanding of DNA damage markers and, more specifically, the functions of each protein kinase within the «holy trinity». The Google AI overview was the only source of artificial intelligence I consulted. Still, I found it helpful for summarizing the article and the major points I needed to understand regarding DNA damage signals.

The majority of cancers arise from mechanisms within cells that attempt to prevent DNA damage. In pediatric cancers, the mechanisms underlying cell immortalization remain under investigation. However, in the past 10 years, there has been an increased observation that pediatric cancer survivors experience a secondary cancer diagnosis later in life. While the cancer treatment methods of radiation and chemotherapy can target immediate cancer cells in pediatric patients, they are also indirectly affecting normal cells and excessively shortening normal telomeres. This research on transgenic mouse models utilizes Telomere Restriction Fragment analysis (TRF) to assess telomere length before and after cancer treatments. Along with FISH-flow to identify the presence of «holy trinity» DNA damage markers after cancer treatments.

Telomeres and Telomerase

Imagine you are Juan Ponce de León in search of the infamous Fountain of Youth, and, knowing that this search could be the remedy for aging and immortality, your thirst for it drives you. You finally find the fountain itself and drink from its magical waters, obtaining something other people have only dreamed of. Your body has been restored to full youth, and you will never have to spend a day of your life scared by the contraction of a potential illness or disease. In fact, the natural aging of your body has ceased altogether. However, because of your immortality, you are stuck watching the world around you change, and the people you love wither away. You find yourself isolated in a world you no longer recognize or feel you have a place in. Immortality isn›t always what you expect, and it comes with a cost.

The Fountain of Youth is a myth that has been told for centuries, but its message about the consequences of immortality and human desires remains prevalent. In the real world, there are no springs of youth; rather, the immortality we may experience results in a painful and deadly disease. Cancer results when cells in the body are at significant risk of damage, and response systems activate to «save» the cell, inducing immortality. Immortality in cells leads to continued, uncontrolled cell division without the proper death of other cells in that area. The ultimate result is the formation of an abnormal mass of cells within a tissue; this is called a tumor. The uncontrolled cell division leading to tumor formation can also create a greater opportunity for DNA damage and mutation within these cells. If a cell population harbors mutations in its DNA that have the potential to induce disease, these mutations can lead to cancerous cells that form malignant tumors. The diseases that arise from these malignant tumors are cancer, which can present in varying tissues, organs, and cell types. To better understand how to cure cancer and prevent it altogether, it is essential to examine what happens within a cell for it to become immortal and develop damage.

A fundamental process of development throughout a person›s lifetime is cellular replication, which occurs through an evolutionarily conserved, highly regulated process called the cell cycle. The cell cycle is also part of another important process, mitosis, which results in the cellular division of a parent cell into two identical daughter cells. During mitosis, the entire DNA genome is replicated and divided between the two daughter cells. This DNA replication process is highly conserved across all organisms and is essential for proper cell function, which in turn supports tissue and organ function. The cell cycle is constantly occurring in the human body to regenerate dying cell populations and support growing ones. Although the cell cycle is a conserved, foundational process, DNA becomes shorter with each division. This is referred to as «the end replication problem,» in which a small end portion of the DNA sequence is lost with each cycle (Ackermann). The final sequence of DNA codes for the removal of proteins necessary for mitosis to occur, so these end sequences are not actually replicated, meaning the daughter cells receive a shorter DNA sequence.

No important DNA is lost during cellular replication because the ends are capped by telomeres, which are repetitive DNA sequences that do not encode proteins. Simply put, telomeres are protective caps at the ends of chromosomes that prevent end-to-end fusion with other chromosomes or the degradation of the DNA sequence (Ackermann). Since cell division is continuous throughout an organism›s life, telomere length serves as a biological marker for chronological age. The average length of a telomere in an adult human›s somatic cell is between 4-15 kilobase pairs in comparison to the 50,000-300,000 kilobase pairs of a single chromosome (Gorenjak). With every cell cycle, it is reasoned that telomeres shorten by 50-200 base pairs, and newly formed daughter cells inherit shorter telomeres, which is how telomeres serve as a mechanism for determining biological age. The protein complex that binds to the DNA sequence is called telomerase, which creates the initial telomere of the parent cell, which is then replicated in the daughter cells, ensuring that every chromosome in every cell is properly capped with a telomere.

Telomerase solves «the end replication problem» by adding repetitive DNA sequences that do not code for proteins but are solely used to form telomeres. Telomerase is an active enzyme during embryonic development, when cell growth and division are high. However, the enzyme is terminated in somatic cells of an organism once cell differentiation begins. During differentiation, cells lose their potential to become any cell type necessary for development and become specialized to support specific tissue regions. The overall function of telomerase and its termination are regulated by several genes that ensure the cell›s proliferative needs are met, allowing it to function (Gorenjak). As mentioned earlier, telomerase is an enzyme composed of protein subunits, *TERT* and *TERC*, giving it a multifaceted function. *TERC* initiates telomere formation by introducing telomere RNA that interacts with the DNA promoter region to facilitate extension. Then the compound *TERT*, also known as reverse transcriptase, adds nucleotides to the ends of telomeres during cell division, ultimately extending telomeres. Telomerase is essential during cellular division, as it can initiate telomere extension during replication if signals indicate that the telomere is at risk of being excessively shortened, leading to DNA damage. However, overactivation of telomerase or damage signals in the cell can cause excessive telomere lengthening, which becomes a triggered response in all future daughter cells. Mechanisms such as telomerase reactivation and alternative lengthening of telomeres can produce an immortal cell population that often leads to diseases such as cancer.

Telomere Damage and Cancer

A cancerous cell is definitively immortal and experiences no biological aging due to a telomere alteration, making it excessively long and prone to DNA mutations that lead to disease. Telomere lengthening can be triggered by aging factors, which is typically why cancers arise in older populations, as cells have continued to divide and approach the Hayflick limit. The Hayflick limit is the number of times a chromosome

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can be divided and replicated before its telomeres become short and no longer protect against DNA damage. The limit is estimated at around 60 division cycles, and once reached, a cell enters senescence, in which it no longer divides but remains actively involved, serving as structural support for the surrounding tissue. Senescence is a way to prevent DNA damage from excessive telomere shortening; however, rather than signaling a cell to enter senescence, it may signal the need to reactivate telomere lengthening. Telomere extension can occur in two ways: reactivation of telomerase or the alternative lengthening of telomeres (ALT) mechanism.

There is not much currently understood about the mechanisms contributing to ALT. However, extensive research worldwide has been conducted on the mechanisms that reactivate telomerase and the overactive function it acquires. Cancer cells can maintain immortality by activating telomerase, which extends telomere length indefinitely. An upregulation of telomerase can induce cell division leading to tumor formation. At the same time, impaired telomerase function can cause excessively short telomeres, leading to chromosomal instability that may recruit alternative lengthening mechanisms, which also lead to cancer. The occurrence of cancerous diseases is usually not observed until later in life, when cells are more likely to enter senescence and mutations in telomerase or the activation of ALTs occur.

Telomere length is highly variable at birth due to genetic predispositions and environmental teratogens to which a fetus is exposed. As development continues in childhood, telomere lengths begin to normalize and average out as biological processes are increasingly regulated internally and less rapid, major environmental changes occur. Previous studies have shown that gender, race, paternal age, smoking status, physical activity, traumatic events, obesity, and oxidative stress can all impact telomere length and can cause a fetus a predisposition for telomere length abnormalities (Gorenjak). Intrinsic risk factors include maternal age, for which research has observed a 6-15% increase in risk for every 5-year increase in maternal age. Another impacting factor is that structural birth defects consistently increase the risk of childhood cancer, potentially due to cell populations already experiencing replicative stress. Genetic factors, such as germline DNA mutations, chromosomal aneuploidy, and epigenetic disorders, account for 5-10% of childhood cancers (Spector). These genetic determinants of childhood cancer have been heavily researched using genome-wide association studies, which have identified common variants associated with cancer. While researchers can more fully understand the underlying genetic abnormalities contributing to several cancers, there remain over 90% of pediatric cancer types with unidentified or unspecified mechanisms.

As outlined above, telomere shortening is associated with a mechanism promoting cancer, but may also be induced by cancer treatments themselves. Parental exposure or even fetal exposure to high-dose ionizing radiation or chemotherapy increases the risk of pediatric cancers since these treatments are directly purposed to induce cell stress that will lead to cancerous cell death (Spector). When these treatment methods are utilized in such young patients, there is an increased risk of latent disease development because their biological processes are so vulnerable. Development in the first years of infancy occurs at an unprecedented rate, and many biological processes continue to be regulated during these years. The first years of growth are characterized by high cellular turnover, leading to rapid telomere shortening, which later stabilizes in early adulthood (Gorenjak). This is why an average telomere length is difficult to determine in childhood because there is great variability observed across cells. It is not until early adulthood that an average telomere length can be observed.

Telomerase activity, which induces this longer pediatric telomere length, can lead to the occurrence of mutations in *TERT* and *TERC* complexes. A mutation in one of these two foundational complexes is likely to occur in genetic cancers that cause an upregulation of telomerase, which continues to extend telomere length. There is also the potential that in-utero teratogens and environmental stressors induce replicative stress during development, causing early recruitment of cell damage signals such as POT1, ATM, ATR, and DNA-PK. POT1 is directly responsible for recognizing telomere length abnormalities (Richard). The other three singles mentioned are considered the «holy trinity» of DNA damage signaling. ATM is responsible for identifying double-stranded DNA breaks, ATR recognizes replication stress and single-stranded DNA breaks, and DNA-PK repairs the double-stranded breaks through non-homologous end joining (Blackford). Telomeres in pediatric cancer patients are often longer than in healthy individuals because they have less exposure to environmental stressors and decreased aging. However, the nature of chemotherapy treatments is to induce oxidative stress in immortal and proliferating cells to stop cell division. The treatments induce oxidative stress directed towards creating breaks in telomeres that then trigger cell death or senescence (Gorenjak). However, radiation and chemotherapy are not cell-specific treatments, and thus all surrounding cells and tissue receive the same oxidative stress-induction, leading to an accumulation of damage signals in noncancerous cells as well.

Treatments that expose individuals to oxidative stress cause regulated DNA damage to immortal cells in attempts to prevent further proliferation and trigger the DNA damage response of senescence. These are the rationales and mechanisms behind radiation and chemotherapy when treating cancer cells. However, this exposure to oxidative stress using these treatments may induce DNA damage in healthy cell populations as well, putting pediatric cancer survivors at risk for telomere dysfunction leading to subsequent malignant neoplasms (Richard). As mentioned earlier, there is great variability in childhood telomere length due to developmental processes still being regulated. Introducing further reproductive stress to such young patients is putting healthy tissue populations at risk for future dysfunction.

Research in the last ten years has seen a higher prevalence of latent cancer diagnosis in childhood cancer patients, potentially due to the treatment methods. Researchers Richard and Man have investigated that the subsequent tumor formations are more likely to occur in the regions where the previous treatment was directed, such as children who received radiation in the head, throat, and neck regions had greater chances of developing thyroid subsequent malignant neoplasm. Other researchers have shown a higher risk of subsequent malignant tumors occurring with specific radiation treatments rather than chemotherapy due to the ionizing oxidative stress X-rays (Gramatgers). Further, they observed that there was accelerated shortening of telomeres in pediatric survivors compared to control participants in two separate studies by two separate researchers, Aalbers and Gramatges. The research that put the necessity of finding alternative treatments for pediatric patients into perspective was a study conducted on a St. Jude Lifetime Cohort population. This cohort was formed with the initial purpose of checking in on patients post-treatment and monitoring their health. However, after noticing that quite a few survivors were receiving a secondary diagnosis while only in their thirties, researchers began looking at telomere length impacted by treatment methods (Song). These previous studies have shaped the goals and methods that will be proposed in the following section for the purpose of looking at how treatment modalities of radiation and chemotherapy affect telomere length and whether there is an increase in DNA damage signals as a result.

Research Proposal

This research will work to address two specific aims utilizing two different methods of analyzing telomeres. The first aim will be to observe how telomere length is affected before and after cancer treatments, more specifically, chemotherapy and radiation treatments. The second aim is to identify DNA damage markers before and after the above treatments in both cancer and normal cells. There has been an increase in research within recent years on pediatric cancer survivors and the long-term effects experienced from cancer treatments. The aims stated above will aid in addressing why pediatric cancer survivors have a greater chance of developing other forms of cancer later in life. The most common treatments utilized in both adult and pediatric cancers are chemotherapy and radiation therapy, which target telomere lengthening mechanisms. These mechanisms, either telomerase or alternative lengthening mechanisms, become inactivated to prevent the immortality of cancer cells. However, these treatments are inducing oxidative stress in all cells within their exposure range,

both cancer and healthy cells. Oxidative stress induces an accumulation of DNA damage markers within cells to trigger DNA recombination and repair. This research will work to observe both telomere length and DNA damage markers after cancer treatments to determine if these are mechanisms of higher cancer risk later in the life of pediatric patients.

This research will be conducted in juvenile mice models utilizing telomere restriction fragment (TRF) analysis to look at changes in telomere length and fluorescence in situ hybridization (FISH) to observe DNA damage markers. In response to aim 1, I hypothesize that there will be a significant decrease in telomere length for co-treatment mice models due to both chemical agents and ionizing radiation targeting varying telomere lengthening mechanisms. I also expect there to be a greater number of DNA damage markers in co-treatment mice due to multiple mechanisms of DNA replication being targeted via varying treatments.

Cancer mice models will be created by upregulating the activity of *TERT* in telomerase. To accomplish this, a gene will be introduced into the mices genome, which will encode for an upregulation of transcription factors for *TERT*, ultimately causing increased activity of *TERT* and telomerase. A DNA sequence of *TERT* with an upregulated promoter region will be introduced to the nucleus of fertilized mouse eggs using a glass micropipette. Once within the nucleus, this newly introduced DNA will be integrated into the genome during the first several cell divisions. The transgenic mice eggs will be implanted in adult female mice to carry during development and birth (Sharing Laboratory Resources, 1994). Transgenic offspring using an upregulation of *TERT* transcription factors require several generations to produce. Although this method is highly feasible, it will require a longer time to produce the desired homologous genome for upregulation of *TERT*. From these mice offspring, those that express cancer development, malignant or not, in the first several weeks after birth will be used within the experimental conditions. A mice model has been chosen for this experiment because it will provide an understanding of telomere length before and after specific treatment options that are controlled. The majority of research highlighting the latent effects of cancer treatments done on pediatric patients is either specific to the type of initial cancer diagnosis or secondary diagnosis and is thus limited in the extent of cancers they discuss. Research conducted in 2020 by Nan Song was my first insight into the latent adverse effects of radiation and chemotherapy treatments. These researchers utilized the St. Jude Cohort study population to look at how specific treatment types and locations may have induced chronic health concerns later in life. The population addressed by Song was pediatric-specific primary diagnosis, and they observed a significant decrease in leukocyte telomere length when DNA samples were taken almost 20 years later. The cancer types observed were varying, but overwhelming; their results suggested an increase in aging for survivors by 11.4 years (Song, 2020). If these researchers were observing an increase in aging among survivors, then it can be inferred that DNA damage markers were increased in these patients› cells, leading to senescence and telomere lengthening.

These previous results and others lead me to build my current research. To look at telomere length in the mice models before and after varying cancer treatments I will be utilizing a telomere restriction fragment analysis (TRF). Experimental mice are the transgenic mice with increased *TERT* activity, whereas the control mice were non-altered mice born from wild-type parents. Somatic cells will be extracted from the mice one week after birth. If cancer or tumor formation is already prevalent at this time, then cells will be taken from that specific site. DNA will be extracted using a DNeasy Tissue and Blood Kit. For more accurate readings, it is recommended that a cell pellet of about $1x10^{\wedge 6}$ cells be used, which will then be quantified using a spectrophotometer such as Nanodrop. Next, the DNA must be digested using a combination of reaction enzymes and separated on an agarose gel, which was loaded with radiolabeled TRF marker and Gel Red. The radiolabeled TRF marker can be visualized after hybridization with a telomere-specific probe, indicating specific telomere length, while the remaining genomic DNA is visualized with Gel Red. Next, the DNA undergoes hybridization and denaturation, during which the C-rich probe is introduced to identify telomere repeat sequences. The gel undergoes a series of washes and then is prepared for scanning. The gel is wrapped in the cassette, and a screen is placed on top. Exposure is for 4 hours or overnight using an imaging software such as Typhoon PhosphorImager. The TRF lengths are then calculated (Mender & Shay, 2015). The end product of TRF analysis is a Southern Blot with DNA appearing as a «smear» on the gel, which is what is quantified. The Southern Blot is also run with a DNA molecular weight ladder in between every 10 lanes. Previous research has shown that TRF length analysis of Leukocyte telomere length is typically performed over a range of 3-20 kilobases, so the DNA ladder will span this entire range to observe the varying telomere lengths. The controls of this TRF will include a positive control of the wild-type mice and a negative control where no DNA sample is added to a well to ensure there is no contamination of the samples or gel. Following the controls on the Southern blot would be DNA samples from all experimental mice prior to cancer treatment, allowing for initial telomere length determination.

After determining telomere lengths prior to treatment, the experimental groups would be separated and receive varying treatments between 3-4 weeks of age, when they are still in the juvenile stage. As of now, the estimated number of mice per condition is 5. Conditions would include five mice that undergo chemotherapy, five that undergo radiation treatment, and five co-treatment mice that experience both treatments. The procedures described above for TRF analysis would be repeated when mice are approximately 3 months old to determine the latent effects of treatments on telomere length. The calculation of telomere length would indicate whether telomere shortening occurs and, if so, to what extent, depending on the treatment type. These results would then be compared to control mice to see whether the treatment caused increased shortening outside of natural shortening from cellular division and aging. I predict that mice that underwent radiotherapy will have shorter telomere length than chemotherapy- or control-treated mice, because radiotherapy can infiltrate a larger tissue region that may include healthy cells. I also expect that co-treatment will result in the greatest telomere shortening compared to all other conditions.

Since these mice are being studied in their juvenile stages, we would also look for any markers that could suggest latent development of cancer during adulthood. The three major DNA damage markers looked at in this research are ATM, ATR, and DNA-PK because these three kinases are the major regulators of damage response mechanisms (Blackford & Jackson, 2017). To identify these markers in post-treatment cells, FISH-Flow analysis will be utilized to visualize their mRNA presence in the cell. Somatic cells from all mice are obtained, cancer treatment mice cells are collected from the region of treatment, and placed in a cell wash device that standardizes the preparation of all cells for analysis. These cells are suspended and fixed, to permeabilize the cells that are resuspended in 70% ethanol so that when probes are introduced, they can hybridize. To introduce the probes, the cells are rehybridized with a buffer containing the specific FISH probes coding for ATM, ATR, and DNA-PK mRNA. The cells are transferred to a plate and sealed, then incubated in a dark space in a static position. After a minimum of six hours, the plates are removed, and the cells are washed to remove excessive FISH probes. The cells are then resuspended in DAPI solution and left to incubate once again. Then, using flow cytometry, the FISH probes can be visualized along with the DAPI stain, and analysis is run using software such as FloJo (Antony et al., 2023). The final result of the FISH-flow analysis is both a visual display of DAPI and FISH probes within the cell, and will be run with a no-serum control in which no probes were introduced, which will identify potential background staining from binding to non-specific sites. A visual result occurs along with a graph from software analysis, suggesting the quantity gradient of DAPI and probes present in the cell.

From these results, I would predict that these DNA damage markers would have a greater quantity in mice that received cancer treatments than in the control mice. Previous research has highlighted that the therapies involved in cancer treatment utilize some of the direct triggers of DNA damage, such as UV light and platinum chemotherapies (Mouw et al., 2017). Also, pediatric patients are receiving treatment when key

biological processes are still being regulated and may impair normal DNA repair capacities. Even in research conducted on pediatric cancer survivors, those who received radiation treatments near the head, throat, and chest regions or received alkylating chemotherapy had an increased likelihood of developing thyroid cancer or subsequent malignant neoplasms five or more years after their initial treatment and diagnosis (Richard et al., 2020). From these previous findings of latent tumor formation post-cancer treatments in human patients, I would expect to see an increase in potentially all three DNA damage markers in comparison to the control, as these could serve as the potential triggers for the reactivation of telomere extension.

Conclusion

The two major mechanisms inducing cell immortality include telomerase reactivation and alternative lengthening of telomeres. Both processes induce cell proliferation and accumulation, leading to tumor formation that can be benign or malignant, with malignant formations being the production of cancer. The most commonly used cancer treatments are radiation therapy and chemotherapy, which target the identified cancerous cell population through tissue layers to induce DNA replication damage, preventing further cellular division and marking the cancer cells for degradation. In order to achieve a cancer-free state, treatments must be continuous and repetitive, and often occur in tandem to increase stress on the cancer cells. However, while inducing oxidative stress in the cancerous cells, the surrounding non-cancer cells are also experiencing this stress dysregulation in normal processes.

The cancer treatments used for oxidative stress remain quite similar across cancer and patient types. While it is rare to develop cancer before the age of 20, it is still possible and is the sad reality of pediatric cancer, with the most common therapies including surgery, radiation, and chemotherapy. During any cancer treatment, the amount of exposure is calibrated to the amount of targeted cancerous cells; however, even with treatments regulated for patient type, these methods put the overall body under increased stress. Children are extremely vulnerable to the side effects of these treatments because their biological processes are still undergoing regulation and could experience lifelong dysregulation if damaged.

The methods behind radiation and chemotherapy target mitotically active cells, cells that continue to proliferate. To stop further cell division, the cell›s immortality is targeted, which will allow cancer cells to be prone to degradation during treatments. The key mechanism allowing the cell its immortality is the maintenance of excessively long telomeres, so treatments aim to shorten telomeres by targeting and terminating the regulatory mechanism of immortality. The use of TRF analysis in the mice model explained above allows the somatic telomere length before and after treatment to be determined alongside a control to observe the true extent of telomere shortening that occurs. Cancer survivors tend to have shorter telomere lengths in general compared to non-cancer individuals, potentially making these patients more prone to DNA damage. Pediatric survivors are especially prone to increased DNA damage while also having a longer aging period remaining post-treatment, in which latent malignant tumor formation may occur. Should telomere shortening occur in pediatric patients after treatment, they may exhibit increased DNA damage signals in cells within the previous treatment area, which could trigger a second cancer diagnosis in later years.

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Microbe Hunters Review

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In a cultural and political landscape that often fails to appreciate the incredible possibilities and knowledge that scientific progress offers, books like de Kruif's *Microbe Hunters* remain important. While his writing is certainly dramatized and can oversensationalize the work of the first pioneers of microbiology, de Kruif does manage to convey the key facts of their discoveries while maintaining a thoroughly entertaining narrative.

Learning about the meticulous work of, for example, the stalwart Robert Koch in *Microbe* Hunters is fun and exciting. The reader is swept into the curious mind of an innovator who, through countless experiments, proves the causes of deadly diseases such as anthrax and tuberculosis. De Kruif manages to walk you through these experiments in a way that is entertaining, one by one, explaining how they were designed to answer an important question (such as "What are these things . . . are they microbes . . . are they alive?"), showing how new techniques to answer these questions were first developed (like cell plating and microscopy), and then finally how that led to the discovery (de Kruif, 1953, p. 103). By going through this process, the reader learns about the foundations of modern biological science and understands how critical it was to develop it so that the world wouldn't be plagued by so many deadly diseases.

Although de Kruif employs dramatization—depicting Koch as a "backwoodsman", Behring's risky experiments as "murderous gropings", and Mechnikov as a "wild Russian"—he uses these characterizations to underscore the magnitude of their scientific contributions. Even if readers receive a somewhat stylized view of these scientists, de Kruif ensures their crucial discoveries and impact on modern science remain clear, emphasizing the enduring importance of their work.

*This author wrote this paper for Biology 485:The Nobel Prizes taught by Dr. Brett Palmero.

How Ethical is CRISPR-Cas9 and What is it?

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Artificial intelligence was used during the writing of this essay. In the "Applications" section, AI helped brainstorm examples of CRISPR applications, including treatments for leukemia, improving crop yields, and the modification of malaria-carrying mosquitoes such as *Anopheles gambiae*. Moreover, AI helped summarize my 3 primary research papers. In the "Ethics" section, AI suggested I first discuss the ethics of the research papers and then expand to broader issues. This guidance improved the flow and clarity of my ideas. AI also assisted with finding credible sources and formatting citations. Overall, artificial intelligence was used to help organize, generate ideas, and strengthen the academic quality of this essay.

Introduction

Imagine waking up one day to discover that an incurable disease you have mysteriously vanished. This is exactly what happened to a woman with a rare hereditary disorder called WHIM syndrome (Doudna, 2017). Researchers studying the disease were astounded when the illness miraculously disappeared. It seems that a single stem cell underwent a spontaneous change that rid the cell of the disease. What happened was essentially a naturally occurring gene edit: her body had genetically modified its DNA, eliminating the disease (Doudna, 2017). Such a spontaneous cure is extraordinarily rare. Natural gene editing like this remains a medical anomaly. But what if gene editing no longer had to rely on chance?

Gene editing is something scientists have long aspired to achieve. With the discovery of a revolutionary technology called CRISPR, they are now closer than ever. CRISPR-Cas9 stands for *Clustered Regularly Interspaced Short Palindromic Repeats*. While the full name is technical and lengthy, the acronym refers to a powerful tool that enables precise gene editing. Interestingly, the CRISPR mechanism was not invented by humans; it was adapted from bacteria. Scientists observed that bacteria used CRISPR as a defense mechanism against viruses and later repurposed it for gene editing in other organisms.

This paper will explore the CRISPR mechanism in detail, beginning with its biological origins and its adaptation for gene editing. It will then examine a specific real-world application: the use of CRISPR to genetically modify malaria-transmitting mosquitoes. Finally, the paper will examine the ethical dilemmas that inevitably arise from gene-editing technologies. Overall, this paper will analyze CRISPR from three key perspectives: scientific, examining its mechanism; practical, through its real-life applications; and ethical, through a discussion of the controversies it raises.

CRISPR Mechanism

Often, when creating new things, humans draw inspiration from nature. For example, the design of Velcro was inspired by burrs and their ability to stick to animal fur with their small hooks (Science Reference Section, 2019). Similarly, an aspect of gene editing was inspired by a naturally occurring immune response in bacteria. Bacteria are prokaryotes whose greatest threat comes from viruses known as bacteriophages. To defend themselves against viral attacks, bacteria have developed a fascinating immune system called CRISPR. Just as burrs inspired the development of Velcro, this system of bacterial immunity inspired an aspect of gene editing. To understand the connection between the bacterial immune system and gene editing, it is first necessary to examine how the bacterial immune system functions.

Bacterial immunity against viruses was researched in the Danisco study (Doudna, 2017). In the dairy industry, products like cheese are made using *Streptococcus thermophilus*, a bacterium that ferments milk. Danisco, a major dairy company, noticed that its production was suffering because large numbers of its milk-fermenting bacteria were being killed by bacteriophages. To address this problem, they funded a study to understand how some bacteria seem to survive viral attacks. In the study, scientists mixed *S. thermophilus* with bacteriophages and found that while 99.9% of the bacteria died, a small fraction of mutant strains survived. After isolating genomic DNA from each mutant strain, the researchers found that all had a DNA sequence matching that of the bacteriophage. These matching copies conferred immunity on the bacteria. Moreover, this immunity, since it is stored in the bacteria's DNA, is heritable. This discovery meant the Dancisco's company could increase its production yields by selecting bacterial strains resistant to bacteriophages (Doudna, 2017).

Now that scientists understood why some bacteria were resistant to bacteriophages, they aimed to determine the precise mechanisms responsible for this immunity. As previously mentioned, scientists realized that the surviving bacterium shared some DNA with the virus. This is because when a bacterium survives a viral attack, it copies some viral DNA and stores it in a specific area of its genome, called the CRISPR array. This region contains a sequence that alternates between repeated bacterial DNA and foreign viral DNA. Essentially, it works as a filing system, keeping track of all the virus infections that the bacteria have survived. If the same virus tries to attack again later, the bacterium can defend itself. It does this by first creating a guide RNA (gRNA) from the stored viral DNA. As its name suggests, this guide RNA directs the Cas9 protein to the matching sequence in the invading viral DNA. Once Cas9 binds to the target viral DNA, it makes a double-stranded cut, destroying the virus before it can cause harm. This system acts like an immune memory, allowing bacteria to quickly recognize and fight off repeat infections (Prillaman, 2024). Therefore, scientists often describe this process of recognition as "a molecular vaccination card" (Doudna, 2017).

The Cas9 protein, which cuts viral DNA, is the key link between the bacterial immune system and modern gene editing. Scientists such as Jennifer Doudna were struck by Cas9's ability to recognize specific viral DNA sequences. They hypothesized that this natural precision could be repurposed to target almost any chosen DNA sequence. The first step in this process is designing the appropriate gRNA, which guides Cas9 to the target DNA. To do this, the gRNA must match the target DNA sequence, typically with a sequence of about 20 nucleotides (Thurtle-Schmidt & Lo, 2018). This level of specificity is significant because, while short DNA sequences are often repeated throughout the genome, a 20-nucleotide match reduces the risk of targeting the wrong site. However, even a perfect match between the gRNA and target DNA is not sufficient on its own. Cas9 will only cut the DNA if a short sequence known as PAM (Protospacer Adjacent Motif) is located immediately next to the target site, as illustrated in *Figure 1*. Cas9 can therefore be used to both locate and cut any desired gene.

Having established Cas9's ability to precisely locate and cleave specific genes, the next consideration is how gene editing is carried out. Sickle cell anemia provides a clear example, as it results from a single-base mutation in the HBB gene, in which glutamic acid is replaced by valine (Pattabhi et al., 2019). CRISPR can correct this mutation by using a guide RNA (gRNA) to direct Cas9 to the precise location of the faulty sequence. Cas9 then creates a double-strand break in the DNA. To repair this break, the cell uses a process called Homology-Directed Repair (HDR). A donor DNA template containing the correct gene without the sickle cell mutation is provided alongside Cas9. The cell then uses this template to accurately copy the correct DNA sequence. Through HDR, the mutated segment is replaced with the normal sequence, effectively editing the HBB gene (Pattabhi et al., 2019).

The Cas9 complex used by bacteria has been modified to increase its practicality in gene editing. In the natural CRISPR-Cas9, two types of RNA make up the gRNA: CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). The crRNA contains a sequence complementary to the target viral DNA, enabling precise recognition. Meanwhile, the

*This author wrote this paper for Biology 140: Gene Editing taught by Dr. Karen Kirk.

tracrRNA binds to the crRNA, helping form a stable Cas9 complex. To facilitate gene editing, scientists created a chimeric form of these two RNAs, which they called a single guide RNA (sgRNA) or a chimeric RNA. The chimeric RNA was tested to determine whether it could still function in guiding Cas9 to the target DNA. The results of this experiment are shown in the gel electrophoresis of *Figure 2*. This technique separates DNA fragments by size. If the DNA is cut by Cas9, multiple fragments will appear along a band. 5 versions of the chimeric RNA were tested, as labeled on the x-axis. In lanes containing chimeric RNA, multiple DNA fragments are visible. This indicates that the Cas9 was successfully directed to the target sequence (Jinek et al., 2012). Therefore, scientists were able to significantly facilitate the process of gene editing.

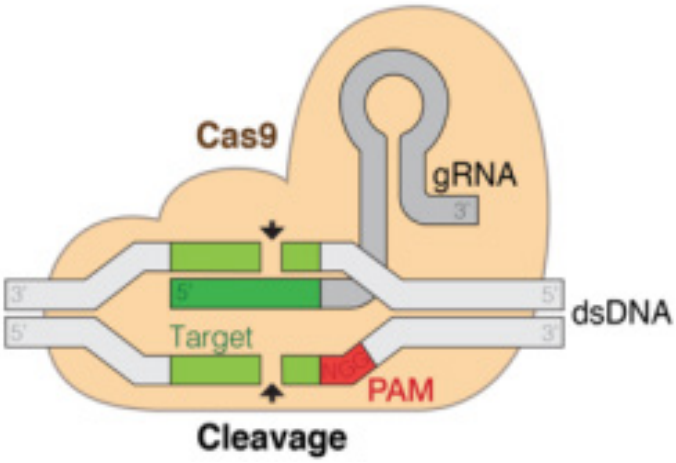

Figure 1: Cas9 protein complex (Lohner, 2021).

Applications

CRISPR technology has been used to genetically modify a wide range of organisms. This paper will examine how CRISPR is used to genetically modify *Anopheles gambiae*, the mosquito species that transmits malaria. Malaria, which kills over 600,000 people annually, is caused by the *Plasmodium* parasite, a type of single-celled eukaryote (WHO, 2024). The parasite enters mosquitoes, and when female mosquitoes feed on human blood, their saliva transmits the parasite into the human bloodstream. The parasite then travels to the liver, where it grows and matures. Once back in the bloodstream, it infects red blood cells, multiplying within them until the cells rupture, releasing even more parasites. This cycle continues, often leading to death. To combat this, scientists are exploring two CRISPR-based techniques. One involves reducing mosquito populations by inducing infertility, and the other focuses on preventing the parasite from infecting mosquitoes in the first place. The following sections will explore how these methods work to decrease the spread of malaria.

CRISPR technology is used to spread infertility in female *A. gambiae* mosquitoes, reducing their population and the spread of malaria (Nolan et al., 2016). A study by Andrea Crisanti and Tony Nolan et al. used CRISPR to insert a gene that causes infertility in female mosquitoes. To increase the chance that this infertility is inherited beyond the usual 50%, this study used a CRISPR-Cas9 gene drive. Once the desired gene is introduced to one chromosome, the gene drive copies it onto the unmodified one. This means that both the paternal and maternal chromosomes carry the gene, ensuring it is passed on to future generations. Since the gene causes infertility in females, it is the males who spread the infertility gene. Over time, as more females become infertile, the mosquito population significantly declines. The study showed that the gene drive was initially effective. However, it was also found that by the 2nd generation, the variants had become resistant to the CRISPR gene drive. This is a significant limitation, as it blocks the gene drive from being inherited, defeating its purpose of blocking the spread of malaria (Nolan et al., 2016).

To overcome resistance to gene drives, the same researchers utilized a different gene: the *doublesex* (dsx) gene (Nolan et al., 2018). This gene is crucial for female development and fertility. The scientist hypothesized that if a mutation in the gene drive with this gene occurred, it would cause infertility. CRISPR gene drives work by cutting the non-modified chromosome and copying the gene drive sequence onto it. To repair this cut, the cell often uses a process called non-homologous end joining (NHEJ), which is error-prone and can introduce small nucleotide insertions or deletions. It is precisely these seemingly small errors that lead to CRISPR resistance. In this study, although mutations would inhibit the gene drive, they would also cause the *doublesex* gene to cause infertility. In this strategy, even if resistance develops and the CRISPR gene drive fails, the female remains infertile. Thus, by incorporating the *doublesex* gene into the gene drive, the researchers created a foolproof system where infertility is inevitable, leading to the eventual collapse of the mosquito population (Nolan et al., 2018).

Introducing sterile insects is not the only way to reduce the spread of malaria; preventing mosquitoes from becoming infected in the first place is another strategy (Dimopoulos, 2018). In this approach, CRISPR is used to make *Anopheles gambiae* mosquitoes less susceptible to *Plasmodium* infection. For the parasite to develop in the mosquito, it requires numerous host factors, called agonists. If agonists are removed, the parasite cannot develop or infect the mosquito. In a study by George Dimopoulos et al., CRISPR is used to knock out the *FREP1* gene, an important agonist. The results showed that mosquitoes lacking the *FREP1* gene were less susceptible to *Plasmodium* infection. Meaning they were not infected by the malaria-causing parasite. However, the gene knockout also caused significant fitness costs, including reduced blood-feeding ability and lower egg hatching rates. These fitness disadvantages suggest that genetically modified mosquitoes may be less competitive in the wild. This reduces their chances of surviving, reproducing, and passing down their increased resistance to parasites (Dimopoulos, 2018).

All three papers shared important similarities: they each used CRISPR to reduce the spread of malaria and employed similar experimental techniques. One key method microinjection to deliver the CRISPR components into the *A. Gambiae* embryos. This process begins by creating the desired Cas9 complex. In each study, the single-guide RNA (sgRNA) was designed with a base sequence complementary to the specific target gene. The microinjection mixes typically included not only the CRISPR-Cas9 construct but also fluorescent marker proteins to help identify successful gene editing. The fluorescent mosquitoes with edited genes were then crossed with wild-type mosquitoes. This process, known as backcrossing, helps maintain phenotypic variability and overall fitness, and serves as a form of selective breeding. All three studies shared significant similarities, the most important being their common goal of reducing the spread of malaria.

Ethics of CRISPR

The genetic modification of *A. gambiae* inevitably raises profound ethical concerns. Spreading infertility throughout a species, potentially leading to its extinction, must be carefully weighed. On one hand, disrupting the mosquito population could have ecological consequences that may affect other species. On the other hand, malaria is responsible for over 600,000 deaths annually (WHO, 2024). While driving *A. gambiae* to extinction may have risks, humans "may consider it unethical not to use germline editing to alleviate human suffering" (Isaacson, 2021) (p.355). This raises the question: Should scientists be able to control the viability of another species if they pose a threat to human health?

Moreover, the ethics surrounding gene editing often depend on the perspective one takes. An environmental scientist might be more opposed to gene drives, given their understanding of the potential ecological consequences. In contrast, a biomedical scientist may be more inclined to support gene editing, focusing on the medical benefits of eliminating diseases like malaria. Policymakers face the challenge of balancing competing interests to satisfy both environmental concerns and public health goals. Additionally, perspectives vary across countries; nations where malaria poses a greater public health threat may be

more supportive of gene editing, while others may view it as too great a risk. These differing viewpoints contribute to intense debates, raising difficult questions about which perspectives should take precedence.

As we evaluate the ethical boundaries of gene editing, it is important to ask which traits should be edited. As previously mentioned, sickle cell disease is due to a genetic mutation that affects red blood cells, impairing their ability to transport oxygen efficiently. Individuals who inherit two copies of the sickle cell gene (homozygous) experience severe symptoms requiring treatments such as monthly transfusions. However, individuals with only one copy (heterozygous carriers) are largely asymptomatic. In both cases, nevertheless, the patient gains partial immunity to malaria (Mayo, 2025). Using CRISPR to correct the sickle cell mutation would alleviate symptoms, but it would also eliminate protection against malaria. While increasing malaria risk might be acceptable to relieve severe symptoms in homozygous individuals, removing malaria resistance from asymptomatic carriers raises more complex ethical questions. How do we choose which to prioritize? Is gene editing sickle cell anemia simply replacing one problem with another?

Another example that highlights the ethical dilemmas surrounding gene editing is deafness. Deafness can be a disadvantage in various scenarios, especially for families with limited resources. While society might label deafness as a disability, many "consider deafness to be part of who they are rather than something to be cured" (Isaacson, 2021) (p.334). Society's view of deafness as a disability has led some to argue that gene editing could be used to "fix" it. Thus, it is important to ask: "How do we distinguish between traits that are true disabilities and ones that are disabilities because society is not good at adapting to them?" (Isaacson, 2021) (p.336). Is it morally acceptable to use gene editing to change traits that cause no inherent harm, but are harmful due to society's perception of them?

As the examples above illustrate, gene editing often exists in an ethical gray zone, neither wholly right nor wrong. Given human nature, gene editing will inevitably occur, making the real question how society chooses to respond to these ambiguous cases. A recent example involves Dr. He Jiankui, who genetically modified the first human embryos, an experiment that led to his imprisonment (Normile, 2019). Although his achievement was scientifically groundbreaking, it was conducted prematurely and therefore unethically. In response, the scientific community issued a statement condemning his work, declaring that "the procedure was irresponsible and failed to conform with international norms" (National Academies of Sciences, 2018). As dilemmas surrounding gene editing continue to emerge, scientists must hold each other accountable for unethical research, regardless of how impressive the results may be.

Overall, the ethics surrounding gene editing are highly nuanced. In my opinion, ethical guidelines should be adjusted based on the location. While worldwide regulations may be important for ensuring consistency, they risk oversimplifying the complexity of this issue. As claimed by the Harvard Gazette, "It's very hard to deal with a transnational problem with national legislation" (Bergman, 2019). Instead, to address gene editing's ethical challenges effectively, each scenario should be evaluated on an individual basis. Moreover, it's important to "require students to learn the moral dimensions of science and technology", including that of gene editing. This will form much-needed public opinions (Bergman, 2019). Diverse perspectives, the cultural context of the countries, and the views of both the scientific community and the public must be considered in debates about gene editing.

Conclusion

Overall, it is evident that an extremely powerful tool for gene editing has emerged. This tool, CRISPR-Cas9, was inspired by an immune response found in bacteria. In nature, CRISPR enables bacteria to recognize specific viral DNA and destroy viruses before they can cause harm. Scientists were struck by Cas9's ability to recognize specific viral DNA and repurposed it to target almost any gene sequence. In gene editing, Cas9 is used to locate a specific gene and introduce a double-stranded break. The cell then repairs this break through either Homology-Directed Repair (HDR) or Non-Homologous End Joining (NHEJ), essentially editing the genome. Although this process exhibits off-target effects, minimizing them is a priority amongst researchers.

Considering the pace of technological advancements, the use of CRISPR in the future is inevitable. The question is no longer whether gene editing will occur but rather when and how (Doudna, 2017). The story of the woman cured of WHIM syndrome by chance is no longer just a medical marvel; it is a symbol of what science has made ordinary. Scientifically, CRISPR's repurposing was nothing short of revolutionary. But just as Doudna realized she could not explore CRISPR without also confronting its ethical implications, we too must recognize that scientific progress and ethical responsibility are inseparable. Gene editing holds incredible power, and therefore immense responsibility.

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An Ethical Analysis on CRISPR-Cas9 Technology

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Use of Artificial Intelligence

For this paper, I used ChatGPT for several purposes. When breaking down my research articles for the application section, I used AI to clarify terminology and simplify some lab techniques. I also used AI to find the early study in 2013 for my mechanism portion of the paper. Lastly, I used ChatGPT as a writing support that checked over my grammar and sentence structure. I did not use AI to write any paragraphs; instead, I used it to achieve a more professional tone and a refined style throughout my paper.

Introduction

What if we could disarm cancer at the genetic level before it has the chance to take over? In recent years, CRISPR technology has made the unimaginable within reach. Scientists have unlocked the genetic code that underlies every living organism on Earth, and with it, new possibilities for precise genetic manipulation. Originally discovered as a component of a bacterial adaptive immune system, CRISPR-Cas9 has been repurposed as a groundbreaking tool that can cut and edit DNA with unprecedented accuracy and efficiency.

With this powerful mechanism, the alteration or deletion of just a few nucleotides can disrupt or repair gene function, leading to profound effects on protein synthesis, cell behavior, and ultimately, the development or suppression of disease. Such capabilities are particularly transformative in oncological studies, where researchers are leveraging CRISPR to uncover new targets for cancer therapy and enhance the immune system's ability to recognize and destroy tumors. This paper explores the application of CRISPR-Cas9 to achieve gene knockouts in advancing oncology—particularly in developing cancer immunotherapies—while also addressing the ethical challenges arising from its use, including concerns about germline editing.

Mechanism of CRISPR

Bacteria and archaea have existed on Earth for over 3.5 billion years, living in all types of environments you could imagine (Doudna, 2022). These single-celled organisms can dominate and exponentially grow their populations, evolving specialized defences against their predators. Bacteriophages are viruses that infect bacteria; during infection, the bacterial cell is killed. In nature, there are 10 times as many phages as bacteria, so a defense system against these viruses is crucial for bacterial organisms. A variety of methods to block phage attacks are used, but the CRISPR system is unique to these bacteria. Clustered Regularly Interspaced Short Palindromic Repeats, otherwise known as CRISPR, is the immune system these single-celled organisms have evolved to use. Researchers have found CRISPR to work with different Cas proteins, with the most common system being CRISPR-Cas9 (Doudna, 2022).

To break this down step by step, we can start with a virus invading the bacteria (Doudna, 2017). The bacterial cell will then capture pieces of foreign DNA from the attacking phage as a memory and then store them in its own genome. These "memories" are called "spacers," and over time, they make up the CRISPR region. This process helps the bacteria recognize future attacks against the cell. So, in the case that the virus attacks the bacteria again, the CRISPR region is transcribed into an RNA molecule that gets cut into smaller segments called CRISPR RNA (crRNA). Each crRNA will contain a different spacer that correlates to a past virus. A helper RNA known as trans-activating RNA or tracrRNA pairs up with crRNA to form the guide RNA. This guide RNA binds to the Cas9 protein to form a complex that is able to search-and-destroy foreign DNA. The key to having this Cas9 complex recognize target DNA is the presence of a Protospacer Adjacent Motif (PAM). The PAM site consists of three nucleotides: usually one nucleotide followed by the two bases "GG," which is then placed right next to the target DNA sequence. Potential cut sites are located three base pairs upstream of the PAM (Doudna, 2017).

*This author wrote this paper for Biology 140: Gene Editing taught by Dr. Karen Kirk.

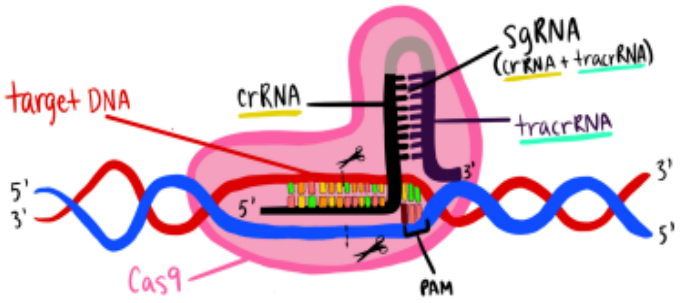

Figure 1. *Note.* This diagram illustrates the CRISPR-Cas9 gene-editing system, showing the Cas9 protein, the single-guide RNA (crRNA + tracrRNA), the target DNA sequence, the PAM site, and the cut sites introduced by Cas9. *Drawn by: Yulia Mercado*

A group of scientists, including Jennifer Doudna and Emmanuelle Charpentier, collaborated to transform the CRISPR-Cas9 bacterial immune system into a gene-editing tool. Their revolutionary paper, "A programmable Dual-RNA-guided DNA endonuclease in adaptive bacterial immunity," published in 2012, illustrates how a combined RNA structure can guide the Cas9 protein. The previously separate crRNA and tracrRNA have now been successfully merged into a single guided RNA strand (sgRNA).

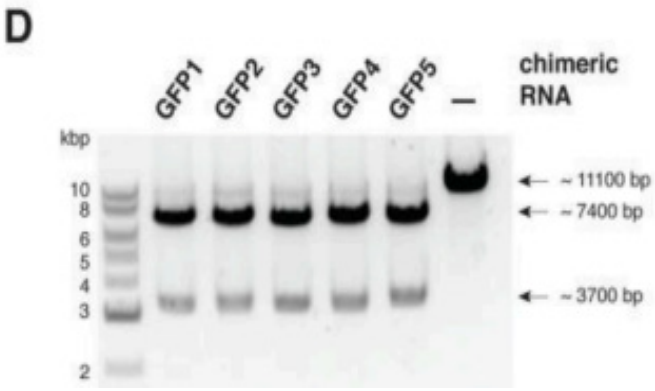

Figure 2. *Note.* Figure 5D from "A programmable dual RNA-guided DNA endonuclease in adaptive bacterial immunity".

Figure 2 demonstrates the sgRNA working with Cas9 to induce a double-stranded break in the target DNA (Doudna et al., 2012). The researchers engineered five chimeric guide RNAs targeting a portion of the green fluorescent protein (GFP) gene to determine whether the sgRNA design would be universally applicable. To experimentally test this, researchers conducted agarose gel electrophoresis, with each lane containing a sample of the Cas9 protein complexed with a different chimeric RNA. Figure 2 shows the five GFP-containing plasmid lanes with dark bands at 7400 base pairs and then lighter bands of DNA at 3700 base pairs. All five experimental lanes showed that Cas9 programmed with the chimeric RNA successfully cleaved the target DNA. This study validated the suggestion that single guide RNA strands can efficiently direct the Cas protein to the target DNA site and edit the gene (Doudna et al., 2012).

To elaborate on that, the 2013 study "Multiplex Genome Engineering Using CRISPR/Cas Systems" introduces mammalian cells and uses CRISPR to conduct genome editing(Cong et al., 2013). This experment involved the chimeric RNA targeting the EMX1 gene in human cells. This

particular gene has been linked to brain development and tumor suppression. Two different CRISPR/Cas9 variants were used: SpCas9 and SpCasn. SpCas9 is the traditional Cas protein that induces double-stranded breaks in the DNA, leading to insertions or deletions at the target site. SpCasn is the "nickase" version of Cas9, because it will cut one strand of DNA instead of two. This will usually result in a less efficient mutation, as only one strand will be repaired rather than both being repaired after SpCas9 cleaves. Figure 3 shows an agarose gel electrophoresis with clear cleavage in the lanes with SpCas9 but not in the lanes with SpCas9n. The percent indel in the first three lanes ranges from 3% to 5% with the DNA being cut from 684 base pairs to around 300 base pairs. This study successfully harnessed the RNA-guided functions of Cas9 to enable targeted gene editing in mammalian cells. This new possibility reveals the potential of CRISPR use in medicine, disease, and other applicable fields (Cong et al., 2013).

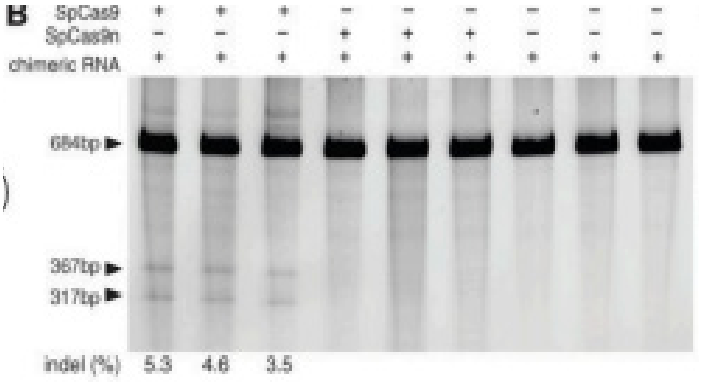

Figure3. *Note: Figure 4B from "Multiplex Genome Engineering Using CRISPR/Cas Systems". This diagram shows that coexpression of an EMX1-targeting chimeric RNA with SpCas9 results in indels, whereas SpCas9n does not.*

Application of CRISPR

Triple-negative breast cancer (TNBC) is an aggressive type of breast cancer that is characterized by the exclusion of two hormone receptors (Sharma, 2016). The cancer cells lack estrogen and progesterone receptors, eliminating hormone therapies as a possible cancer treatment. Another characteristic of TNBC is the lack of the HER2 protein. The HER2

protein plays a crucial role in cell growth, so underexpression or absence of this protein leads to affected growth of malignant cells. In HER2-positive breast cancer, a distinct subset of the disease, the HER2 protein is overexpressed on the surfaces of malignant cells, and anti-HER2 therapies are used to treat this breast cancer. Anti-HER2 therapies are ineffective in TNBC due to the absence of HER2 expression, underscoring the urgent need for alternative treatment strategies to address the challenges TNBC poses. To address this, researchers are interested in figuring out which genes help TNBC cancer cells escape the immune system and continue to proliferate (Sharma, 2016).

The attacking cells of the immune system are called T cells, and they are equipped with receptors that recognize antigens. In a healthy individual, cytotoxic T cells will kill harmful cells, and the immune system will function normally. In an individual with cancer, such as TNBC, the cancerous cells can bypass the immune system's "security" and take over the microenvironment. Three studies conducted in the past five years have used CRISPR gene editing to identify potential immune system targets that could lead to greater success in TNBC treatments.

In the study "In vivo multidimensional CRISPR screens identify Lgals2 as an immunotherapy target in triple-negative breast cancer," the researchers sought to identify genes in triple-negative breast cancer that help tumors evade the immune system, with the ultimate goal of identifying new immunotherapy targets (Ji et al., 2022). In-vivo CRISPR screening is a powerful application that allows scientists to knock out specific genes in cancer cells to see how tumor growth is affected. To identify immune-related genes that contribute to tumor survival, the researchers constructed a DrIM (disease-related immune gene) sgRNA library. This curated collection of single-guide RNAs was designed to target hundreds of immune-associated genes for CRISPR-Cas9 knockout. Inside each cancer cell, the sgRNA directs the Cas9 endonuclease to its matching DNA sequence, where Cas9 introduces a double-stranded break. The cell attempts to repair this break through non-homologous end joining (NHEJ)—a repair process prone to errors. These errors, in the form of insertions or deletions (indels), which frequently disrupt the gene's function, effectively knocking it out.

After the gene knockouts were introduced, researchers implanted the genetically engineered TNBC cells into the mammary fat pads of mice and allowed the tumors to grow for 14 days. Following this period, the mice were euthanized, and the tumor tissues were harvested. These cancerous tissues were then analyzed using Next-Generation Sequencing (NGS) to identify the effects of the gene knockouts on tumor progression. This modern, highly efficient method can simultaneously sequence millions of small RNA fragments, which scientists can then use to observe how gene knockouts affect the expression of specific genes. By observing the tissue samples with reduced tumor growth, researchers could identify which gene knockouts led to the reduction. One key gene found through this screening process was *Lgals2*, which has since been recognized as a contributor to immune evasion and tumor progression in triple-negative breast cancer. In this example, using CRISPR-Cas9 to knock out Lgals2 would improve the immune response in a TNBC patient (Ji et al., 2022).

Meanwhile, the study "In vivo CRISPR screens identify the E3 ligase Cop1 as a modulator of macrophage infiltration and cancer immunotherapy target" addressed that some tumors can resist immune checkpoint blockades (ICB), which is a form of cancer immunotherapy designed to help T cells kill cancerous cells (Wang et al., 2021). The scientist's goal was to identify genes that regulate how the immune system interacts with these tumors, and whether inhibiting those genes positively impacts the microenvironment. Similar to the last study, an in vivo CRISPR screen in mice was done to see the immune response after specific gene knockouts. For their experiment, researchers engineered cancer cells using plasmids that carried different variants of the ovalbumin (OVA) gene. The OVA gene, which encodes a protein found in egg whites, is frequently used in cancer immunology as a model antigen.

This model antigen acts as a molecular "tag" that allows scientists to monitor how T cells recognize and respond to tumor cells. Using a molecular biology method called Gibson Assembly, scientists could join multiple DNA fragments together. This enabled them to prepare the OVA gene fragments to have overlapping end sequences with another genetic plasmid called lentiCRISPR-V2-blast vector. The lentiCRISPR-V2-blast vector is a lentivirus system that uses a type of retrovirus to deliver genetic material into desired cells. Now that the scientists have created the lentiviral vectors needed for carrying out targeted gene edits, they are used to infect the TNBC cells from mice in vitro.

These modified cancer cells were implanted into mice, and tumors were allowed to grow in normal mice and immunodeficient mice. By comparing which gene knockouts lead to enriched or depleted tumors in each group, they identified genes (like Cop1) that influence how cancerous cells interact with the immune system. The Cop1 gene, when "silenced," resulted in depleted tumor growth, suggesting a promising gene target for immunotherapy in triple-negative breast cancer (Wang et al., 2021).

Finally, in the study "In vivo CRISPR screens identify Mga as an immunotherapy target in triple-negative breast cancer," researchers used CRISPR-Cas9 to knock out specific genes throughout the entire genome of cancer cells in order to identify tumor-intrinsic regulators that influence immune responses (Feng et al., 2024). The in vivo CRISPR screening method started with obtaining tumor cell lines from the mouse models. The Cas9-expressing tumor cells were infected with lentiviruses that carried the guide RNA and CRISPR so that CRISPR-Cas9 could take effect. After tumors grew for one week, DNA was taken from the cancerous tissue and used to determine which sgRNA was still present. Depleted

tumor growth meant that knocking out that specific gene helped fight the cancer, while an enriched tumor growth suggested avoiding knocking out that gene. It was found that the Mga gene significantly depleted tumor growth when knocked out and therefore has the potential to contribute to a successful immunotherapy treatment for TNBC (Feng et al., 2024).

All three studies shared commonalities in their use of CRISPR-Cas9 for in vivo screening to advance cancer immunotherapy. Cas9's high efficiency and broad PAM recognition made it the most practical editing tool. Researchers were able to encompass a wide variety of cancer-related genes and use CRISPR to systematically disable them. The choice to use mice as model organisms was consistent across the studies due to their genetic and physiological similarities to humans. This allowed researchers to observe gene knockouts in a realistic biological context, making the findings more applicable to human cancer treatment.

Ethics of CRISPR

Germline editing refers to the alteration of DNA in reproductive cells or early embryos. This raises a profound ethical dilemma, as any genetic alterations made are not confined to one individual but are instead passed down to every future generation. For patients burdened with genetic diseases, the ability to spare future children from inherited suffering feels like a miraculous opportunity—one that brings hope. And truly, if given the chance to erase pain from your family's legacy, wouldn't you feel a moral responsibility to act?

From a physician's perspective, germline editing is seen as unpredictable in the long term and ethically too risky. Doctors are more inclined to approve gene editing in somatic cells, like skin cells, as these are not inherited, but changing something that takes effect for generations after is a whole different story. On the other hand, from a patient's or parent's perspective, gene editing may be seen as a beacon of hope—the only way to break the cycle of inherited disease. Many families struggle to afford the long-term medical costs that accompany chronic illness, and the promise of a permanent solution through genetic intervention offers both emotional and financial relief. Policymakers would have the challenging task of balancing the ethical concerns, the potential for medical breakthroughs, and public health implications. They would focus on establishing laws and regulations for safety, advocating for global compliance, and making sure there is equity in access to germline editing.

While germline editing offers hope to future generations and the potential to save lives, I believe germline editing should not be permitted at this moment in time. There are several issues still unresolved that suggest more work needs to be done before allowing CRISPR in reproductive cells. To name a few, there is a lack of consent from editing embryos, the genetic edits done would be irreversible, and there should be a clear understanding of the distinction between treatments and enhancements before proceeding any further.

In Part 7 of *The Code Breaker* (Isaacson, 2021), the distinction between treatment and enhancement is considered, and valid points are raised. Isaacson says, "Genes might predispose or predetermine certain kids to be short or obese or have attention deficits or be depressive. At what point do genetic modifications to fix such traits cross the line from health treatment to enhancement?" (Isaacson, 2021). I support future germline editing that alters health diseases or conditions, but not cosmetic preferences. It is integral to honor our natural appearance and characteristics, which, unless it interferes with daily living, does not

qualify for the use of CRISPR. Not only would it create societal gaps between the wealthy and the poor, but it would also open the door to dangerous procedures performed for cosmetic reasons.

There is also the risk that if germline editing were made available to all, it would be improperly used. When Isaacson discussed a hypothetical situation in which a parent picks and chooses heritable genes for their child, it seemed too easy and accessible to quickly request a handful of desirable traits. " Without any gates or flags, we might all go barreling down at uncontrollable speed, taking society's diversity and the human genome along with us." (Isaacson, 2021). To avoid all these possible implications, I believe it is necessary to continue the topic on regulations that can be implemented if germline editing exists and have a plan for strong scientific oversight with this ethical consideration.

Conclusion

CRISPR-Cas9 has proven to be a groundbreaking tool capable of altering nature itself, offering the potential to cure diseases and transform medicine. What started as a bacterial immune defense got revolutionized into arguably the most powerful gene-editing tool known to man. Currently, 80% of patients with triple-negative breast cancer have no good option for therapy (Wofford, 2024). The application of in vivo CRISPR screens in model organisms, such as mice, demonstrated successful reduction of TNBC tumors, promising new avenues for targeted cancer immunotherapies.

The identification of the Lgals2, Mga, and Cop1 genes plays a major role in uncovering targets to enhance the effectiveness of cancer immunotherapies. Without disrupting the function of these genes using CRISPR-Cas9, their implications on immune regulation would remain unknown; for instance, in the case of Lgals2, continued signaling promotes macrophage recruitment that protects and sustains tumor growth. But with knowledge of the depletion of tumor growth when Lgals2 is knocked out, carefully curated immunotherapies can be developed to inhibit its expression and enhance anti-tumor immune responses.

Looking ahead, CRISPR holds immense potential to revolutionize medicine and our understanding of biology. However, as its capabilities expand, so does the need for careful ethical oversight. Balancing scientific advancement and ethical responsibility will require global collaboration, strict regulations, and a commitment to using gene-editing technologies to alleviate suffering rather than to enhance traits for non-medical purposes. In the case of germline editing, the stakes are even higher with possible off-target effects that cause more harm than good in future generations. Upholding ethical standards while embracing innovation will be key to ensuring CRISPR's future serves humanity responsibly.

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A Review on Ivan Petrovich Pavlov

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Ivan Pavlov has, in a way, become a household name, as phrases like “Pavlov’s dogs” and “Pavlovian reflex” are still used today and are common examples in psychology classes. It’s interesting that while he had a massive and lasting impact on psychology research, the Nobel Prize he received was actually for the “physiology of digestion”. This might show just how vital his research was for further development in different scientific fields.

The circumstances of his Nobel Prize award in 1904 are interesting, considering his competitors. He seems to have been nominated only 4 times that year, while Elie Metchnikoff was nominated 19 times for notable discoveries in cellular physiology and pathology, especially phagocytosis, which seems much more important than simple digestion. However, previous nominations shed more light on this. In 1901, Pavlov was nominated a whopping 33 times. There were, of course, a lot of other notable nominees for physiology and medicine that year, such as Ramon y Cajal, von Behring, Golgi, and many more. It’s not that Pavlov’s research wasn’t impactful, but that there were so many other important discoveries in medicine early on that his award was delayed. Pavlov did achieve widespread recognition in his home country and had formal laboratory training (Rozo, 2017), which may have given him an edge in technique and opportunities that less formally trained candidates lacked. This relates to the ideas of greatness vs. great discoveries discussed in *Road to Stockholm*. A great discovery may seem relatively small if competition is fierce, and merit alone is not enough to secure an award.

Pavlov’s research most commonly relied on dogs as test subjects, as described in his own Nobel lecture and published work. He studied the activity of digestive glands and the secretion of digestive juice in response to various stimuli and diets. Surgery was performed on the subjects to collect their secretions and measure them. This involved diverting the digestive glands from the stomach canal into external tubes, and cutting the stomach nerves to create an “isolated stomach-wall pouch” (Pavlov, 1904). Various stimuli were then presented to the dogs, focusing on taste, sound, and sight, such as showing them a loaf of bread. The sight of the food would stimulate the salivary glands, but if the dog was not given the bread to eat, this salivation would stop (Pavlov, 1904). Feeding the dog the bread would then reignite the salivation reflex. This demonstrated a conditioned reflex, which later became a major topic in psychological studies known as classical conditioning.

Pavlov expresses sympathy for the dogs during his Nobel lecture and mentions the use of anesthesia during surgery, coupled with sanitation and wound care, but there may be some wonder whether this was done specifically for a public speech and to present a nicer image of the experiments. While the dogs were described as enthusiastic to participate, this still raises a number of ethical concerns that limit the replication of experiments like this today. In other experiments he performed, fistulas were also created in the dog’s stomach and esophagus, leading to an external opening from which digestive juice could be collected during “sham feedings” (Wood, 2004), as the dogs were unable to swallow the food they had eaten. This was used to collect pure gastric juice for other studies. Modern summaries and pop culture often omit the details of Pavlov’s experiments and sometimes describe the dogs only as drooling, failing to mention the operations performed on them. The more gruesome aspects of Pavlov’s legacy were filed down because they might sit poorly with modern audiences. However, these techniques were still used in recent experiments, such as a 2021 study. A study on the effects of the peptides bombesin and neurotensin on gastric secretion included 14 dogs that underwent gastrointestinal surgery and received a “Pavlov pouch” for gastric juice collection (Tsalis et al., 2021). While the methods may appear unpleasant, it seems that Pavlov’s techniques were precise enough to still be used today and carry his name and his legacy.

Just like his surgical procedures, Pavlov’s methods of stimulus and behavior association are still relevant today. Classical conditioning and the conditioned reflex are still very prevalent in psychology teachings, and are even commonly used as therapy for human patients. A conditioned reflex control technique (CRCT) based on classical conditioning can be used to treat behavior addictions, including drug addiction, kleptomania, obsessive-compulsiveness, and many others (Park, 2023). Pavlov’s research pinpointed a mechanism that not only applied to dogs and their digestion but also had effects far outside his initial experiments.

Pavlov’s research and discoveries contributed greatly to both physiology and psychology, and his techniques are still in practice today. While his methods can be considered controversial, the same is true of many early Nobel laureates. Our scientific progress exists on a foundation that can’t be forgotten just because it’s unpleasant, and the same is true of the people responsible for it. However, this begs the question of whether great discoveries would really be possible without these early unethical experiments, and whether Greatness as a scientific achievement really corresponds to the greatness of personal moral character.

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*This author wrote this paper for Biology 485:The Nobel Prizes taught by Dr. Brett Palmero.

The Lateral Line System of *Pristella maxillaris*

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Introduction

The lateral line system, present in aquatic amphibians and over 30,000 fish species, is a mechanosensory system—a sensory structure that responds to mechanical changes such as water movement and pressure fluctuations—and is crucial for behaviors such as prey and predator detection, navigation, and intraspecific (within-species) interactions (Bleckmann & Zelick, 2019; Faucher et al., 2010; Webb, 2011). The lateral line system consists of neuromast receptors—clusters of sensory hair cells—that detect water flow along the head, trunk, and tail (Webb, 2011).

Neuromasts are sensory organs found within the skin's epithelial layer. They contain two main cell types: sensory hair cells, which detect movement, and nonsensory support cells (Webb, 2011). In bony fish such as *Pristella maxillaris*, there are two classes of neuromasts: canal neuromasts, which are located in fluid-filled canals and detect changes in water acceleration, and superficial neuromasts, which are found on the skin surface and detect water velocity (Webb, 2011). The combined input from these neuromasts helps fish sense their environment and guide their behavior.

Pristella maxillaris, known as the X-ray fish due to their translucent body, is a shoaling, or grouping, fish inhabiting the Amazon, Orinoco, and Guianas Rivers (Bian et al., 2019). Due to morphological diversification and mutations, the coloration of *Pristella maxillaris* varies from wild-type black-gray to mutant I silver-white or mutant II transparent (Bian et al., 2019). Furthermore, genetic modification has produced fluorescent varieties known as Glo Pristellas. While research exists on their growth, development, and coloration, limited studies have examined the lateral line system of *Pristella maxillaris*, particularly the fluorescent Glo Pristella.

The experiment examined Glo Pristellas, a genetically modified *Pristella maxillaris* that expresses fluorescent proteins. The standard lateral line examination protocol involves fluorescent staining and imaging, which may pose unique challenges with the fluorescent variant. Furthermore, variation in the lateral line system across species, based on habitat and behavior, suggests potential differences in Glo Pristellas compared to their unmodified counterparts.The experiment uses fluorescence staining techniques to observe the lateral line system of the Glo Pristellas, aiming to identify the external anatomy and the lateral line system along the head and body. Due to their shoaling behavior, it is hypothesized that Glo Pristellas will exhibit a high density of neuromasts on their heads, as observed through fluorescence staining.

Methods

The following procedure was followed based on the "Lab 5 – Fish Lateral Line System Student Protocol" provided by Dr. Schwalbe and *Lateral Line Structure* by JF Webb (Schwalbe, 2024; Webb, 2011).

Apparatus preparation

Before obtaining the fish, the apparatus was prepared. Prior to the lab, the digital camera was mounted on a dissecting microscope, which was connected to a monitor via an HDMI cable and to a laptop via a USB cable to produce the visual output. Therefore, the laptop was turned on, and the CaptaVision+ software was opened. Once the camera was on and connected, the microscope's field of view was visible on the monitor. After testing the camera, the apparatus was prepared for the experiment.

Preparing the fish

First, the fish was selected from Dr. Schwalbe's Lab, Lillard 176. For this experiment, a *Pristella maxillaris*, commonly known as Glo Pristellas, was selected. Using a net, the fish was carefully removed from the fish tank and transferred to a 200 mL beaker of conditioned tap water.

Once back in the lab room, the fish was transferred, using a net, into a 100 mL beaker containing 4-di-2-ASP for 5 minutes. This solution was orange and served as the fluorescence stain that was imaged later in the experiment. After 5 minutes of staining, the fish was transferred, using gloved hands to prevent staining the net, to a beaker containing 100 mL of MS222. This solution was a muscle relaxant that humanely euthanized the fish; therefore, it was important to take precautions and prevent skin contact. After five minutes and verification by Dr. Schwalbe that the fish was no longer moving or moving its gills, the fish was transferred, using gloved hands, to a sylgard-lined petri dish prepared with 1:1 conditioned tap water and MS222.

Using a ruler placed beneath the petri dish, the total length, from the mouth to the edge of the caudal fin, was measured and recorded in Excel. The fish was then pinned in the middle of the petri dish using insect pins. While the pin placement varied across views, the first view imaged was the lateral view. Therefore, the fish was pinned with its mouth facing left and its left side facing upward. When pinning, it was important to take precautions to avoid piercing the body and disrupting neuromasts. The fins tore easily; therefore, pins were placed on the edge of the body and at an angle to prevent obstructing the camera view.

Imaging the fish

Three views of the fish were imaged: lateral, dorsal, and ventral. After the fish was secured to the petri dish with insect pins, the petri dish was placed under the microscope. For the lateral view, the fish was pinned with its mouth facing left and its left side facing upward, as previously described, with the pins placed along the edge of the body. For the dorsal and ventral views, the pins were placed in a "V" formation to create a supportive structure that prevented the fish from moving while avoiding pinning through the fish.

Picture information was recorded in Excel for record-keeping and easy retrieval. Images were captured in both bright and fluorescent light for all views, as outlined below.

First, a fiber-optic light or a bright light was used. The microscope was focused, and the fish was observed by moving it around underneath the microscope. The external anatomy was identified, using the bright light to examine the eyes, gills, scales, fin rays, and tail. Images were taken to record the anatomy.

Second, the fluorescent light was used. To prepare the microscope, the provided yellow filter was placed underneath the microscope objective lens. With the Glo Pristellas used in the experiment, it was challenging to observe the fluorescent neuromasts due to their color. Therefore, a bright light was kept on in the background to help with visualization. The exposure was set to 3 seconds. The microscope was focused, and the fish was observed by moving it around underneath the microscope. Important lateral line structures in each view included head canals, trunk canals, and clusters of superficial neuromasts. Images were taken to record the anatomy and lateral line structures. When finished with fluorescence imaging, immediately turn off the light, do not look directly into it, and remove the yellow filter.

The fish was re-pinned for each new view, and imaging was repeated with both bright and fluorescent light. Images were saved to the computer, then used for data analysis. Picture information was recorded in Excel for proper record keeping and easy image retrieval.

Clean-up

Once all images were obtained and properly organized, the bright field and fluorescence lights were turned off. For disposal of the fish, it was unpinned and placed in the provided Ziplock bag for proper disposal by Dr. Schwalbe. The 4-di-2-ASP and MS222

*This author wrote this paper for Biology 340: Animal Physiology taught by Dr. Margot Schwable.

solutions were disposed of in the provided containers, not in the sink. Lastly, the station was wiped completely with 70% ethanol, including personal devices, to prevent transfer of MS222 out of the lab.

Data analysis

For this experiment, no quantitative data were collected. As a result, data analysis involved manually stitching images of the fish together for full-length views. This was done in PowerPoint, where multiple images were uploaded and properly aligned to create a cohesive image of the fish rather than individual pieces. While CaptaVision+ offers a stitching feature, some views remained difficult to obtain with it.

Results

Results summary

Fluorescent staining and imaging were performed for the lateral line system, including the canal and superficial neuromasts, of the Glo Pristellas variant of the *Pristella maxillaris*. The distribution of the lateral line system along the body, from rostral to caudal, was examined in lateral, dorsal, and ventral views. When examining the distribution of the lateral line system, as exhibited in Figures 2, 3, and 4, a high density of canal neuromasts was observed in the naris, infraorbital, postotic, and mandibular regions surrounding the eyes, nares, and mandible.

Neuromasts were less prominent in the ventral view of the fish than in the lateral and dorsal views. In the trunk, neuromasts were less dense, but superficial neuromasts were visible along the lateral view. Neuromast visibility was lower in the tail and fins. Based on fluorescent staining and imaging, the lateral line system of *Pristella maxillaris* is widely distributed throughout the body, with a high density towards the rostral, dorsal end.

Tables and figures

Lateral Line Distribution, Neuromast Abbreviations	Lateral Line Distribution, Neuromast Abbreviations
Infraorbital	IO
Mandibular	MD
Naris	N
Otic	OT
Preopercular	PR
Postotic	PO
Supraorbital	SO
Supratemporal	ST
Trunk	T

Table 1. Lateral line distribution and neuromast abbreviations. The table above lists the neuromasts labeled in the following pictures of the *Pristella maxillaris,* along with the abbreviations used to denote them. In summary, the definitions for the abbreviations are as follows: IO, infraorbital; MD, mandibular; N, naris; OT, otic; PR, preopercular; PO, postotic; SO, supraorbital; ST, supratemporal; and T, trunk.

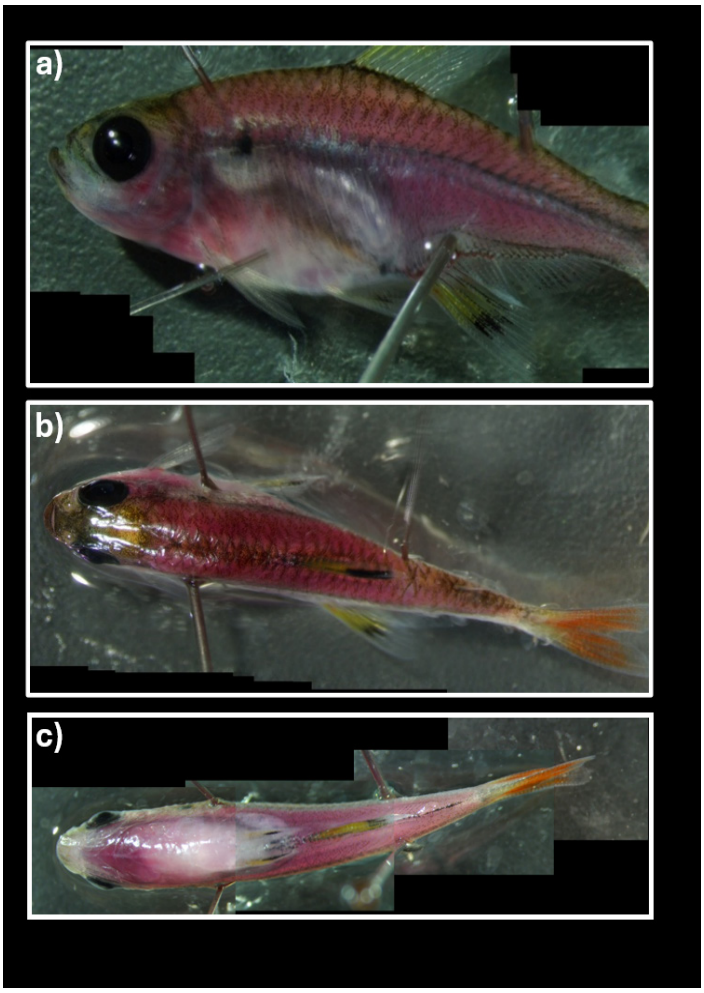

Figure 1. Bright light view of *Pristella maxillaris*. The figure above shows the bright-light lateral, dorsal, and ventral views of *Pristella maxillaris* (Glo Pristellas) examined in the experiment. The fish was placed in a petri dish of 1:1 conditioned tap water and MS222 and pinned using insect pins. The lateral line system was not visible in the bright field. Figure 1a) is the lateral view of *Pristella maxillaris*. The image was stitched using CaptaVision+ and a microscope at 6.3X magnification. Figure 1b) is the dorsal view of *Pristella maxillaris*. The image was stitched using CaptaVision+ and a microscope at 6.3X magnification. Figure 1c) is the ventral view of *Pristella maxillaris*. Stitching was performed manually on PowerPoint.

Discussion

This experiment hypothesized that Glo Pristellas would exhibit a high density of neuromasts on their head, potentially helpful with their shoaling behavior. Using fluorescence staining, the experiment visualized the lateral line system and confirmed a significant concentration of canal neuromasts in the naris, infraorbital, postotic, and mandibular regions, as shown in Figures 3 and 4, supporting the hypothesis.

Glo Pristellas are a social shoaling fish that potentially rely heavily on their lateral line system. This aligns with research by Faucher et al., which examined the importance of the lateral line system for accurate shoaling behavior in *Hemigrammus bleheri (firehead tetras)*. The experiment concluded that fish deprived of the lateral line system cannot maintain a shoal, causing them to move further apart rather than closer, indicating the importance of the lateral line system in shoaling behavior (Faucher et al., 2010). The observed neuromast density in Glo Pristellas may support their social interactions.

Given the limited research on the lateral line systems of *Pristella*

maxillaris, this experiment offers valuable insights into new fish species. Specifically, it introduces experiments regarding the relationship between the color variations, such as translucence and fluorescence, and lateral line function. In particular, it would be interesting to conduct further research investigating how fluorescence affects lateral line distribution and shoaling behavior under varying light conditions in Glo Pristellas.

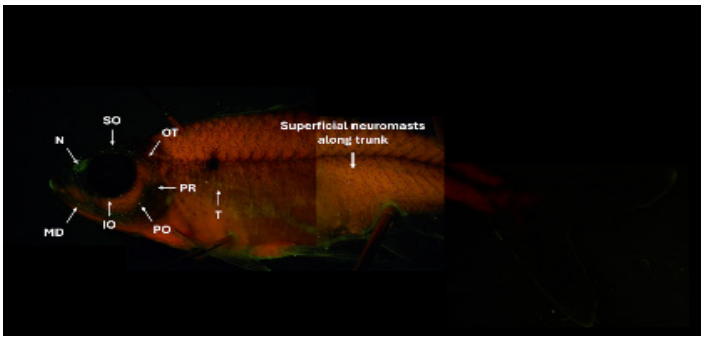

Figure 2. Lateral distribution of the lateral line system in *Pristella maxillaris.* The image above is the fluorescent light lateral view of the *Pristella maxillaris* (Glo Pristellas) examined in the experiment. The fish was placed in a petri dish of 1:1 conditioned tap water and MS222 and pinned using insect pins. The image was captured using CaptaVision+ and a microscope at 6.3X magnification. Stitching was performed manually on PowerPoint. The lateral line system was visible throughout the body in the fluorescence view, as labeled in the image above. Not observed in the lateral view were ST, supratemporal, which are expected across the top of the head behind the eyes.

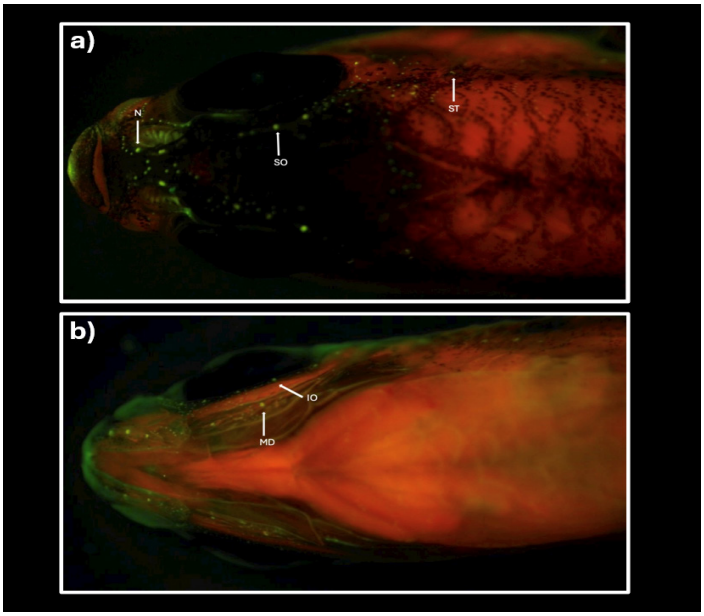

Figure 3. Distribution of the lateral line system in *Pristella maxillaris*. The figure above is the fluorescent light dorsal and ventral views of the *Pristella maxillaris* (Glo Pristellas) examined in the experiment. The fish was placed in a petri dish of 1:1 conditioned tap water and MS222 and pinned using insect pins. Figure 3a) is the dorsal view of *Pristella maxillaris*. The image was captured using CaptaVision+ and a microscope at 10X magnification. Several neuromasts were visible on the dorsal view of the head. Figure 3b) is the ventral view of *Pristella maxillaris*. The image was captured using CaptaVision+ and a microscope at 8X magnification. In the ventral view, the lateral line system was not as visible compared to lateral and dorsal views, though several neuromasts were still visible towards the rostral end of the fish, such as the mandibular and infraorbital neuromasts.

In addition, this experiment has broader scientific implications. An experiment on *Danio rerio (zebrafish)* lateral line systems conducted by Young et al. revealed that stormwater containing toxicants damages neuromasts, leading to fewer neuromasts with fewer hair cells (Young et al., 2018). This indicates the potential of the lateral line system as an environmental health indicator. Additionally, Holmgren et al. used zebrafish lateral line hair cells, which are structurally and functionally analogous to cochlear hair cells, to examine damage caused by noise, aging, and ototoxic drugs (Holmgren & Sheets, 2021). This underscores the value of the lateral line system as a model for developing therapies for human hearing loss.

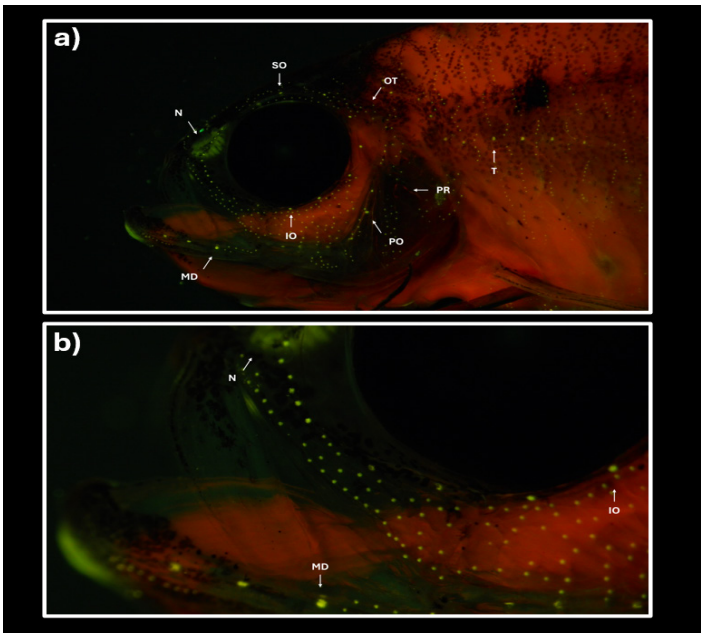

Figure 7. Close-up of the lateral head distribution of the lateral line system in *Pristella maxillaris*. The image above is the fluorescent light lateral view of the *Pristella maxillaris* (Glo Pristellas) examined in the experiment. The fish was placed in a petri dish of 1:1 conditioned tap water and MS222 and pinned using insect pins. The image captured CaptaVision+ and a microscope. Figure 7a) is a close-up of the head of *Pristella maxillaris*. The image was captured using CaptaVision+ and a microscope at 8X magnification. Figure 7b) is a further examination of the infraorbital region. The image was captured using CaptaVision+ and a microscope at 20X magnification. Not observed in the lateral view were ST, supratemporal, which are expected across the top of the head behind the eyes.

Therefore, this experiment validates the hypothesis regarding neuromast distribution in the heads of Glo Pristellas and provides a foundation for future studies on variations in the lateral line system and their broader applications in conservation and human health.

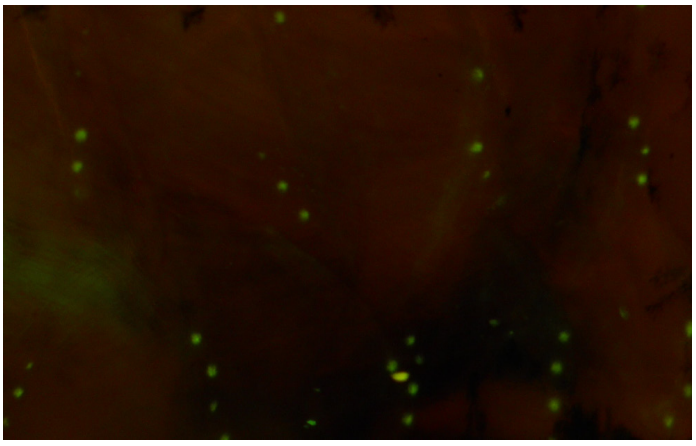

Figure 8. Close-up of the lateral trunk distribution of the lateral line system, specifically the superficial neuromasts, in *Pristella maxillaris.* The image above is the fluorescent light lateral view of the *Pristella maxillaris* (Glo Pristellas) examined in the experiment. The fish was placed in a petri dish of 1:1 conditioned tap water and MS222 and pinned using insect pins. The image captured CaptaVision+ and a microscope at 40X magnification.

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The Effects of Food Distance and Quality on Eastern Gray Squirrel Foraging Preferences

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Introduction

To survive, prey organisms must both find enough food and avoid predators, and there are trade-offs between these two necessities. To find food, an organism must forage in its environment, risking predation. Organisms balance food intake and this danger, which is the basis of the "optimal foraging theory," which posits that organisms will maximize energy gains while minimizing energy costs (including predation risk) while foraging (St. Juliana et al., 2017).

For animals that are frequently preyed upon, like rodents, optimal foraging is especially important, and the risk of predation dictates their foraging behavior. For example, white-footed mice (*Peromyscus leucopus*) forage more during darker nights (Jacob et al., 2017) and gerbils (*Gerbillus andersoni allenbyi* and *Gerbillus nanus*) forage more in higher vegetation and in nights with less illumination (St. Juliana et al., 2017). Both conditions (darkness and increased vegetation) lower predation risk, thereby reducing foraging costs and allowing greater risk-taking and longer foraging periods. However, it remains unknown whether eastern grey squirrels (*Sciurus carolinensis*) also exhibit this optimal foraging behavior, especially when they are offered food in different forms, are frequently disturbed by predators in a suburban environment, and must travel varying distances to reach their foraging area.

In this study, we attempted to resolve some of these questions regarding the eastern gray squirrel by measuring their giving-up densities (GUDs), or the weight of food at each feeding spot (density) at which the squirrels stopped foraging (St. Juliana et al., 2017). A lower GUD implies more foraging and more food eaten. We hypothesized that foraging squirrels would prefer food from a tray that was closer to a tree more than food from a tray that was farther from a tree, and we predicted that the squirrels would have lower GUDs in the closer tray than in the farther tray. We hypothesized and predicted this because food farther from a tree has a higher predation risk for squirrels, and, according to optimal foraging theory, squirrels should spend less time foraging in areas with higher predation risk and thus have higher GUDs in these areas. We expected this to be especially true given the location of this experiment, which is exposed and frequently disturbed by domestic dogs (*Canus familiaris*). The presence of domestic dogs and cats has been shown to reduce rodents' foraging activity (Mahlaba et al., 2017).

Additionally, we hypothesized that squirrels would prefer food from a tray that was close to a tree and had unshelled peanuts more than food from a tray that was far from a tree and had shelled peanuts. We predicted that these squirrels would have lower GUDs in the close and unshelled peanut tray, and higher GUDs in the far and shelled peanut tray. We hypothesized and predicted this because although shelled peanuts are a more attractive food source because they require less energy to consume, they are farther from a tree and thus pose a greater risk of predation. In an area frequently disturbed by predators such as domestic dogs, we predicted that the risk of predation would outweigh the benefits of eating shelled peanuts, and that squirrels would prefer a lower-energy reward with lower predation risk by eating unshelled peanuts close to a tree.

Methods

We performed these experiments in Northbrook, Illinois, United States of America (42°7'15" N 87°51'33" W) in a suburban backyard. The yard was fenced in and dominated by short grass, with intermittent shade from large trees. In both experiments, we filled two 60 x 30.5 x 6.5cm trays one-third full of sand. We placed one tray close (0.5 meters) and one far (8.5 meters) from a large sugar maple (*Acer saccharum*) in one of the yard's corners, which connected the tree to the fences and to multiple other large trees and dense vegetation. We placed specific amounts of peanuts in both trays, burying most and leaving some partially exposed so the squirrels knew food was present. We left the squirrels to forage, and at the end of each trial, we sifted through the trays and weighed the remaining peanuts in each tray using a Pelouze X1 kitchen scale to determine the GUDs. We also noted the start and end times, the temperatures, and the weather for each trial. We discarded the data from a trial if both trays had no peanuts, if one tray had all its peanuts and the other had none, or if both trays had all their peanuts remaining. Finally, between (never during) trials at random intervals for both experiments one and two, we set three domestic dogs free in the yard to disturb the squirrels as part of the dogs' regular routine.

Experiment 1

We placed 45 grams of unshelled peanuts in each tray. After an initial 2-hour acclimatization period, we began with 30-minute trials and gradually decreased the duration to 15-20 minutes as the squirrels were conditioned to eat from the trays. We conducted 32 trials, averaging 25 minutes each, over eight days in November (11-5-24 to 11-12-24), in the late mornings and afternoons. We used a one-tailed paired T-test in Microsoft Excel to analyze this data.

Experiment 2

We buried 29 unshelled peanut kernels (with between one and three kernels per peanut shell) in the tray close to the tree, and 22.7 grams of shelled peanuts in the tray far from the tree. We conducted 19 trials, averaging 20 minutes per trial. We did this experiment over four days in November (11-14-24 to 11-17-24) in the late mornings and early afternoons. We used a one-tailed paired T-test in Microsoft Excel to analyze this data.

Results

For the first experiment, we placed two trays with unshelled peanuts at different distances from a tree. Averaged over 32 total trials, with the average trial length being 25 minutes, the tray close to the tree had significantly lower GUDs than the tray far from the tree (Fig. 1) ($p < 0.001$).

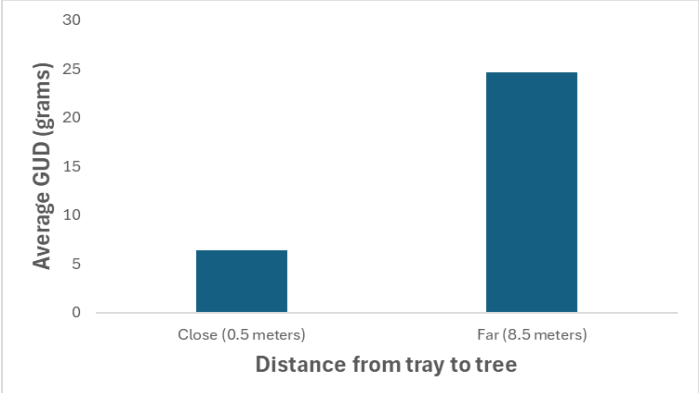

Figure 1. The average GUDs for trays at varying distances from tree cover. We filled two trays with sand, one-third full, and buried 45 grams of unshelled peanuts in each. We placed one tray close (0.5 meters) to a tree, and one tray far (8.5 meters) from a tree. We measured the GUDs for each tray after each trial for 32 total trials (with an average trial length of 25 minutes) and performed a paired one-tailed T-test via Microsoft Excel. We discarded trials with no peanuts eaten in either tray, all peanuts eaten in both trays, or all eaten in one and none in the other. The tray close to the tree had significantly lower GUDs than the tray far from the tree ($p < 0.001$).

For the second experiment, we placed one tray with unshelled peanuts close to a tree and one tray with shelled peanuts far from a tree. Averaged over 19 trials, with the average trial length being 20 minutes, the tray close to the tree with unshelled peanuts had significantly lower GUDs than the tray farther from the tree with shelled peanuts (Fig. 2) ($p=0.028$).

*This author wrote this paper for Biology 220: Ecology and Evolution taught by Dr. Josh Hedge.

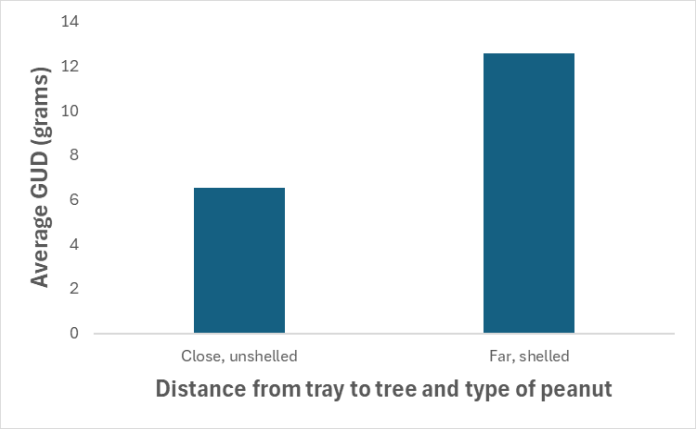

Figure 2. The average GUDs for trays at varying distances to tree cover and varying food rewards. We filled two trays with sand, one-third of the way. We buried 29 unshelled peanuts (each kernel inside a peanut shell counted as one peanut) in the tray close (0.5 meters) to a tree, and 23 grams of shelled peanuts in the tray far (8.5 meters) from a tree. We measured the GUDs for each tray after each trial for 19 total trials (with an average trial length of 20 minutes) and performed a paired one-tailed T-test via Microsoft Excel. We discarded trials with no peanuts eaten in either tray, all peanuts eaten in both trays, or all eaten in one and none in the other. The tray close to the tree with unshelled peanuts had significantly lower GUDs than the tray farther from the tree with shelled peanuts (Fig. 2) (p=0.024).

We did not observe any other predators or instances of successful hunting by the domestic dogs. Additionally, during both experiments, we saw blue jays (*Cyanocitta cristata*) taking peanuts (mostly unshelled) from the trays, but these were rare events. Finally, we observed intense competition among the squirrels: sometimes five were foraging at once, and only one would use a tray at a time.

Discussion

In the first experiment, we observed that the GUDs of the close tray were significantly lower than the GUDs of the far tray. Thus, we reject the null hypothesis that there would be no relationship between tray distance and GUD. In the second experiment, we observed that the GUDs of the close and unshelled peanut tray were significantly lower than the GUDs of the far and shelled peanut tray. Thus, we reject the null hypothesis that there is no relationship between tray distance, food quality, and GUD. We observed only minor sources of error in this study, mainly weather variation, blue jays taking peanuts, and intense intraspecific competition among the squirrels. The first two errors likely did not affect our results, as blue jay sightings were rare and the weather varied only slightly. With regards to the intraspecific competition, we are unsure what effect it had on the experiment, if any. Most probably, it had a leveling effect, lowering the GUDs for both trays in both experiments. Because food was scarcer due to competition, the squirrels had to forage more in both trays. However, more experiments would be needed to confirm this. In any case, these errors are unlikely to have changed the overall trends in our results.

These results are what we expected. From both experiments, we saw that squirrels optimize their foraging for low risk by foraging close to tree cover. The squirrels' preference in the second experiment for the close, unshelled peanuts is also what we expected according to this logic. The increased cost and predation risk for the far, shelled tray were amplified by frequent disturbances in the area from three domestic dogs. While these individuals were extremely inefficient predators with zero recorded captures, they still represented a threat that increased GUDs at the far tray. Thus, the risk of predation outweighed the benefits of a more energy-efficient food source, as we predicted. This result agrees with research reporting that rodents forage less in areas disturbed by domestic cats and dogs (Mahlaba et al., 2017). These results also agree with other research on optimal foraging in rodents. For example, the behavior of white-footed mice in response to illumination levels and vegetation cover (Jacob et al., 2017) and the behavior of gerbils in response to moonlight and cloud cover (St. Juliana et al., 2017) follow optimal foraging theory. Illumination, vegetation, and cloud cover all affect predation risk, and these organisms increased their GUDs in response to this increased predation risk.

Thus, this experiment adds to a growing body of research reporting the optimal foraging behavior of rodents, specifically their decreased foraging in response to increased predation risk. This is especially important in a world that is increasingly developed, urbanized, and deforested. As cover for rodents and indeed any animals is removed, individuals will be forced to forage farther away and subject themselves to increasing predation risk and decreasing energy gain, which will harm populations and thus their biodiversity. Increased vegetation, especially tree cover, could be added to open, deforested, or urban areas to provide organisms with safe places to forage at lower predation risk. Thus, planting and conserving vegetation would allow us to align with the optimal foraging strategies of endangered animals by providing foraging areas where they can forage at lower GUDs to gain more energy with lower risk.

Future studies could determine whether predation risk or the cost of travel is the more important factor for GUDs, as this was unclear in our study. Although we assumed predation to be the more important factor, because the area is frequently disturbed by predators, and the eight-meter distance between the trays represents a relatively small energy cost, this is not necessarily true. This is because the farther trays cost more energy to reach *and* are more open to predation (both of which would raise GUDs). Furthermore, studies could be conducted to determine the effects of optimal foraging behaviors in endangered populations and areas, particularly in fragmented habitats where organisms may need to cross dangerous edge areas to forage. This would allow us to determine what we can do to increase their energy gains and decrease their costs, for example, by planting vegetation or restoring habitat.

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Growth of Fungal Endophytes Isolated from *Quercus macrocarpa* Roots and *Acer rubrum* Bark

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Plants harbor rich microbial communities both internally and externally. Endophytes are fungi and bacteria that colonize internal plant tissues without causing harm and may even benefit their hosts. To better understand endophyte ecology, this study compared the growth of fungal colonies isolated from Quercus macrocarpa root and Acer rubrum bark tissues under different culture conditions. Samples were collected from Lake Forest College and cultured on Potato Dextrose Agar (PDA) or Malt Extract Agar (MEA) media to test the hypotheses that 1) endophytes would exhibit greater growth (mm^2) on MEA compared to PDA and 2) endophytes coming from different tissues would have differing competitive abilities, influencing the growth they show on differing media. The results contrasted the first hypothesis as nutrient media had no effect on endophyte growth in general, or for these two specific species on these specific media. However, our second hypothesis was supported, showing significant interactions for growth between the two tissues on PDA compared to MEA. This suggests that competition between distinct species may yield significant difference in growth on various media. These findings suggest that while growth media may not affect endophyte growth uniformly, the tissue source significantly influences competitive interactions. Future research should explore the mechanisms underlying these competitive dynamics, including potential resource partitioning and allelopathic effects. This study contributes valuable insights into endophyte ecology, emphasizing the need for further investigation of their interactions across diverse plant species.

Introduction

Plants are complex in many ways, and one facet in which this can be observed is through their ecology. Many interactions take place throughout the plant body. Some may be visible, while others are not. Zooming in, plant-microorganism interactions are taking place both internally and externally throughout the plant body. These interactions can impact either organism in this relationship in various ways. Some interactions may cause harm to either of the individuals, but in other cases, both individuals may benefit, only one may, or none (Berg, 2009; Evert & Eichorn, 2012). Endophytes are fungi that occur ubiquitously within all known plants, occasionally creating mutualisms with their hosts (Sun & Guo, 2012). Recently accepted descriptions of endophytes characterize their colonization as occurring within internal plant tissues without harming the host (Compant et al., 2010; Petrini, 1991). This contrasts with epiphytes, which are characterized as organisms living on the external surfaces of the host plant (Petrini, 1991). Previous studies researching the diversity of endophytes have found that *Ascomycetes* and *Basidiomycetes*, 2 major phyla of fungi, commonly colonize deciduous and coniferous trees (Arnold et al., 2007). This means that endophytes can be found in even the most suburban neighborhoods of the United States.

Studies have shown that fungi, such as endophytes, harbor pharmaceutical potential, and being ubiquitous in all known plants, there are many reasons why the study of endophytes is warranted (Arnold et al., 2000). Endophytic fungi have also been noted to produce beneficial bioactive chemicals for their hosts and to decompose leaf litter (Sun & Guo, 2012), making them important for ecosystem services and energy recycling. Endophytes are commonly isolated utilizing sterilization techniques of the tissue sample of interest. While the epiphytes are removed, the prevailing endophytic fungi are then left to grow as colonies on nutrient-rich media, often consisting of agar. Understanding the essential growth factors that are best suited to cultivate a particular species, such as an optimal growth medium, can aid research in efficiently producing substantial endophyte colonies of interest.

Other research has shown that cultivation-dependent techniques most commonly utilize standard Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), and Sea Water Agar (Sun & Guo). Once transferred onto this media, colonies may reveal themselves to be fast- or slow-growing endophytic fungi. Growth of endophytic communities has been shown to display not only primary and secondary colonizers, but facilitation and inhibition (competition) between species as well (Saunders & Kohn, 2008). This begs the question of not only whether nutrient media affects the growth of a given species, but also whether nutrient media affects the competition of two distinct endophytic species as well.

The goal of this study was to compare the growth of two distinct endophyte colony isolates individually placed upon PDA and MEA, as well as the growth of the two species competing on each medium. It was predicted that endophyte growth would be higher on malt extract agar (MEA) than on Potato Dextrose Agar (PDA) for both root and bark isolates. It was also predicted that root and bark isolates would compete on both MEA and PDA. Previous studies have shown that Malt Extract Agar (MEA) support greater endophytes growth out of all commonly used growth media (Torta et al., 2022), so it was hypothesized that MEA would show higher average surface area growth (mm^2) than PDA. Additionally, it was hypothesized that since the bark and root tissues come from different tissues/organs of the plant body, they will have differing competitive abilities influencing the surface area (mm^2) of endophyte growth, which will influence their competition results, regardless of what media they were grown upon.

Results

2.1 Endophyte Growth for Differing Tissue Locations as a Function of Growth Medium

Statistical analyses indicated the average surface area of fungal growth at day 9 was significantly higher for bark than for root tissue, regardless of growth medium ($p = 4.09E-12$). No significant difference was found for the average surface area of growth between MEA and PDA, regardless of tissue location ($p = 0.32$). There was also no significant interaction found for the average surface area of growth between root tissue growing on the two media compared to bark tissue growing on the two media ($p = 0.32$; Figure 1).

2.2 Competition Between Endophytes from Different Tissues on Differing Media

No significant difference was found for the average surface area of growth between MEA and PDA across tissues ($p = 0.24$; Figure 1). However, fungal growth at day 9 was significantly higher from bark than from roots in both media ($p = 1.36\text{-}27$; Figure 2). Furthermore, there was a significant interaction between tissue type and media ($p = 0.01$; Figure 2).

DISCUSSION

This study aimed to compare how nutrient media may affect the growth of cultivated endophytes, alone and in competition. The data did not support the first hypothesis that cultivation on MEA would result in higher average surface area growth for either species. The results show that the nutrient media had no effect on endophyte growth in general, or for these two specific species on these specific media. This aligns with some previous work showing minimal effect of media on endophyte growth (Saunders & Kohn, 2008). However, there was a significant difference in fungal growth from different tissues, suggesting that fungi isolated from roots and bark species may exhibit different growth patterns (Sun & Guo, 2011) and perhaps grow at varying rates.

Similar to the first experiment, fungal growth was significantly

*This author wrote this paper for Biology 348: Plant Biology taught by Dr. Camilla Pizano.

higher in bark than in root endophytes during competition, again showing the distinction between these endophytes, regardless of where or what they grow on. No significant difference was found between growth on either medium, suggesting these fungal species do not exhibit nutrient media preferences. However, a significant interaction between tissues and media suggests that competition between endophytes isolated from these tree species resulted in different outcomes on PDA than on MEA. This suggests competitive abilities may vary between endophyte species or source organs. While growing strains in isolation did not distinguish media performance, biotic interactions revealed growth impacts.

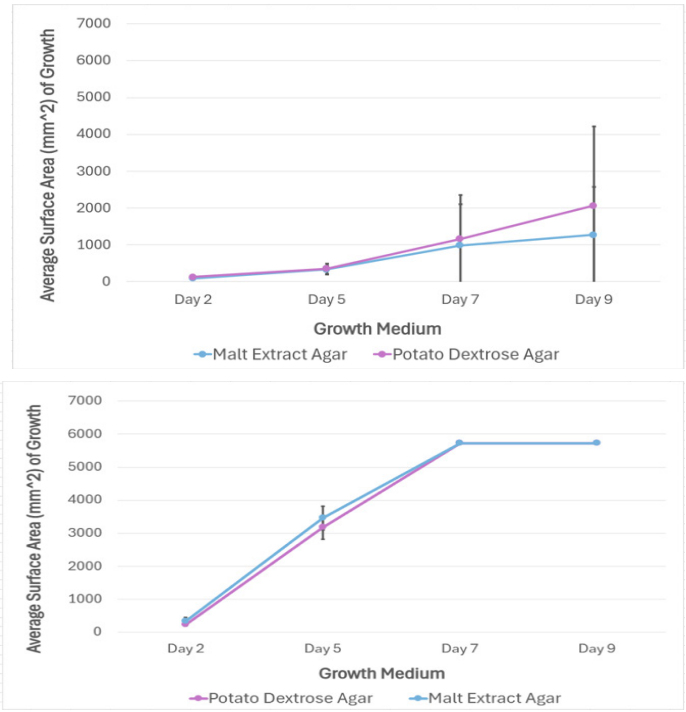

Figure 1. Average surface area (mm²) (± st. deviation) of fungal endophyte growth across 9 days as a function of growth medium for (A) *Quercus macrocarpa* root tissue and (B) *Acer rubrum* bark, with the blue line representing Malt Extract Agar (MEA) growth medium (n=10) and the pink line representing Potato Dextrose Agar (PDA) growth medium (n=10).

These findings provide insight into how interspecific competition could structure endophyte communities in plant tissues (Arnold et al., 2003). However, other mechanisms remain unclear, such as the presence/absence of stressors (Saunders & Kohn, 2008). Future work should examine competitive strategies, such as resource use or the measurement of allelopathic compound production. Identifying endophyte species and investigating interaction mechanisms may help explain their community assembly patterns.

In conclusion, this study advances understanding of endophyte population regulation by demonstrating how competition may influence growth outcomes. While not all hypotheses were confirmed, the results provide direction for additional experiments aimed at resolving the ecological forces that shape endophyte diversity and abundance within host plants. Elucidating these complex multitrophic interactions can enhance research aimed at utilizing endophytes in beneficial ways.

MATERIALS AND METHODS

4.1 Sampling Location

This experiment was originally set up on March 20th, 2024, at Lake Forest College in Lake Forest, Illinois. The sampling area was chosen by sight, upon visual confirmation of deciduous tree cover. One healthy, deciduous tree was chosen at random, which was identified as *Quercus macrocarpa* at coordinates 42□15'04"N, 87□49'40". Using gloves and sterile tools, the research team cut out a small portion of an exposed root of the tree and placed it within a sterile bag. Another healthy deciduous tree was chosen at random, which was identified as *Acer rubrum* at coordinates 42□00'08"N, 85□00'02". Using the same precautions, a small portion of the bark was collected.

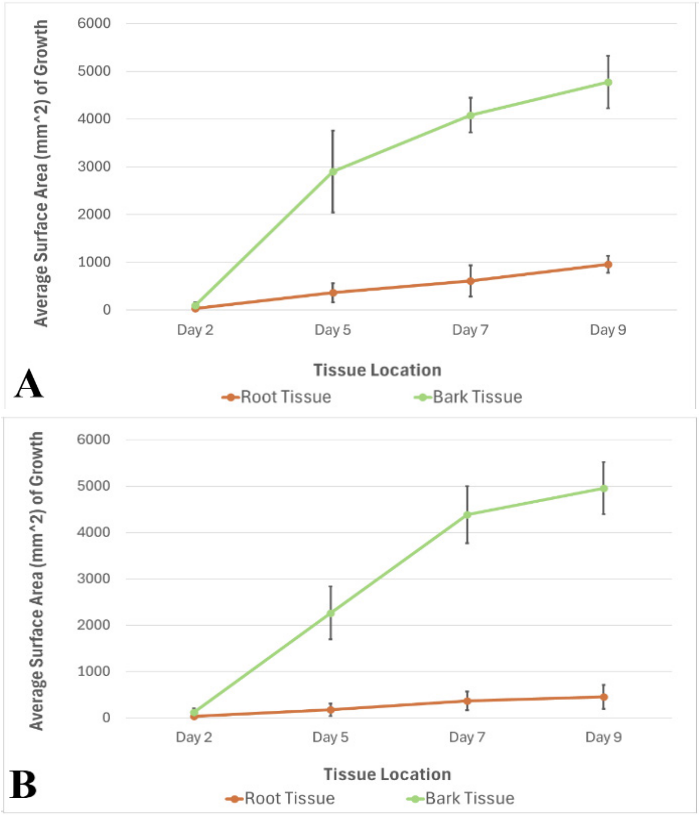

Figure 2. Average surface area (mm²) (± st. deviation) of fungal endophyte from *Quercus macrocarpa* root (n=10; brown) and *Acer rubrum* bark (green) in competition across 9 days on **(A)** Potato Dextrose Agar (PDA) and **(B)** Malt Extract Agar (MEA).

4.2 Experimental Setup

To culture endophytes, researchers must first sterilize their samples. This step is critical for isolating endophytic fungi and is commonly used in endophytic studies (Sun & Guo, 2012). The purpose of this sterilization is to eliminate epiphytic microorganisms within the sample (Petrini et al., 1992). Many studies have used these sterilization techniques to research the spatial affinities of endophytic fungi within their host plants.

Within lab 014 of Lillard Hall on Middle Campus of the college, the research team immediately prepared the samples once they were taken from the trees. To prepare the sterilization treatment, two pieces of glassware large enough to accommodate the samples were used. One glass was filled with 0.5% bleach, while the other was filled with 70% ethanol. Using sterile forceps, the samples were first submerged in 0.5% bleach for 2 minutes. Once this time had elapsed, these samples were transferred to the other glassware and submerged in 70% ethanol for 2 minutes. Following this, each sample was left to grow on Potato Dextrose Agar for 7 days.

On March 27th, 2024, these two colonies were used to create all 60 replicates for the media and media/competition experiments. Each tissue was first separated into 40 equal-sized samples. Sterilizing tweezers in bleach in between each placement, 10 replicates containing only the root tissue were placed upon PDA. Another 10 replicates were then created for the root tissue using MEA. Using a separate set of tweezers, this was repeated using the bark tissue, creating 10 replicates for PDA and 10 for MEA.

The competition experiment consisted of 10 replicates using PDA and another 10 replicates using MEA. To ensure consistent sample placement, each petri dish was marked with positioning labels 3mm from the outer edge. To ensure proper identification, the placed samples were each marked with 'R', designating root tissue, or 'B', designating bark tissue. Using the same protocol as before, 10 replicates were created upon PDA, each containing one sample of root tissue and one sample of bark tissue. This was repeated with MEA to create the 10 MEA competition dishes.

Once created, all the experimental replicates were covered with a petri dish lid, sealed, and labeled. All the replicates were left to grow within lab 014 of Lillard Hall, which is climate-controlled at room temperature.

4.3 Data Collection

After the experimental setup, data was collected by the researchers for 9 days. This took place on days 2, 5, 7, and 9, resulting in 4 data collections. To collect data, researchers spread all experimental replicates on a tabletop, grouping them by tissue and treatment. Each replicate was flipped upside down to allow researchers to determine clear boundaries for fungal growth on the agar substrate. Within the frame of each picture, a ruler marked in mm was laid out next to the experimental replicates to establish a measurement calibration for data processing. These photos were labeled by day, tissue, and treatment to ensure proper organization of the data.

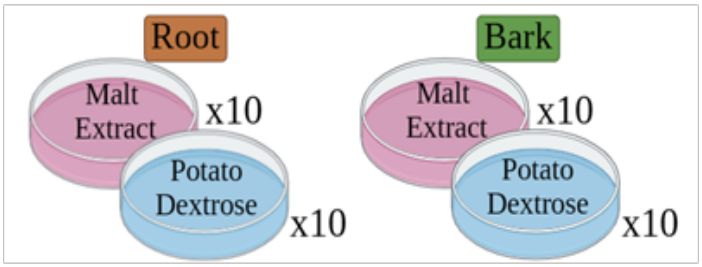

Figure 3. Setup for endophyte growth for differing tissue locations as a function of growth medium. Root and bark tissue colonies were separated into 20 equal samples each using sterilized tweezers. 10 replicates of root tissue alone were cultured on potato dextrose agar (PDA) plates. Another 10 replicates of root tissue were cultured on malt extract agar (MEA) plates. In the same manner, 10 replicates of bark tissue alone were cultured on PDA and another 10 on MEA.

4.4 Data Analysis

Data processing for this experiment was completed using the digital software ImageJ. Photos of each plate were imported into the software, and a measurement calibration was completed using the ruler present in the photos. This measurement calibration enabled the software to accurately calculate the growth's surface area. The area of growth was outlined in the photo, and the software was able to produce a measurement of the surface area in mm^2 of the fungal growth. The analysis for this experiment was completed in Microsoft Excel. Excel was used to record and calculate data, as well as to produce figures encapsulating the results. The measured growth across all 10 replicates for each treatment and tissue was used to calculate an average surface area of growth and a standard deviation for each day of data collection. These calculations were used to produce Figures A, B, C & D. The data was also used to conduct a Two-Factor ANOVA Test with replication. The first factor considered was the location of the tissue, either from the bark or root, and the second factor considered was the treatment the tissue received: MEA or PDA. This test was conducted with an alpha value of 0.05, with any p-value below 0.05 being considered a significant difference, therefore rejecting the null hypothesis that growth media had no effect on the growth of either tissue, or on the competition between them.

Acknowledgments and References

We would like to express our deepest gratitude to Dr. Pizano for her guidance and support throughout this research project. Dr. Pizano went above and beyond to help us with our experiment, provide feedback on our methods and analysis, and connect us with additional resources. Her enthusiasm for research and learning was motivating, and we could not have completed this work without her mentorship. We are also thankful for the Lake Forest College Biology Department and its staff for providing the facilities and materials necessary to conduct our laboratory experiments.

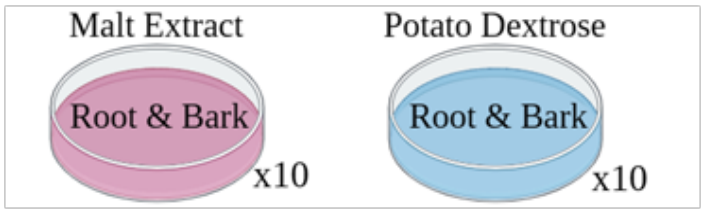

Figure 4. Setup for Endophyte Growth on Differing Growth Media as a Function of Tissue Location. 10 replicate petri dishes were prepared for each growth medium: potato dextrose agar (PDA) and malt extract agar (MEA). Within each petri dish, positioning guides 3mm from the outer edge ensured consistent placement of endophyte samples. Each sample was labeled 'R' or 'B' to designate where the endophyte came from, root or bark tissue. 10 replicates on PDA contained one sample each of root and bark endophytes. Another 10 replicates on MEA also contained a paired root and bark endophyte sample. This resulted in 20 total competition replicates to analyze potential interactive fungal growth between tissue types on the two media.

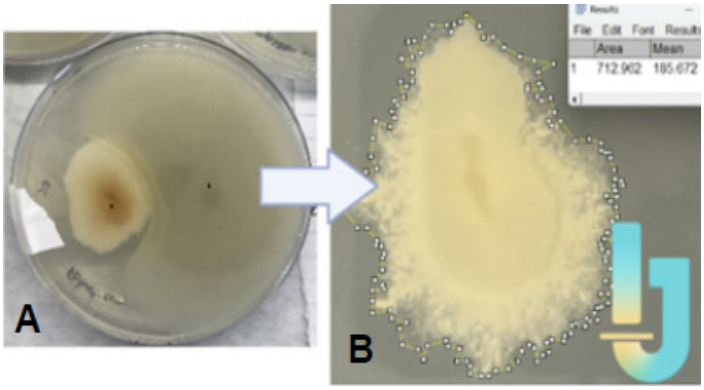

Figure 5. Measurement of fungal growth surface area using ImageJ software. A) Photograph of a petri dish containing a fungal culture plate. **B)** Screen capture from ImageJ showing digital representation of a culture plate with dotted outline indicating the measured area of interest. Photos were imported into ImageJ and calibrated with an included ruler to allow accurate area measurement determination by outlining the growth border.

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Effect of Water Temperature on Heart Rate in Human Mammalian Dive Response

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Introduction

The Mammalian Dive Response (MDR) is one of the most fascinating reflexes that occur in virtually all mammals, including humans. MDR refers to the ability of mammals to hold their breath underwater for a sustained period, which is reflected by further changes in underlying physiology. Marine mammals such as whales and seals can hold their breath for an hour or more, depending on the species. The most extended mammal breath held was reportedly a Cuvier's beaked whale that held its breath for 2 hours and 17 minutes in 2014 (1). In humans, this response exists in a less developed form, as the average person can hold their breath for only 2 minutes (1). Some cultures, such as the Bajau, who have been free-diving for food for many years, have been found to potentially have genetic and physiological adaptations for more sustained water submersion (up to 12 minutes or so) as part of their lifestyle (2). While it is still incredible compared to regular humans, it is far below that of marine mammals. These animals have increased blood volumes, greater vasoconstriction, more apnea, and a better ability to handle carbon dioxide in their blood, allowing them to handle long-term dives better than humans do (3).

In any case, the underlying physiology is very similar across mammals during MDR and should be considered. When the face becomes wet underwater, sensory receptors in the nasal cavity send signals to the brainstem, which in turn stimulates additional receptors and nervous pathways that trigger a parasympathetic response. This then leads to changes such as apnea (holding the breath), bradycardia (a slower heart rate), vasoconstriction, reorganization of blood supply to prioritize the brain and heart, and a decreased metabolic rate. These efforts collectively preserve the body's oxygen supply while the mammal is underwater (3,4). In general, this response is stronger in colder water than in warmer water, as seen in recent research, and heart rate is often a dominant metric for showing this, although others are tested as well (5,6).

The goal of the present study is to compare and confirm the effects of different water temperatures on the heart rate in different temporal intervals before, during, and after an MDR. It is hypothesized that heart rate will decrease more during cold-water submersion than during room temperature or warm water submersion.

Methods

The heart rate of one participant was measured throughout the duration of the experiment. A finger pulse transducer and a respiratory belt transducer were connected to the participant and configured to PowerLab to display respiratory rate and heart rate in real time in LabChart7. To ensure proper setup and accurate data acquisition before the start of the experiment, a test run was conducted for about a minute, during which the participant's heart rate was monitored at rest and during a breath hold. Once deemed successful, the participant began the submersion process in room temperature water, cold water (6 degrees Celsius), and warm water (30 degrees Celsius). Once data recording began, the participant remained at rest for approximately 30 seconds to obtain a resting heart rate. After 30 seconds, the participant submerged their face in a medium-sized tub of tap water for roughly a minute or until they needed to come up. After another 2-3 minutes of recording the recovery period, the recording stopped, and the trial would be finished. The next recording would begin in approximately 3-5 minutes or when the participant was ready to proceed. This process was repeated for each submersion. Means of heart rate data (only) were calculated using LabChart7 for resting heart rate, 15 seconds after water submersion, 15 seconds before the participant came up, post-breath hold (immediately after coming back up), and during the 2-3 minute recovery period, when data were still being collected. Graphing and data input were done in Microsoft Excel.

*This author wrote this paper for Biology 340: Animal Physiology taught by Dr. Margot Schwable.

Results

Sample recordings of resting heart rate and respiratory rate were successful, and no major technical issues occurred after test trials(Fig.1). For experimental results, the cold and control (room temperature) water had some similarities and differences in data trajectory, whereas the warm water had the largest gap between the cold and room temperature conditions. The cold water dive had a large initial drop in heart rate, followed by fluctuations (increases followed by decreases) throughout the duration of the trial. The control showed less fluctuation than the cold water and decreased steadily until the participant came up from the water. Both the cold and room temperature heart rates remained below resting heart rate, even during the recovery period (Fig. 2). The warm water dive had a markedly steeper upward trajectory than the cold and control water dives. The heart rate was always higher than the resting heart rate. It fluctuated throughout the dive but started to stabilize somewhat after the dive. (Fig.2)

Figure 1. Sample 11-second recording screenshot of heart and respiratory rate at rest using a finger pulse transducer and a respiratory belt transducer.

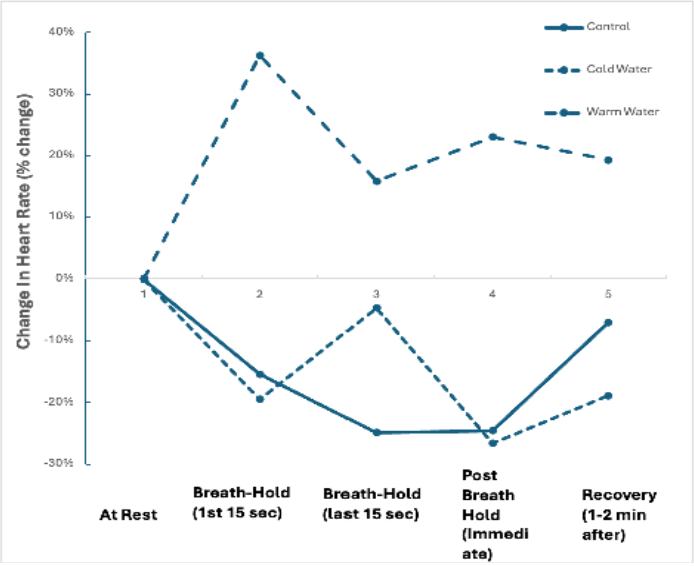

Figure 2. Percent change (%) in heart rate from one volunteer in different temporal intervals before, during, and after placing the face in a tub filled with room temperature, cold, and warm water. Heart rate was monitored with a finger pulse transducer.

Discussion

Our initial hypothesis for this experiment was that cold water would cause larger drops in heart rate than room temperature or warm water. This hypothesis was partially supported in the comparison between

cold and warm conditions. The warm water heart rate did not decrease as much as the cold water, and this signified differences that have been observed in previous research (5,6). However, when comparing the cold water to the control, these results were more similar than expected, and the control water decreased more than the cold water during the dive. This would likely contradict research where cold water has been seen to have the most pronounced decreases in heart rate

In this data, however, there were a few factors and limitations to consider that minimize the conclusiveness of the results. The main limitation is a very small sample size of just one participant. This would need to be increased in future experiments that examine this phenomenon to obtain more robust data. Another factor was some anxiety during trials due to holding breath. This may help explain fluctuations in heart rate during the experiment, and it is important to consider future studies. The last factor to consider is the inclusion of more diverse physiological measures beyond heart rate. Respiratory rate was measured but not analyzed in this experiment. As there are several physiological changes besides heart rate, further additions would be beneficial for understanding what happens during MDR and its other important changes. Even so, this data has still provided insights into heart rate changes during water submersion in humans

In addition to improving the experiment, there are intriguing studies that could further explore MDR. Since the participant was also an athlete, comparing MDR between athletes (those who play sports) and non-athletes would be of interest to determine whether MDR is heightened or suppressed in athletes. Also, age and gender differences would be another possible direction. MDR is a fascinating phenomenon, and expanding research in humans can further advance understanding of how it works and its developmental stages.

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The Lateral Line System of *Tanichthys albonubes*

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Introduction

The lateral line system is a mechanosensory system in fish that detects physical and hydrodynamic changes in the environment, allowing the organism to process this information and use it to guide its behavior (Kasumyan, 2003; Webb, 2011). The body is covered with a spatial array of neuromasts, the functional units of the lateral line system. Consisting of hair cells covered by a cupula, these mechanosensors are stimulated as the cupula is displaced relative to these hair cells (Webb, 2011). Many experiments inhibiting this function have shown that integral behaviors depend heavily on the lateral line system (Wunder 1927, Pitcher *et al.* 1976; Schwartz and Hasler 1966), including detecting water movement to sense prey and predators, navigating the physical environment without sight, and schooling properly (Montgomery and Pankhurst 1997; Campenhausen *et al.* 1981). Fish taxa display great diversity in the lateral line system, including differences in complexity, neuromast distribution, and quantity. For example, neuromasts are only observed on the trunk of certain groups of fish, but groups that do may display varying distributions of these neuromasts along this region (Kasumyan, 2003; Webb, 2011). Numerous studies have described the lateral line morphology of various fish taxa, using robust fluorescent staining techniques to visualize neuromasts. This study aims to describe the morphology of the neuromasts in *Tanichthys albonubes*, a minnow species commonly found in the aquarium trade but with limited research on its lateral line system. It is hypothesized that neuromasts will be distributed along both the head and the trunk, but also that distribution may resemble that of *Carassius auratus,* a well-studied fish in the same order: Cypriniformes.

Methods

This experiment was conducted on February 28th, 2025, within Lillard Hall on the campus of Lake Forest College. One live adult *Tanichthys albonubes* was chosen as the specimen of interest for this study due to its small size and its availability. The selected specimen was removed from its housing tank and gently transferred into a beaker containing conditioned tap water. A beaker containing approximately 100 ml of 63 µM 4-di-2-ASP was prepared to stain the neuromasts of the specimen, making them appear a bright fluorescent yellow under fluorescent light. After preparation, the specimen was transferred to this beaker for approximately 5 minutes. The specimen was then transferred into a separate beaker containing approximately 100 ml of 0.1% MS222, a solution used for humane euthanasia of fish. After five minutes, the specimen was placed onto a petri dish lined with a sylgard liner once proper euthanasia was confirmed. Insect pins were placed into the dorsal and ventral fins to stabilize.The specimen was placed in the petri dish. This was done to straighten the lateral side of the specimen and make it fully visible. A small amount of MS222 solution was poured into the petri dish to fully cover the specimen.

Once the body length was measured from the mouth to the caudal fin, the specimen was placed under a dissecting microscope with a camera attached. Using the program CaptaVision+ allowed for high definition observation and photography of the freshly euthanized specimen under a fiber optic light source and under a fluorescent light source (NightSea) to locate superficial and canal neuromasts. Using this technology, the distribution of neuromasts (dependent variable) was assessed as a function of fish species (*Tanichthys albonubes*) and of fluorescent staining/imaging technique (independent variables). Photographs of the specimen in the lateral position were first taken under fiber optic light, followed by fluorescent light. Once this was completed, the specimen was re-pinned so that its dorsal side could be seen directly under the microscope. Photography under fiber-optic light, followed by fluorescent light, was repeated. For the last set of photographs, the specimen was re-pinned so that the ventral side was directly under the microscope camera. Photography under both light sources was again repeated for this view.

*This author wrote this paper for Biology 340: Animal Physiology taught by Dr. Margot Schwable.

Figures and Tables

IO	Infraorbital
PO	Preoperculum
SO	Supraorbital
MD	Mandibular
ST	Supratemporal
RO	Anterior strip of neuromasts on the head
VO	Posterior strip of neuromasts on the head
T	Trunk

Table 1.1. ***Abbreviations for neuromast locations on the lateral line system.***

Results

The following images were taken under the dissecting microscope with a camera attached. Images include lateral, dorsal, and ventral views of the *Tanichthys albonubes* specimen under fiber-optic and fluorescent light. Neuromasts are visible under the fluorescent light and appear in the images as bright yellow spots.

Figure 1.1. ***Lateral Imaging of Tanichthys albonubes.*** A lateral view of the front half of Tanichthys albonubes (White Cloud Mountain Minnow) under bright light at 8x magnification, freshly euthanized with MS222.

All images depict only clear superficial neuromasts. Canal neuromasts cannot be seen within these images. The lateral line system was composed of IO (Fig. 1.2; Fig. 3.2), SO (Fig. 1.2; Fig. 2.2), MD (Fig. 1.2; Fig. 3.2), PO (Fig. 1.2; Fig. 3.2), ST (Fig. 1.2; Fig. 2.2), RO (Fig. 1.2), VO (Fig. 1.2), and T (Fig. 1.2; Fig. 3.2) superficial neuromasts. It is clear that there are over 100 superficial neuromasts located on the head alone (Fig. 1.2). From lateral and ventral views, it can be seen that there is a clear line of superficial neuromasts located along the trunk of the specimen. The entire line runs ventrally across the trunk and is composed of repeated lines of 3-4 superficial neuromasts arranged vertically.

Discussion

The photographs produced in this study show that *T. albonubes* exhibits a lateral line system comprising a significant number of superficial neuromasts on the head and trunk, visible in lateral, dorsal, and ventral views.

While canal neuromasts are present in *T. albonubes*, they are embedded in bony canals that are not immediately visible in this external imaging method. Further investigation and imaging are required in order to accurately depict and describe canal morphology. Given the imaging quality and the lack of replicates, a detailed description is not yet possible. Additional studies, including canal descriptions, may accurately depict the entire morphology of this lateral line system. It must also be noted that with time.

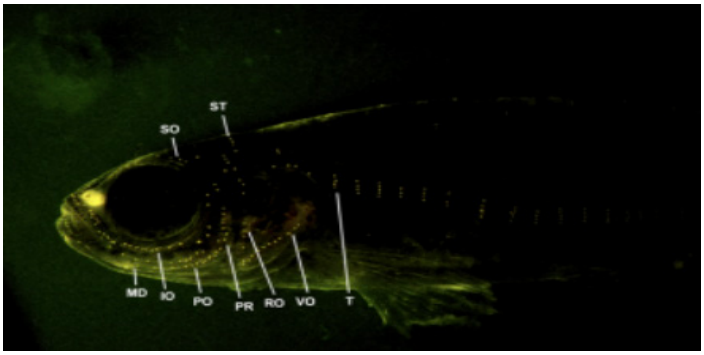

Figure 1.2. ***Lateral Line Imaging of Tanichthys albonubes.*** Distribution of the lateral superficial neuromasts of Tanichthys albonubes (White Cloud Mountain Minnow), visualized under fluorescent lighting using 4-di-2-ASP staining and magnified at 8x magnification. While present, canal neuromasts are not clearly visualized in this image.

The fluorescence of neuromasts decreases, requiring Figures 2.2 & 2.3 to be supplied by other researchers conducting the same experiment. It can be seen that the localization of neuromasts along the head mirrors that of *C. auratus*, with ST, RO, and VO neuromasts occurring on the head, which are not present in all fish (Webb, 2011). Additionally, the neuromasts along the trunk mirror those of *C. auratus,* with linear rows of superficial neuromasts ventrally along the trunk scales. This finding supports the hypothesis that the lateral line morphology of *T. albonubes* resembles that of its distant relative, *C. auratus, rai*sing the question of whether other relatives in the Cypriniformes order exhibit these similarities as well.

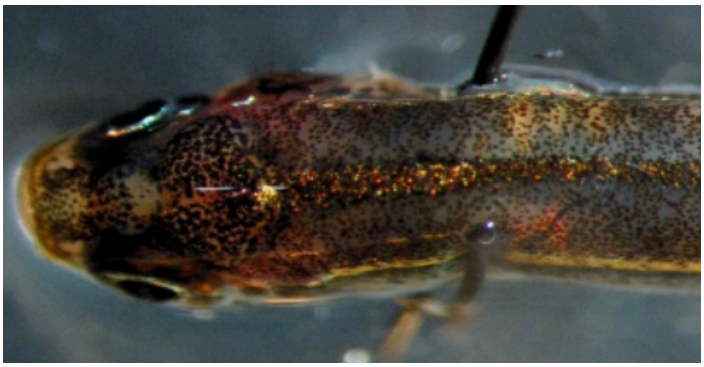

Figure 2.1. Dorsal Imaging of Tanichthys albonubes. A dorsal view of the front half of Tanichthys albonubes (White Cloud Mountain Minnow) under bright light at 12x magnification, freshly euthanized with MS222.

C. auratus has been described as possessing roughly 1000 superficial neuromasts across the body, with roughly 52-60 canal neuromasts (Kasumyan, 2003). However, a full-grown *C. auratus* is much larger than a full-grown *T. albonubes*, suggesting the potential for the number of neuromasts to differ with size. Alternatively, other species of minnow in the Cypriniformes order have also shown similar localization of superficial neuromasts along the head. *Ericymba buccata* displays hundreds of superficial neuromasts distributed across the head, suggesting that mechanosensory input received near the head may contribute to the detection and localization of prey (Jones et al., 2024). The morphology of the lateral line system of *T. albonubes* may serve a similar function.

Data collected in this experiment provides insight into the lateral line system of *Tanichthys albonubes*, showing similarities to distant relatives *Carassius auratus* and *Ericymba buccata*, specifically in the distribution and localization of superficial neuromasts on the head. Despite the limitations of this study, this data contributes to understanding lateral line morphology, particularly for this species and for small cyprinids. This experiment contributes to the growing body of research on the sensory adaptations of freshwater fish.

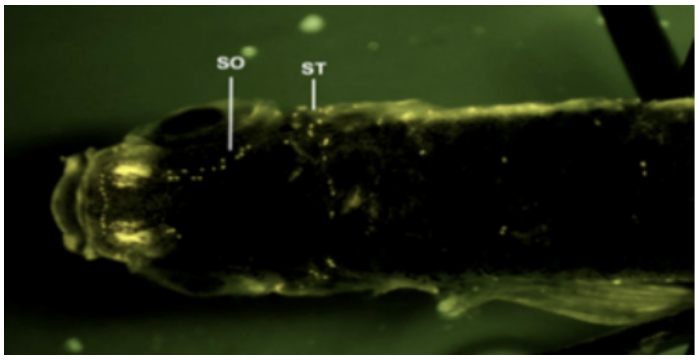

Figure 2.2. ***Lateral Line Imaging of Tanichthys albonubes.*** Distribution of the dorsal superficial neuromasts of Tanichthys albonubes (White Cloud Mountain Minnow), visualized under fluorescent lighting using 4-di-2-ASP staining and magnified at 12x magnification. While present, canal neuromasts are not clearly visualized in this image.

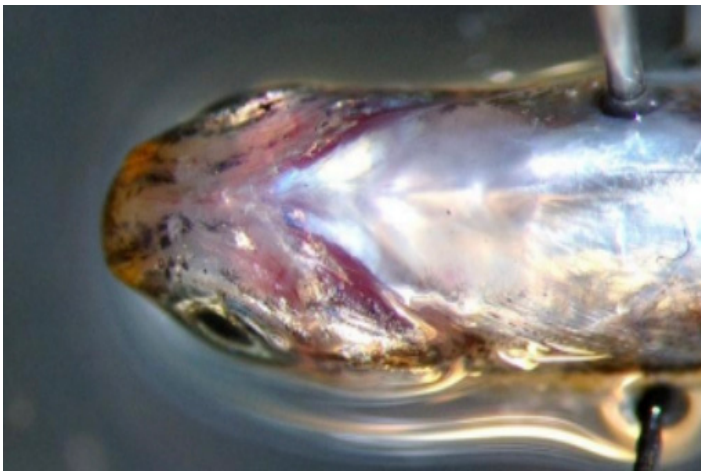

Figure 3.1. ***Ventral Imaging of Tanichthys albonubes.*** A ventral view of the head of Tanichthys albonubes (White Cloud Mountain Minnow) under bright light at 12x magnification, freshly euthanized with MS222.

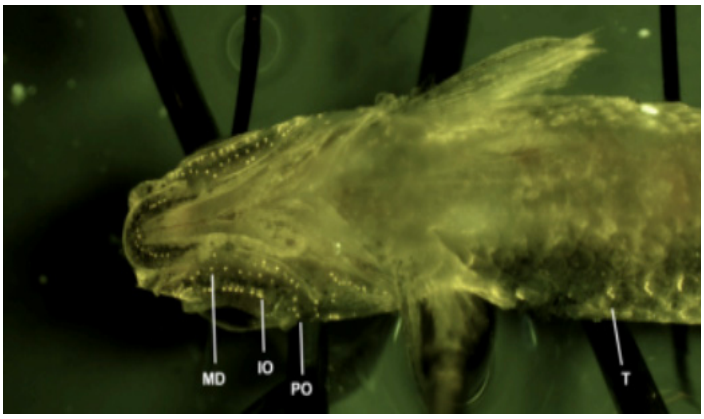

Figure 3.2. ***Lateral Line Imaging of Tanichthys albonubes.*** Distribution of the ventral superficial neuromasts of Tanichthys albonubes (White Cloud Mountain Minnow), visualized under fluorescent lighting using 4-di-2-ASP staining and magnified at 12x magnification. While present, canal neuromasts are not clearly visualized in this image.

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Environmental and Nutritional Trade-Offs in Squirrel Foraging Strategies

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Introduction

Optimal foraging theory suggests that animals have evolved to maximize their net rate of energy intake by balancing foraging efficiency with environmental constraints. Specifically, animals are predicted to leave a depleting patch when an alternative patch offers either more or faster food. However, natural conditions are rarely ideal, and factors such as uncertainty about the value of alternative patches, travel time, and potential risks associated with switching patches can influence decision-making, potentially delaying optimal departures (Chandel, 1983). This theory highlights the central question of how animals choose the most effective strategy for nutrient acquisition while accounting for environmental variability.

Despite criticisms of being tautological (Pierce, 1987), optimal foraging theory has been extensively validated through experimental research. Various studies have demonstrated that species adjust their foraging strategies in response to diverse factors. For instance, pigeons may alter their foraging patterns depending on the time of day (Chandel, 1983), and gerbils are influenced by moonlight levels that affect predator visibility (St. Juliana, 2017). Pollinators, such as bees, modify flower choices in the presence of predators like crab spiders (Huey, 2017), while ants make decisions based on nutrient availability ratios (Adams, 2002). These findings underscore the complexity and adaptability of foraging behaviors across species, influenced by competition, stress, predator detection, and other ecological pressures.

In this study, we focus on squirrels as model organisms to investigate how environmental stressors shape foraging decisions. The experiments were conducted in the backyard of a residence in Northbrook, Illinois, which is home to Eastern Gray Squirrels (*Sciurus carolinensis*). While squirrels have a varied diet, nuts represent a staple food source, with notable differences in nutritional value and accessibility depending on nut type and condition (Lewis, 1982). Previous research suggests that squirrels prioritize nutrient-rich food sources, but the specific ranking of their preferences remains unclear.

Based on prior information, we hypothesize that squirrels will prefer foraging in covered trays placed under trees, regardless of nut type, because these locations likely reduce perceived predation risk. Additionally, we predict that squirrels will favor nuts with shells in covered areas over nuts with no shells in uncovered areas, prioritizing the safety of a covered environment over the ease of access provided by nuts with no shells, which they would not need to invest time or energy in removing. Our rationale is that unshelled nuts require less effort and energy to consume, allowing for quicker nutrient intake. However, when exposed to potential threats in uncovered areas, the perceived risk may outweigh the benefits of faster consumption, driving a preference for safer, covered environments. Therefore, we anticipate that the safety of a covered environment will outweigh the accessibility of food when unshelled nuts are available. The goal of this research is to better understand how environmental stressors influence foraging behavior in squirrels. Specifically, we aim to determine whether they prioritize reduced visibility of predators in covered foraging areas or the effort required to consume food, such as shelled versus unshelled nuts. By addressing these factors, this study aims to provide insights into the ecological pressures shaping squirrels' foraging strategies in their natural environment.

Methods

Our study consisted of two experiments designed to assess squirrel foraging preferences under varying environmental conditions. Both experiments employed a within-subjects design, as each squirrel was exposed to all conditions. This approach allowed us to assess general preferences rather than tracking the behavior of specific individuals. Our analysis focused on overall choice patterns using giving-up density (GUD) as the primary metric. GUD, a commonly used measure in foraging ecology, assesses animals' perceived predation risk by quantifying the proportion of food left behind after foraging (McMahon, 2018). This method assumes that animals have fixed food preferences, allowing higher GUD values to be interpreted as higher perceived risk. For clarity, specific terms were operationalized in this study. For example, "eat at tray" (EAT) refers to the squirrel's tray choice for foraging. No trials were conducted under rainy conditions or at night, given the squirrel's diurnal foraging preference.

The first experiment tested our hypothesis about squirrels' foraging location preferences, based on whether the foraging trays were in covered or uncovered areas. In the first experiment, we prepared two large trays by filling each approximately one-third full of sand, ensuring consistent sand levels across trays. The tray has dimensions of 23.5 cm (length) x 12 cm (width) x 2.5 cm (height) and was supplied with 45 grams of peanuts in shells. An acclimatization trial of approximately 2.5 hours was conducted to familiarize the squirrels with the tray's placement, which were approximately 15 meters apart to minimize overlap in their foraging areas. A total of 32 trials were conducted, each lasting approximately 15 minutes. During these trials, we allowed the squirrels to forage freely, ensuring that trials ended before all food was consumed to retain sufficient GUD data. These data were used to infer patterns in location preference under consistent nut conditions.

Results

To investigate whether squirrels prioritize environmental protection or nutritional reward when foraging, we conducted a second experiment using the identical trays from Experiment 1. Each tray measured 23.5 cm (length) x 12 cm (width) x 2.5 cm (height) and was filled approximately one-third full with sand. The food type and placement were modified to create a contrast between energy investment and environmental safety. One tray, located under a tree in a covered area, contained 23 grams of peanuts with shells.

These peanuts not only provided a greater nutritional reward due to the inclusion of the shell but also offered the squirrels an opportunity to file their teeth, which continuously grow, as is typical of rodents. The second tray, placed in an uncovered area closer to the house, contained 29 grams of peanut kernels, offering easily accessible food with less energy required for consumption. This design allowed us to compare the influence of safety (provided by the covered area) with the reduced energy investment required when harvesting from uncovered areas. After each trial, the GUD was measured for the different conditions.

We conducted 19 trials over four days, with trials occurring between late morning and afternoon. Each trial lasted approximately 15 minutes, and environmental conditions, including temperature and weather, were kept relatively constant. The data collected from both experiments were analyzed using Jamovi. Two paired-sample tests were conducted: the first evaluated whether there was a statistically significant relationship between the location of the trays (covered vs. uncovered) and the squirrels' giving-up densities (GUDs), while the second assessed their preference for peanuts with shells versus without shells.

Results:

Two paired-sample t-tests were conducted to analyze the relationship between squirrels' giving-up densities (GUDs) and the positioning of food trays under different conditions in Experiments 1 and 2.

Experiment 1:

In the first experiment, the analysis revealed a statistically significant difference in GUDs between the uncovered and covered areas. On average, 6.38 g of peanuts were left in the uncovered area compared to 24.6 g in

*This author wrote this paper for Biology 220: Ecology and Evolution taught by Dr. Josh Hedge.

the covered area. The paired-sample t-test showed a significant effect, $t(31) = -7.40$, $p < .001$, indicating that squirrels foraged more intensively in the covered area (see Fig. 1).

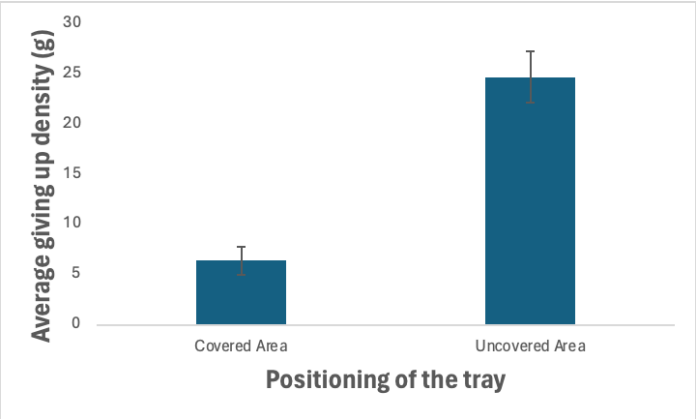

Figure 1. Mean giving-up density (GUD) as a function of tray location (covered vs. uncovered) in Experiment 1.

Experiment 2:

In the second experiment, a paired-sample t-test was conducted to compare GUDs for two conditions: peanuts with t shells located close to the tree (covered area) versus peanuts without shells in the uncovered area. Results revealed a statistically significant difference, $t(18) = -2.12$, $p = .048$. Squirrels exhibited a preference for the covered area, leaving an average GUD of 6.52 g for the peanuts with shells in the covered area compared to 12.6 g for the peanuts without shells in the uncovered area (see Fig.2).

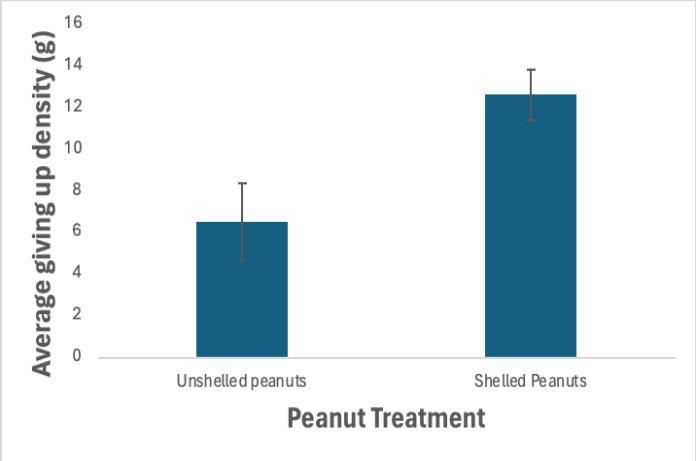

Figure 2. Mean giving-up density (GUD) as a function of peanut type and whether the peanuts were unshelled in a covered environment or shelled in an uncovered environment in Experiment 2.

Discussion

The results of both experiments supported the hypotheses, with squirrels demonstrating a clear preference for covered food. This outcome aligns with the predictions of the first experiment, in which squirrels consistently selected covered food at a higher rate. One possible explanation for this behavior is the type of tree under which the covered tray was located. The sugar maple (Acer saccharum), a species that can reach heights of 27–37 meters (90–120 feet), may have been perceived as offering significant protection from aerial predators, such as eagles, hawks, falcons, and owls, which use high vantage points to spot prey (MIT Press, 2002). Future studies could explore how tree height or alternative vegetation, such as dense bushes, might influence perceived safety and foraging duration.

Another variable to consider is the presence of backyard dogs (*Canis lupus)*, which are known predators of squirrels (Tobajas, Ramos-López, Piqué, & Sanchez-Rojas, 2023). While the squirrels in this study appeared accustomed to the dogs, as they typically ran away when they saw them, they also used the tree to protect themselves. The dogs' absence during the trials and the greater distance of the covered tray from the house may have influenced squirrel behavior. This highlights the need to account for predator presence and environmental context in similar studies.

The trial durations were shorter than anticipated, averaging approximately 15 minutes, whereas previous studies have shown squirrels foraging for more extended periods. This deviation may be attributed to the seasonal timing of the experiments, conducted during the transition from fall to winter. During this period, squirrels intensify their foraging and caching activities to prepare for winter scarcity, engaging in scatter hoarding by burying nuts and seeds in multiple locations.

This behavior is driven by hormonal and metabolic changes, as well as the need to secure high-calorie foods for colder months when mobility is reduced (van der Merwe et al., 2005; Michigan State University, n.d.). When assessing the Giving-Up Density (GUD), it is also essential to consider the potential impact of other species. For example, blue jays (*Cyanocitta cristata*), which inhabit the study area and also consume nuts, may have influenced the results. Although their impact is unlikely to have been substantial, their presence underscores the need to control for interspecies interactions in future research to ensure accurate data interpretation.

Lastly, this summer's rare double emergence of the *Magicicada septendecim* and *Magicicada tredecim* cicadas likely altered squirrel foraging behavior. This unique event, which occurs only once every 221 years, created an unusual abundance of cicadas, providing a significant food resource (Clay, Shelton, & Winkle, 2009). The temporary surplus during their reproductive period may have reduced squirrels' reliance on experimental food trays and influenced their caching and feeding patterns later in the season. This highlights the importance of considering unique environmental events when analyzing animal behavior.

This study is significant because it demonstrates that squirrels prioritize perceived threats in their foraging, often valuing safety over greater nutritional rewards. The focus on urban squirrels is particularly relevant, as these animals navigate environments with diverse challenges, including human activity that can indirectly hinder their foraging. Although dogs were the perceived predator in this study, urban squirrels are exposed to a range of disturbances, highlighting the complexity of their risk assessments.

Squirrels play a vital ecological role by dispersing seeds and pollinating plants—services essential for ecosystem stability. Disruptions to their foraging activities could have cascading effects, potentially altering plant regeneration and broader ecological interactions (Lurz, Garson, & Rushton, 1995). Moreover, a deeper understanding of squirrels' foraging habits not only informs conservation efforts for these rodents but also enhances our knowledge of animal behavior and decision-making under environmental stress, contributing to the broader field of wildlife ecology and management.

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Effects of Interspecific Competition on the Foraging Habits of Eastern Grey Squirrels

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Introduction

Optimal foraging theory states that organisms will want to maximize their energy gain relative to energy expenditure when foraging. This process can result in a variety of different strategies undergoing adaptation for foraging. These often involve trade-offs, such as having to forage for a longer amount of time if the journey took longer.

Competition would theoretically affect on foraging behavior. Since competition affects resource availability, it alters the amount of energy that organisms can obtain when foraging in an areas with high competition. Previous studies have found that Sciurus carolinensis (eastern gray squirrels) will interrupt foraging and exhibit alert behaviors when competitors are heard nearby (Jayne et al., 2015). They will also return to low-density food patches more rapidly in the presence of intraspecific competitors (Hopewell et al., 2008). Intraspecific competition between squirrels has also been shown to reduce foraging efficiency and speed (Teichroeb et al., 2024). However, competition can also have a positive effect, such as reducing predation risk and making it easier to find food (Jayne et al., 2015). These trade-offs may lead to competitioneither increasing or decreasing net energy and risk, depending on the situation.

Our experiments examine the effects of competition on Sciurus carolinensis (eastern gray squirrel) foraging, measured by the giving-up density (GUD) of feeding trays. The first experiment tested whether or not intraspecific competition would affect the GUD. The null hypothesis was that there would be no difference in GUD between trays with no intraspecific competition and the trays with intraspecific competition (represented by a mirror). The alternative hypothesis is that there is a difference in GUD between the trays where there was no intraspecific competition and the trays where there was intraspecific competition. Our prediction was that the GUD would be higher in trays with a mirror, as the perceived intraspecific competition would lead to less optimal foraging, as another squirrel being there would lead to fewer peanuts in that tray. The second experiment tested whether or not interspecific competition (represented by a crow statue) or intraspecific competition (represented by a squirrel statue) would have a stronger effect on the squirrel's GUD. A crow was used to represent interspecific competition because it competes for similar resources (Jayne et al., 2015). The null hypothesis was that there would be no significant difference in GUD between the tray with intraspecific competition and the tray with interspecific competition. The alternative hypothesis was that there would be a significant difference in GUD between the tray with intraspecific competition and the tray with interspecific competition. Our prediction was that the GUD would be higher in the tray with intraspecific competition, as thistypically has a stronger effect, leading the squirrels to avoid it more.

Methods

Experiments were conducted behind Nollen Hall on the south campus of Lake Forest College, Illinois. The experiments began on April 3rd, and the final trial was run on April 29th. Each trial consisted of two stations side by side, approximately 8 feet apart. Each station had a plastic tray filled to about 1/3 with sand. Approximately 60 grams of unshelled peanuts were mixed within. Stations were set out for 45 minutes to 2 hours. Afterwards, the remaining peanuts were collected and weighed to determine the GUD. The grams of peanuts lost per hour were then calculated for each trial by comparing the initial versus the final weight.

Experiment 1:

For the first experiment, one tray was accompanied by a large mirror that leanedagainst the side of the tray. This mirror was used to assess intraspecific competition, as the squirrel would see another squirrel when it looked at the mirror. The other tray had no modifications.

To analyze the data, a two-tailed unpaired t-test was performed. This was chosen as the alternative hypothesis was a significant difference in either direction, and the treatments were two unrelated trays.

Experiment 2:

The second experiment had one tray with a life-size squirrel statue placed within (image 1). The second tray contained a model crow placed within (image 2). This modeled a choice between perceived intraspecific and interspecific competition. To analyze the data, a two-tailed unpaired t-test was performed. This was chosen because the alternative hypothesis would be a significant difference in either direction, and the treatments were two separate trays.

Image 1. Treatment 1 for the second experiment: a realistic squirrel statue.

Image 2. Treatment 2 for the second experiment: a realistic crow statue.

Results

Experiment 1

There was no significant difference in mean grams lost per hour between tray types for experiment 1 ($t(18) = .5042$, $p = .6202$). The tray without a mirror had a mean loss of 27.19 grams per hour, while the tray with a mirror had a mean loss of 24.64 grams per hour (Figure 1). There were also no apparent behavior differences between the two trays.

*This author wrote this paper for Biology 220: Ecology and Evolution taught by Dr. Josh Hedge.

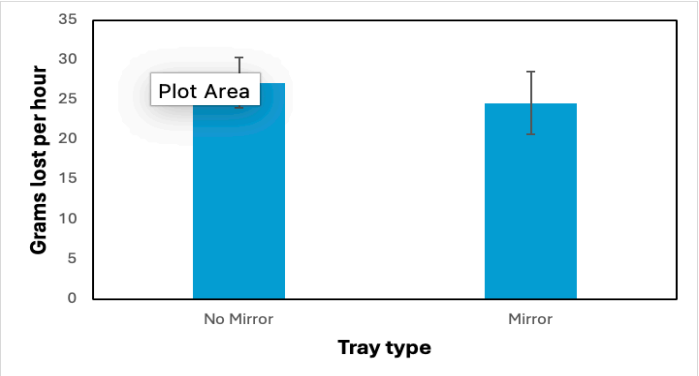

Figure 1. Mean results for the first experiment. Grams lost per hour in the tray with a mirror accompanying versus the tray without a mirror. The difference was found to be insignificant (t(18) = -.5042, p = .6202)

Experiment 2

The second experiment also yielded no significant difference ingrams lost per hour between the tray with a crow statue and the tray with a squirrel statue (t(20) = -.4352, p = .6681). The tray with a crow statue had a mean loss of 15.85 grams per hour, while the tray with a squirrel statue had a mean loss of 18.92 grams per hour (Figure 2). When approaching the trays, squirrels seemed hesitant and curious. They mostly chose to eat on the side oppositethe statues. The mean grams lost per hour was taken for both experiments 1 and 2 and compared (Figure 3). The results werenearly statistically significant, with the first experiment having a higher mean grams lost per hour (25.91) than the second experiment (17.39) (t(40) = -1.9666, p = .05619).

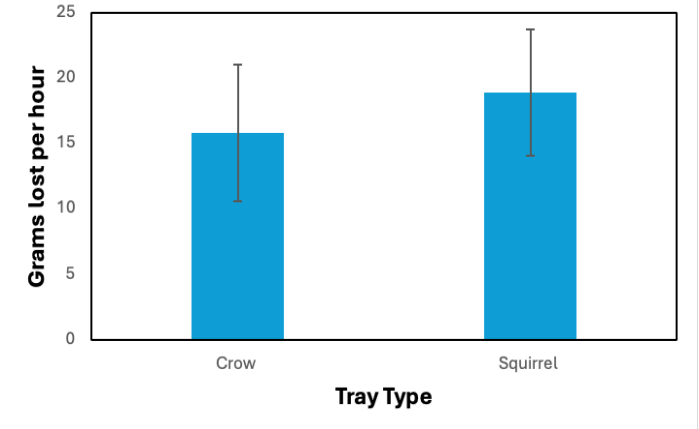

Figure 2. The mean results for the second experiment. Grams lost per hour in the tray with a squirrel replica versus tray with crow replica. Results were statistically insignificant (t(20) = -.4352, p = .6681).

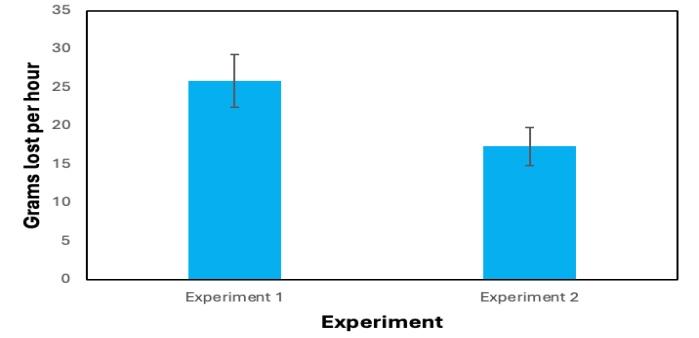

Figure 3. Mean results for both tray types in experiment 1 versus those in experiment 2. Results were near significant, with more grams being lost per hour from the trays in experiment 1 (t(40) = -1.9666, p = .05619).

Discussion

The data failed to reject either null hypothesis. In the first experiment, there was no significant difference between trays, indicating that the squirrels have no preference for the blank tray or the tray with intraspecific competition represented by a mirror (t(18) = .5042, p = .6202). The second experiment also had no significant difference between trays. This would indicate that the squirrels have no preference between the tray with a crow statue (interspecific competition) and the tray with a squirrel statue (intraspecific competition). The comparison between the two indicated a nearly significant difference in grams lost per hour in the first experiment compared to the second experiment (t(40) = -1.9666, p = .05619). This may indicate that the squirrels were possibly discouraged from foraging when they spottedthe visual competitors (statues). However, given only a near-significant value, major conclusions should not be drawn.

If the statues were perceived as competitors, the results of the first experiment suggest that the positive benefits of other squirrels balance out the decreased foraging efficiency that competitors bring (Teichroeb et al., 2024). While it has been found that the sounds of competitors cause squirrels to pause and engage in alert behaviors, it is possible that this behavior does not necessarily mean that squirrels perceive competitors as an inherent negative (Jayne et al., 2015).

The data for the second experiment are consistent with the finding that there was no difference in squirrel foraging behavior when corvid sounds were compared with squirrel sounds (Jayne et al., 2015). While this may seem intuitive, as intraspecific competition is typically thought to be stronger than interspecific competition, there is evidence that organisms can gather important information about food sources from heterospecifics just as well as conspecifics (Avargues et al., 2013). If squirrels are utilizing either to gather information about the food source, it may not matter which species is present.

Some of the significant issues that this study faces arise from the fact that the representations of competition were possibly not very convincing. Since they were simply realistic statues, they did not move, smell, or sound like typical competitors. Additionally, in the first experiment, some squirrels did not even pause to look into the mirror, so they were unaware of the potential intraspecific competition. If the squirrels did not view these representations as competitors, the results would be an inaccurate measure of how competition affects foraging behavior. Another issue was that the representation of intraspecific competition was changed from the first to second experiment. After seeing that many squirrels were not even looking into the mirror during the first experiment, the intraspecific indicator was switched to the squirrel statue.

For future studies, it would be helpful to look more into the effect of competition with more convincing stand-ins. Due to the near-significant difference between the first and second experiments, it is possible that the statues discouraged the squirrels' visits because they looked more like realistic competitors. With this in mind, studies in which one tray has no statue and the other tray has a squirrel statue would help clarify this result. Adding certain things to the statues to make them more realistic, such as the smell of a typical squirrel, or some type of sound production.

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Examining the Lateral Line System of Captive-Bred *Pristella maxillaris*

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Introduction

The lateral line is a mechanosensory system present in all fish species and is characterized by the ability to detect external water movement. It consists of larger canal neuromasts embedded in the bone canal and smaller superficial neuromasts found on the skin's surface. The lateral line system plays a crucial role in predator avoidance, schooling, navigation, and prey detection, making it essential for survival. The lateral line system is relatively conserved across species, yet research has shown that it remains plastic, as its reliance can change under different ecological conditions (Schwalbe et al., 2012). Furthermore, the ability to acquire sensory information is often specialized for an animal's behavioral needs (Spiller et al., 2017), suggesting that the lateral line system can evolve and adapt to specific needs and environmental pressures, or, possibly, the lack thereof. A study conducted by Fischer et al. (2013) investigated developmental plasticity in the lateral line system in response to predation. They found that guppies (Poecilia reticulata) from high-predation sites had more superficial neuromasts than those from low-predation sites. Additionally, a study by Vanderpham et al. published in 2013 found that fish from coastal rivers had more head canal pores than those collected upstream from rivers or lakes.

These studies highlight how increased predation and varying water flow can influence the density and distribution of neuromasts in wild fish, however, there is a gap in knowledge regarding the effect on the lateral line in long term captive-bred fish experiencing a prolonged lack of pressure. This experiment aims to investigate this gap in knowledge by examining the lateral line system of a genetically modified and long-term captive-bred species, Pristella maxillaris. To achieve this, a fluorescent microscopy technique will be used to visualize and identify the various components of the lateral line in Pristella maxillaris, enabling further investigation into the distribution of the superficial and canal neuromasts. Due to the lack of long-term environmental pressures in captivity, I hypothesize that the lateral line system will be less complex.

Methods

The experiment was conducted on Friday, February 21. A camera was mounted on a dissecting microscope, with the camera's visual feed displayed on the monitor. The camera was connected to the monitor via an HDMI cable and to the laptop via a USB cable. The software utilized for this experiment was CaptaVision+.

A fish was selected from a tank in the room LI 176. The fish selected for this experiment was the Pristella maxillaris. It was gently collected with a fish net and placed in a beaker of ~100-200 mL of conditioned tap water. Once the fish was isolated, it was gently transferred into a beaker containing ~100 mL of 4-di-2-ASP, using gloved hands. The fish remained in this solution for 5 minutes. After 5 minutes, the fish was then transferred with gloved hands to a beaker containing ~100mL of MS222 for 5 minutes. This solution humanely euthanized the fish. Both the 4-di-2-ASP and the MS222 solutions were not disposed of in the drain or trash; they were properly disposed of in their designated waste containers. After 5 minutes, the fish was placed onto a petri dish lined with sylgard containing 1:1 conditioned tap water and MS222. Throughout the experiment, the fish was handled with gloved hands to protect the skin from the MS222.

The fish was then placed under the microscope to obtain its total length. This was done by measuring from the mouth to the edge of the caudal fin. This length was 32 mm and was recorded in the "Lab 7 - Fish Lateral Line System spreadsheet". With gloved hands, the fish was positioned and secured with pins to expose the lateral view. This view displayed the mouth to the left with the left side of the body pointing up. Extreme caution was taken when pinning the fish so that it was not impaled and would not potentially damage the neuromasts. The fish was then placed under the microscope and focused using the fiber-optic light. In the software, the exposure was adjusted so that the fish was well lit but not overexposed. The fish was first observed at low magnification under fluorescent light. This was done by switching off the fiber optic light, turning on the fluorescence light, and placing a yellow filter under the microscope. A lateral full body view was captured using the live stitching feature.

This displayed the fluorescently lit lateral line system of the fish along the head and body. An image of a close-up view of the head was also captured to provide better visualization of the neuromasts. The fluorescence light was then switched to the fiber optic light, and the yellow filter was removed, allowing images under bright light to be captured. The live stitching feature did not work in bright light, so three separate images of the fish's body were taken and later cropped together. These steps of image capturing were repeated for both the dorsal and ventral views of the fish. For each image taken and saved to the computer, information on the image number, the type of light, the view, the magnification, and other relevant notes was recorded in the spreadsheet. After sufficient images were obtained, they were transferred to our personal devices via email.

After the experiment, the fish was disposed of in a designated Ziplock bag, and the workstation and equipment were disinfected using 70% ethanol. Later, the images were cropped and pieced together, and the various components of the lateral line system were labelled using PowerPoint.

Results

Lateral View

Figures 1, 2, and 3 display the lateral view of the Pristella maxillaris. These images display the supraorbital (SO), otic (OT), mandibular (MD), infraorbital (IO), postotic (PO), and trunk (T) canals. As seen in the fluorescent images in Figures 1 and 2, the trunk canal runs roughly 5 mm along the fish's body. Additionally, a higher number of neuromasts is observed on the head compared to the side of the body.

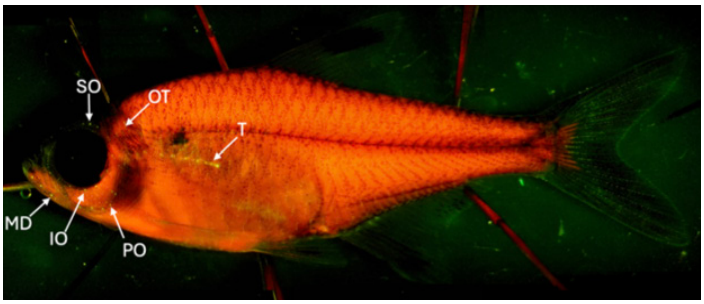

Figure 1. Lateral full body view of Pristella maxillaris under fluorescent light. Labelled are various canals of the lateral line system. These include the supraorbital (SO), otic (OT), trunk (T), mandibular (MD), infraorbital (IO), and postotic (PO) canals. The image was taken with live stitching under X6 magnification.

Dorsal View

Figures 4 and 5 display a dorsal view of the Pristella maxillaris. The supratemporal (ST) and supraorbital (SO) canals are labelled in Figure 4.

Ventral View

Figures 6 and 7 display the ventral view of the Pristella maxillaris. The mandibular (MD) canal is labelled in Figure 6.

*This author wrote this paper for Biology 340: Animal Physiology taught by Dr. Margot Schwable.

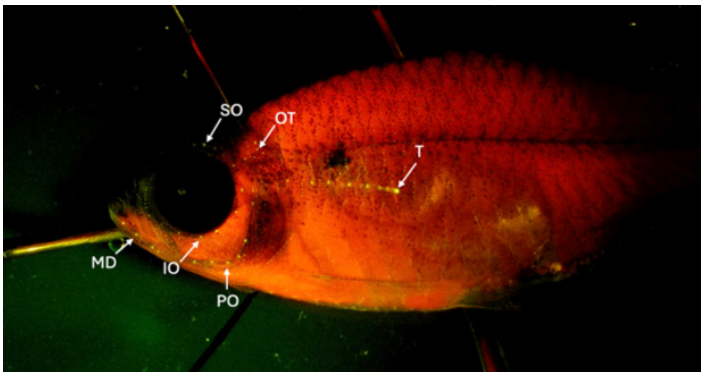

Figure 2. Close-up lateral view of the head with the labelled canals of the lateral line system. The image was taken with X6 magnification.

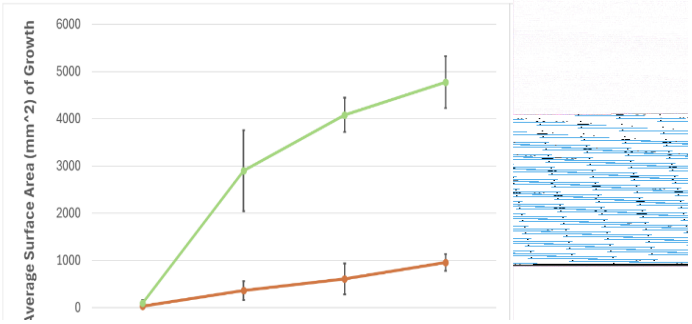

Figure 3. Lateral full body view image of Pristella maxillaris under bright light. Two images were taken separately and then stitched together. The images were taken under X6 magnification.

Figure 4. Dorsal full body view of Pristella maxillaris under fluorescent light. The supratemporal (ST) and supraorbital (SO) canals are labelled. The image was captured with live stitching under X6 magnification.

Discussion

Fluorescent images of the Pristella maxillaris revealed a shorter trunk canal along the body, supporting the original hypothesis that there will be a less complex lateral line. Past literature has shown that the lateral line system is adaptable, as behavioral needs and environmental factors can affect its development and specialization. However, continuous research is needed to understand the lateral line system in long-term captive species. A 2013 study by Brown et al. was among the first to investigate lateral line differences between captively bred and wild-origin fish. In this research, they found that wild steelhead trout (Oncorhynchus mykiss) juveniles had significantly more superficial neuromasts than hatchery-reared juveniles. This study highlights how a loss of environmental factors, such as predation and strong water currents, may cause the lateral line system to undergo evolutionary modifications due to decreased selective pressure, supporting the findings of this experiment.

Figure 5. Dorsal full body view of Pristella maxillaris under bright light. Two images were taken separately and then stitched together. The images were taken under X6 magnification.

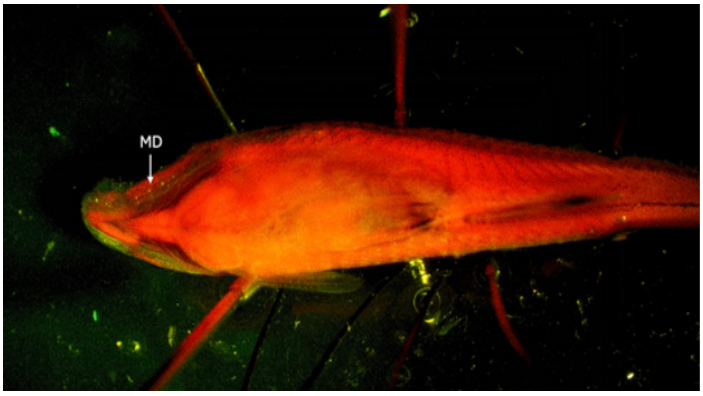

Figure 6. Ventral full body view of Pristella maxillaris under fluorescent light. The mandibular (MD) canal is labelled. The image was captured with live stitching under X6 magnification

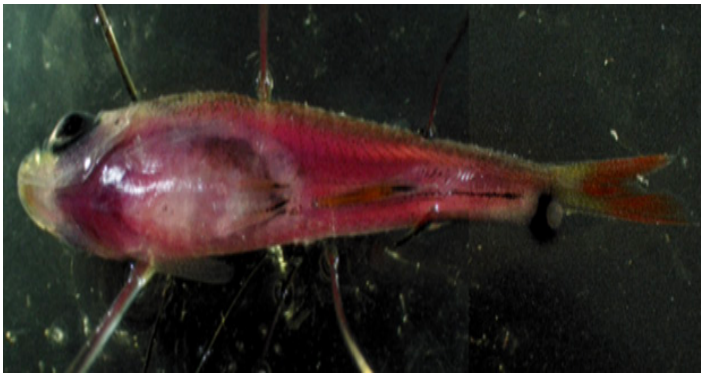

Figure 7. Ventral full body view of Pristella maxillaris under bright light. Two images were taken separately and then stitched together. The images were taken under X6 magnification.

This experiment provided further investigation of the lateral line in a captive-bred species and highlights the importance of continued research to better understand the effects of a domesticated environment on a fish's lateral line system. The findings of this experiment are vital to creating a more holistic understanding of how the lack of environmental pressures may affect the adaptation and development of the lateral line system. Since the lateral line is species-specific, future research can investigate its effects on the lateral line system in species that have experienced prolonged pressure relief. This research can be applied to future sustainability and conservation efforts, particularly in fisheries.

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Pathogenicity Assessment of Three Newer α-Synuclein Mutants Under Varying Expression Levels in Yeast

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Parkinson's disease is the second most common neurodegenerative disease, resulting from the misfolding and aggregation of the α- synuclein protein. The majority of Parkinson's cases occur sporadically; however, there are familial cases of PD resulting from genetic mutations which induce an early onset form of the condition. Additionally, research has shown that various conditions and alterations within the cellular environment can significantly alter the characteristics of α- synuclein toxicity and the rate of disease progression. There are six mutations to the α-synuclein gene that have been extensively evaluated by researchers, however there are three newer mutant variants of α- synuclein (A18T, A29S, and A53V) that haven't been assessed to the same degree. Here I investigate the intrinsic and extrinsic factors driving the toxicity of these three newer mutants using budding yeast as a model organism. I report that 1) these mutants show differential levels of toxicity in a concentration dependent manner; 2) the intrinsic property driving the toxicity of each mutant is more complicated than either loss or gain of the amino acid; 3) the A29S position dominates the phenotype of combined mutants; and 4) the mutants show differing degrees of sensitivity to altered cellular environments. This study reinforces the importance of understanding extrinsic and intrinsic factors that affect the toxicity of wildtype and mutant α- synuclein.

INTRODUCTION

Neurodegenerative diseases are conditions characterized by the death of neural cells and progressive degeneration of the brain. These disorders can vary in the age of onset although typically occur later in life and cause a variety of symptoms dependent on what brain region is degenerating. Synucleinopathies are one such group of degenerative disorders that includes conditions such as Multiple System Atrophy (MSA), Dementia with Lewy Bodies (DLB), and Parkinson's Disease (PD) which is the most common synucleinopathy in addition to being the 2nd most prevalent neurodegenerative disorder globally. PD is characterized by severe motor symptoms including tremors, rigidity, and bradykinesia as a result of degeneration of dopaminergic neurons within the substantia nigra (Jankovic, 2008) which has been linked with the presence of Lewy Bodies, aggregated plaques of the α- synuclein protein (Spillantini et al., 1998).

Most cases of PD are obtained sporadically late in life, the exact causes for which aren't well understood although it's been believed that environmental toxins are a factor (Chin-Chan et al., 2015), however there are familial cases of PD which are linked to genetic mutations. Mutation to the *PINK1* gene for instance is shown to increase the expression of α- synuclein and cause an early onset form of PD that presents that same as the sporadic form (Gandhi et al., 2009). Through studying these familial cases researchers have gained significant insight into the mechanisms underlying the pathology of PD, improving our understanding of α- synuclein and the various intrinsic or extrinsic mechanisms that affect the pathology of the protein.

α-synuclein is protein expressed throughout the brain however it is more highly expressed in certain regions such as the substantia nigra (Taguchi et al., 2016). The exact function is unknown but research indicates that its potential involvement in neurotransmission and interacts with the SNARE complex (Burré et al., 2010) among various other potential roles such as plasticity and membrane trafficking (Bendor et al., 2013). In synucleinopathies like PD however α-synuclein misfolds and becomes pathological due to several environmental and genetic factors. Altered environmental conditions within neural cells have shown to affect the extent in which PD progresses and how α-synuclein aggregates. High levels of nitration and oxidation is shown to be heavily involved with α-synuclein fibrillation and toxicity (Giasson et al., 2000), while other processes such as

SUMOylation can be protective and reduce the accumulation of misfolded proteins (Krumova et al., 2011). As mentioned earlier, increasing levels of α-synuclein alone can cause PD, for instance a family with a gene duplication of the *SNCA* gene that codes for α-synuclein experienced the classic symptoms of PD at an earlier onset (Kara et al., 2014). Other genetic factors like mutations also can drastically alter the toxicity of α-synuclein, six mutations on α-synuclein itself (A30P, E46K, H50Q, G51D, A53T, and A53E) cause early onset PD.

Many PD researchers have evaluated mutants of α-synuclein to better understand its underlying mechanisms and the unique properties that contribute to pathogenicity, such as A53T having highest affinity for membrane binding while E46K has high affinity for negatively charged lipids in particular (Liu et al., 2021).

In contrast to these however, there are three newly identified mutants, two sporadic (A18T and A29S) and one familial (A53V), that haven't received as much attention, possibly due to the sporadic nature of A18T and A29S (Hoffman-Zacharska et al., 2013) while A53V is linked to later onset PD as opposed to early onset (Mohite et al., 2018).

Previous work in our lab has investigated these novel mutants within a yeast model system, two thesis students Carris Borland and Amanda Grassel comparatively evaluated the toxicities of these mutants and assessed how they are affected in various altered cellular environments. Both Carris and Amanda found that each mutant was more sensitive to a particular condition compared to the others, however their findings regarding the natural toxicities of the mutants were inconsistent. Carris reported that the new mutants were differentially toxic from one another while Amanda instead found no difference between them. Knowing that increased expression is a key component of α- synuclein toxicity, I aimed to determine how a higher level of expression would affect the toxicities of these mutants.

Most work in the lab, including the work done by Carris and Amanda, was done using a PYES2 vector, however more recent work in the lab uses a P426G vector provided by Dr. Tiago Outeiro that has a higher level of protein expression. I hypothesize that with P426G expression vector, the three new mutants will show differential levels of toxicity, using a yeast model organism. Yeast serves as a good model due to their manipulatable genome which can be used to induce altered cellular conditions like nitration through *COX5A* and *COX5B* knockouts (Chung et al., 2013; Costello et al., 2008). Furthermore yeast have conserved protein folding and modifying pathways as in our own cells which makes them useful to model protein accumulation and aggregation that's common in neurodegenerative diseases (Mille-Fleming et al., 2008).

To evaluate the toxicities of the three new mutants A18T, A29S, and A53V, I have four aims. First, I predict that the mutants will be differentially toxic when under increased expression, and that their toxicities are dependent on the level of expression. To do this I will create the mutants within the P426G high expression vector and then comparatively evaluate them with one another, and with the mutants in the PYES2 vector. I additionally will compare the new mutants to the older six mutants A30P, E46K, H50Q, G51D, A53T, and A53E, as well as within a second strain of yeast. Second, is to determine the intrinsic properties of each mutation that make them toxic, from which I predict that either the loss of the original amino acid (Alanine) or the gain of the mutant amino acid (Threonine, Serine, or Valine) is key. To do this I aim to create and evaluate various amino acid substitution mutations at those three positions, exchanging the amino acid for another that belongs to one of the four large categories of amino acids: hydrophobic, hydrophilic, acidic, or basic. My third aim was to see how the properties of the mutants might affect one another when combined and what that might reveal about the

*This author wrote this paper as a senior project under the direction of Dr. Shubhik DebBurman.

locations of these mutations, from which I expected the mutants toxicity to either be averaged or enhanced when combined. To assess this, I aimed to create and evaluate three double combinatorial mutants as well as one triple mutant and assess how the various combinations affect overall pathology. My fourth and final aim is to assess how these mutants are differentially affected in altered cellular conditions. Carris and Amanda had previously found each mutant to be particularly sensitive to certain conditions within the PYES2 vector so the goal for this aim is to reassess this within a higher expression vector, of which I've chosen to assess altered nitrative, SUMOylation, and mitochondrial dysfunction conditions.

To view the remainder of this project, visit: https://www.lakeforest.edu/academics/student-honors-and-research/student-publications/eukaryon

The Impact of Pregnancy on Mood, Cognition, and Microglia Function in Alzheimer's Disease Mice

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Alzheimer's disease (AD) is a prominent neurodegenerative disorder (NDD) affecting the aging population. Of the 6 million people affected, 2/3rds are women. Evidence suggests women are more susceptible to AD due to a variety of social stress factors, differences in immune response, and a decline in estrogen levels. Notably, estrogen levels are prolonged in pregnancy and offer protection against neurodegeneration. However, the cellular mechanism for this protection remains unclear. This study aims to understand the impact of pregnancy on mood, cognition, and microglia function in Alzheimer's disease mice. We hypothesized that pregnancy would decrease the levels of the biological hallmarks of AD, therefore reducing anxiety-like behaviors and improving cognition. To test this hypothesis, we used control and AD mice between the ages of 7-8 months. The mice were run through a behavior paradigm to measure anxiety-like behavior and memory. Immunohistochemistry was used to stain for amyloid plaques and microglia.

Introduction

The central nervous system (CNS) consists of the brain and spinal cord. Together, they can sense, integrate, and direct our behavior (Thau et al., 2025). The brain senses our environment through touch, vision, audition, olfaction, and taste and converts this sensory information into a chemical signal sent through neurons that the brain can then interpret (Nervous System, 2023) (Figure 1). Essentially, it is the brain that coordinates the release of neurotransmitters and hormones which influences our behavior, emotion, and cognition. At the outermost layer of the brain is the cerebral cortex, which is divided into four lobes—the frontal, parietal, occipital and temporal lobe (Thau et al., 2025). The frontal lobe is typically associated with voluntary motor function, problem-solving, attention, memory, and language (Thau et al., 2025). Sensory information is then processed in the parietal lobe (Jawabri & Sharma, 2025). Responsible for processing visual information is the occipital lobe. The temporal lobe is associated with processing auditory stimuli (Jawabri & Sharma, 2025). Within these four lobes there are other important structures such as the thalamus, hypothalamus, and hippocampus (Torrico & Abdijadid, 2025). The thalamus is the brain's relay center that receives information from sensory receptors throughout the body and sends that information to the appropriate cortical area (Torrico & Abdijadid, 2025). Additionally, it can also regulate consciousness and sleep. Connecting the CNS to the endocrine system is a small structure known as the hypothalamus: this small structure controls heart rate, blood pressure, appetite, and the release of various hormones among other functions (Thau et al., 2025). The hippocampus resides in the temporal lobe and is associated with memory encoding, consolidation, retrieval, and decision making. This curved structure has three zones—the dentate gyrus (DG), the Cornu Ammonis (CA), and the subiculum (Fogwe et al., 2025) (Figure 2). Within the hippocampus are subfields CA1, CA2, CA3, and CA4, where CA2 and CA3 border the hilus of the DG (Fogwe et al., 2025). Alongside other temporal structures of the brain, these hippocampal structures work together in memory formation in three steps: registration, storage, and retrieval of information (Fogwe et al., 2025). This seahorse-shaped region of the brain essentially holds a key to our memories and creates new memories as we age.

Aging is defined as the time-related accumulation of molecular and cellular damage that can lead to the gradual decrease in physical and mental capacity (Gilbert, 2000, Ageing and Health, 2025). Although there are a variety of lifestyle factors that can impact aging, around 40% of individuals will experience some form of age-associated memory loss after 65 years of age while 5 to 8% will live with dementia (The Differences between Normal Aging and Dementia, 2024). Age-associated memory loss will not significantly disrupt daily life, but dementia can. Dementia is an umbrella term for symptoms related to behavior and cognitive function that are due to specific diseases (Dementia vs. Alzheimer's Disease, 2025). Specifically, Alzheimer's disease (AD) is known to account for 60-80% of dementia cases (CDC, 2025). AD is a NDD that is typically associated with behavioral and cognitive decline that can progressively worsen (Alzheimer's Disease Fact Sheet, 2023). Initially, AD can present itself with forgetting information that was just read, misplacing an object, and difficulty with planning and organizing (Alzheimer's Disease Fact Sheet, 2023). As the disease progresses, individuals can lose awareness of recent experiences, have difficulty communicating, and can require constant assistance during daily life (Alzheimer's Disease Fact Sheet, 2023). Given that AD can severely impact daily life throughout its progression, it is important to explore factors which can protect against the disease as well as gain insight into how the disease presents itself at the cellular level.

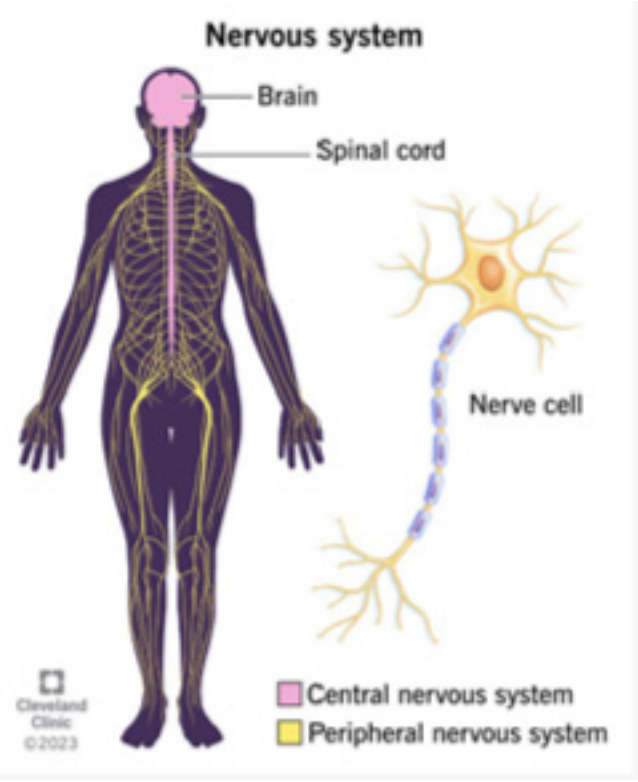

Figure 1. The Central Nervous System and Neuron (Nervous System, 2023).
The nervous system is composed of the CNS and peripheral nervous system. The CNS consists of the brain and spinal cord. The primary unit of the CNS in the neuron.

Pregnancy and Alzheimer's Disease

AD is the leading cause for dementia (Dementia, 2025). Notably, it impacts around 6.9 million Americans, with two-thirds of that population being women (Alzheimer's Association, 2024; Alzheimer's Disease Facts and Figures, 2025). Women are often noted to have a greater susceptibility to developing AD due to a variety of social stress factors, differences in immune response, and a decline in estrogen levels (Moutinho, 2025). Notably, women also experience specific life events such as pregnancy and menopause (Barth & de Lange, 2020). These events are characterized by physical, emotional, and physiological changes that can have life-long impacts (Orchard et al., 2023). Specifically, pregnancy has shown to impact a woman's cognition and inflammatory response which is noted to coincide with an increased in estrogen levels (Barth & de Lange, 2020; Kepley et al., 2025). Given that pregnancy alters the immune response and has similar physiological

*This author wrote this paper as a senior thesis under the direction of Dr. Holly C. Hunsberger

changes to an AD brain, the gradual cognitive renormalization pregnancy provides could provide insight into the molecular representation of AD.

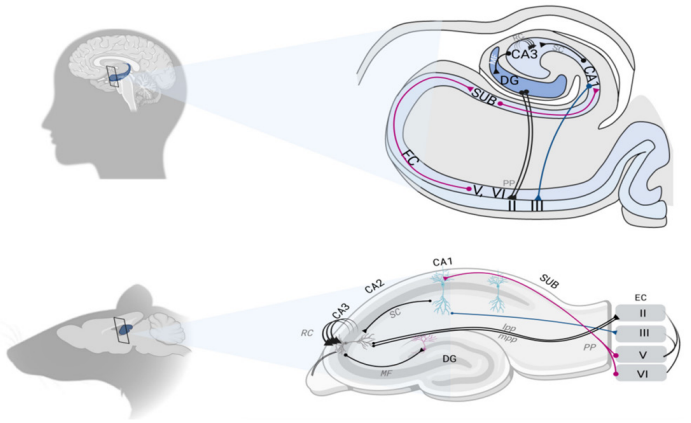

Figure 2. Human and rodent hippocampal divisions

The hippocampus and corresponding division in humans (top) and rodents (bottom) (Roux et al., 2021). Abbreviations: EC: entorhinal cortex; MF: mossy fibers; PP: perforant path; SC: schaffer collateral; RC: recurrent collaterals; SUB: subiculum

Pregnancy involves biological and social changes; it is described as a period where a fetus develops in the uterus and consists of various physiological transformations (About Pregnancy | NICHD - Eunice Kennedy Shriver National Institute of Child Health and Human Development, 2024). This dynamic period in a woman's life creates a challenging environment that promotes cognitive reserve and emotional adjustments to care for newborns (Orchard et al., 2023; de Lange et al., 2019). The researchers note that a challenging environment is seen in the increased cognitive load that caring for a child during their infancy, toddlerhood, early childhood, adolescence and adulthood stages of life brings (Orchard et al., 2023). Cognitive reserve refers to the idea that individual differences in the neural networks that help complete a task can provide reserve against brain pathology (Hindle et al., 2014; Stern, 2006). Here, the brain compensates using alternate networks and can modulate the clinical representation of AD pathology. During pregnancy, postpartum, mid-life and late life, women are exposed to a demanding environment and increased cognitive load that requires continuous adaptations (Orchard et al., 2023) (Figure 3). Women often report experiencing cognitive decline during early motherhood (Davies et al., 2018). These are often self-reported declines in concentration and feelings of absentmindedness. In a study involving 709 pregnant women, significant reductions in cognitive function, memory, and executive function were reported when compared to nulliparous women (Davies et al., 2018). At this time, there are also physical changes in the brain that occur, like decreased grey matter during pregnancy and renormalization of parietal grey matter in the postpartum period (Davies et al., 2018). An MRI study examined 25 female adolescents with no reproductive history and 20 adult first time mother and showed that in both cases, the adolescent girls and mothers experienced a volumetric reduction in cortical thickness and surface area (Carmona et al., 2019). These findings were consistent with the hormonal changes that occur during adolescence and pregnancy (Carmona et al., 2019). Similarly, a study that included 12,021 middle-aged women showed that parous women had less brain aging compared to nulliparous women (de Lange et al., 2019). Despite reporting subtle cognitive decrements and lower grey matter in the brain—women exhibit characteristics of decreased brain aging (Davies et al., 2018). Rodent literature also suggests that there is some cognitive decline during the final week of pregnancy, which would correspond with the third trimester of human pregnancy (Darnaudéry et al., 2007; Galea et al., 2000). Similar to humans, rodents showed a volumetric reduction in brain size and cellular changes (Hillerer et al., 2014). Male and female rats, with or without a breeding history, exposed to a stress-inducing paradigm demonstrated changes in neurogenesis which were measured through dendritic pruning and spine density changes (Hillerer et al., 2014). The study noted that neurogenesis increased during the peripartum period—specifically in the DG of the hippocampus (Hillerer et al., 2014). When comparing parous rats to middle-aged nulliparous rats, a reproductive history was associated with increased neurogenesis (Eid et al., 2019). During the transition to pregnancy, and later to postpartum, the associated reproductive hormones can impact behavioral, emotional, and cognitive responses (Trifu et al., 2019). Changes in reproductive hormones such as estradiol, progesterone, and cortisol during pregnancy are also associated with psychiatric disturbances (Trifu et al., 2019).

Furthermore, the immune response could act as a link between pregnancy and AD, although it is understudied (Fox et al., 2018). During pregnancy, a woman's immune response improves and is noted by the protective window the rise of estrogen provides (Fox et al., 2018; Robinson & Klein, 2012)we investigate the relationship between pregnancy and AD.\n\nMethods:\nCross-sectional cohort of British women (N = 95. This protective window is believed to support the cognitive renormalization that occurs during the postpartum and late-life period in a woman's life (Fox et al., 2018). Notably, AD exhibits an impaired immunoregulatory response which is believed to worsen pathology biomarker, like plaques and tangles. (Fox et al., 2018; Town et al., 2005). Current research lacks an understanding of the role of immunoregulation during pregnancy and how the hightened inflammatory response during this time offers neuroprotection which is not seen in AD (Pregnancy History May Be Linked to Dementia – Alzheimer's Society Comment | Alzheimer's Society, 2018). Here, we predict that looking at the pathological markers of AD, immune related cells like micrgoglia, and behavior changes in an AD rodent model, could provide insight in neuroportection after pregnancy.

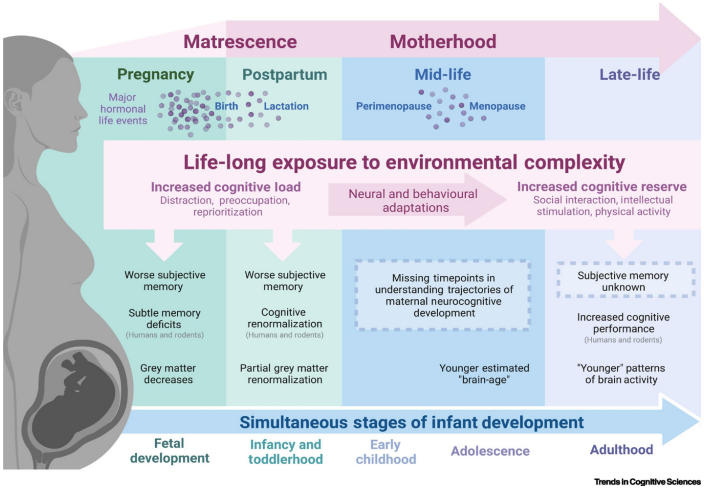

Figure 3. Diagram of the life-long impact of pregnancy and mother hood (Orchard et al., 2023).

Neuropsychiatric symptoms and cognition in Alzheimer's disease

Notably, neuropsychiatric symptoms (NPSs), such as anxiety and depression are associated with accelerated AD pathology and are often observed to a greater extent in women (Lyketsos et al., 2011). Specifically, anxiety has been reported to act as a better predictor of AD progression and has been associated with increased amyloid burden (Hunsberger et al., 2024). Currently, NPSs are considered an early biomarker for AD progression instead of just a risk factor, indicating their significance in modifying disease progression (Donovan et al., 2018). However, it is still unclear how NPSs mechanistically impact AD and whether their manifestation and symptomatology differ between men and women (Hunsberger et al., 2024). Interestingly, there is human and rodent data suggesting that neuroinflammation can influence AD pathology when present alongside NPSs (Holmgren et al., 2014). The immune cells of the brain, primarily microglia, detect neuronal stress such as amyloid burden and increase the production of cytokines and oxidative stress (Holmgren et al., 2014. This defense mechanism can worsen pathology from an

unregulated release of interleukin (IL)-1, leading to neuronal death. On the other hand, microglia can also release anti-inflammatory cytokines that protect against neuronal death and increase the phagocytosis of amyloid beta (Aß) plaques (Holmgren et al., 2014). Interestingly, these inflammatory mediators have been reported to play a role in NPSs encountered during AD progression (Eikelenboom et al., 2002). Cytokine production, like that in neuroinflammation, can modulate psychological stress, neuroplasticity, and neural circuitry involved in mood (Salim et al., 2012). Therefore, the dysregulation of immune signaling can result in anxious behavior, depressive behavior, and cognitive dysfunction.

Anxiety is one of the most common NPSs in individuals with AD (Lyketsos et al., 2011). It is typically associated with brain structures like the hippocampus, amygdala, and prefrontal cortex (N. K. Zhang et al., 2024). There is a positive correlation between anxiety and Aß burden which highlight its importance in disease progression. In human studies, higher levels of anxiety were associated with advanced brain aging (Han et al., 2021). There is a positive correlation between anxiety and Aß burden which highlight its importance in disease progression. In humans, approximately 50% of patients exhibit anxious behavior (Várkonyi et al., 2022). Therefore, using rodent models to understand this NPS is essential to AD research (Várkonyi et al., 2022). Biological brain aging was measured through methods like omics data (e. g. epigenetic clocks) and clinical biomarkers like blood chemistries (Várkonyi et al., 2022). Positron-emission tomography (PET) studies further confirm the positive associations between anxiety and Aß deposition, yet there are still some inconsistencies in the data as amyloid burden does not necessarily correlate with cognitive decline (Várkonyi et al., 2022). Therefore, the use of behavior testing on rodents can allow researchers to explore these complex behaviors (van Meer & Raber, 2005)the laboratory mouse (Mus musculus. In AD rodent models, behavior testing is used to measure anxious behavior, depressive behavior, and cognition (Zhong et al., 2024). Mice are exploratory animals, therefore anxiety-like behaviors are seen through avoidance of areas or latency to approach areas, repeated behaviors, decreased movement, and thigmotaxis (staying near the edges) (Pietropaolo et al., 2012). These behaviors are assessed using the open field (OF), marble bury (MB), and novelty suppressed feeding (NSF) tests. Transgenic (Tg) AD mouse models such as the APP/PS1 model exhibit decreased social investigation, indicating withdrawal-like behavior (Pietropaolo et al., 2012). Anxiety-like behaviors in mice are defined through motivated behavior that is elicited through exposure to a potentially harmful context (Pentkowski et al., 2021). Research exploring the impact of anxiety on the 3xTg AD model, which has both tau and amyloid, noted that mice at 4-, 6-, and 8- months of age exhibited less movement in the OF and groomed themselves more often than the control group (Várkonyi et al., 2022). Similarly, AD mice exhibited anxiety-like behavior through significant decreases in the number of entries into the open arms of an elevated plus maze and significantly less time spent in the light side of the light-dark test (Zhang et al., 2016). All together these results imply that anxiety is a prodromal symptom of AD.

Depression can also play a role in disease progression, both in humans and rodents. Typically, depression is associated with alterations of glutamatergic synaptic transmission, neuroinflammation, and atrophy in the hypothalamic-pituitary-adrenocortical (HPA) axis and hippocampus (Dolotov et al., 2022). There are changes in nerve and glial cells which can lead to neurodegeneration and cause brain pathologies to increase. Similar to pregnancy, depression can lead to reduced gray matter volume and cortical thickness depending on severity (Brommelhoff & Sultzer, 2015). Additionally, a loss of hippocampal serotonergic neurons and abnormal fatty acids in the prefrontal cortex indicate demyelination in the hippocampus (Lai et al., 2011; Luo et al., 2022; Nihonmatsu-Kikuchi et al., 2013). To assess depressive-like behavior in rodents, the OF test, elevated plus maze task, and forced swim test are often used. When investigating depressive-like behavior in mice using the OF test, researchers noted increased depressive-like behavior in AD mice as indicated by less time moving when compared to the control group (Frye & Walf, 2009). Similarly, in the OF test, AD mice were seen to have significantly reduced travel times, reduced number of entries in the center, and less time spent in the center when compared to the control group (M. Zhang et al., 2023). Interestingly, behavioral tests done prior to Aß plaque deposition in AD mice indicated behavioral and cognitive differences (Martín-Sánchez et al., 2021). Here, AD mice had a higher percentage of immobility compared to control mice. The appearance of anxious and depressive-like behavior can also impact cognition and was indicated by a greater latency to reach a platform in AD mice compared to the control group.

Neuropsychiatric symptoms and cognition during pregnancy

Throughout pregnancy and postpartum, there are various fluctuations in reported mood and cognition for women. Pregnancy places women in a vulnerable state, making them more sensitive to stressors and stress related disorders (Pawluski et al., 2011). After pregnancy, around 15% of women worldwide develop postpartum depression and can experience other mood disorders during pregnancy (Pawluski et al., 2011). Additionally, an estimated 10-20% experience anxiety and depression, factors which are associated with detrimental effects on the mother (Pawluski et al., 2011). These mood disorders can create stress for mothers, further increasing anxiety-like behaviors. Although anxiety can exist independent of depression, anxiety holds an 85% comorbidity with depression (Brunton et al., 2015). Similarly, pregnant female rats also display increased anxious behavior when measured on the EPM (Neumann et al., 1998). After parturition, though, female rats exhibit decreased anxious behavior. Interestingly, there have been studies aiming to understand whether parous rodents allocate care preferentially to female or male pups, but results remain inconclusive (D'Amato et al., 2006). Additionally, a woman's cognition is altered during pregnancy (Brett & Baxendale, 2001). Studies examining the subjective experience of subjective and cognitive changes during pregnancy report that women have increased forgetfulness, confusion, disorientation, and poor concentration (Brett & Baxendale, 2001). Additionally, pregnancy is marked by fluctuations in hormones that can impact memory like oestrogens, progesterone, glucocorticoids, and oxytocin. Oestrogens include estrone 1 (E1), estradiol (E2), and estriol (E3) which all show an increase in plasma level during pregnancy (Brett & Baxendale, 2001). A majority of the oestrogen receptors (ERs) are in the hypothalamus while the hippocampus and the basal forebrain are known to have lower ER densities. Despite a reported cognitive decline during pregnancy, a higher number of cumulative months pregnancy is associated with a lower risk for the development of AD (Fox et al., 2018). Mainly, a woman's reproductive history has shown to play an important role in modifying their inflammatory response through the prolonged estrogenic exposure (Fox et al., 2013) (Figure 4). These data demonstrate that pregnancy can place the brain in a challenging environment which can potentially protect the brain against dementia, and dementia-like disorders.

Neurodegeneration in Alzheimer's disease

As mentioned briefly before, biologically, AD is characterized by amyloid plaques, neurofibrillary tau tangles, and neuroinflammation (Parhizkar & Holtzman, 2022). Behaviorally, AD is characterized by cognitive decline, mood changes, and motor dysfunction depending on the brain area that is impacted (Peña-Bautista et al., 2020). Factors such as stress and the subsequent neuroinflammation can play a role in disease progression, although the specific mechanism remains understudied (Peña-Bautista et al., 2020). The neurodegenerative progression is both a psychologically and physiologically stressful event. Individuals tend to present with aberrant emotional and aggressive behavior as more neurons are lost and neuronal circuits mediating stress responses are disrupted (Justice, 2018). Structures such as the hippocampus exhibit amyloid plaque and tangle pathology even in the early stages of AD and mild cognitive decline (Pentkowski et al., 2021). These key characteristics were initially discovered by Alois Alzheimer in 1901, where he noted aggregation of those abnormalities in a 50-year-old female patient (Jeong, 2017; Lopez-Lee et al., 2024).

The cleavage of the amyloid precursor protein (APP) by alpha-, beta-, and gamma secretases lead to either soluble or insoluble product (Simunkova et al., 2019). When APP is cleaved by alpha secretase, a soluble p3 fragment is formed, on the other hand cleavage via beta

secretase results in an insoluble Aß fragment (Simunkova et al., 2019). In both cases, gamma secretase functions to isolate the product of either secretases (Simunkova et al., 2019) (Figure 5). Mainly, the Aß42 form is deposited in the brain and is the most toxic product of beta secretase, with its hydrophobic and fibrillogenic nature (Murphy & LeVine, 2010; Parodi-Rullán et al., 2019; What Happens to the Brain in Alzheimer's Disease?, 2024). This toxic form only accounts for around 10% of the enzymatic process, while the other 90% is accounted for by alpha secretase (Murphy & LeVine, 2010). Clearance of these amyloidogenic deposits occurs through various mechanisms. These mechanisms include the blood-brain barriers, interstitial fluid, cerebrospinal fluid absorption-mediated pathways, most important to this project, glial cells (Ullah & Lee, 2023).

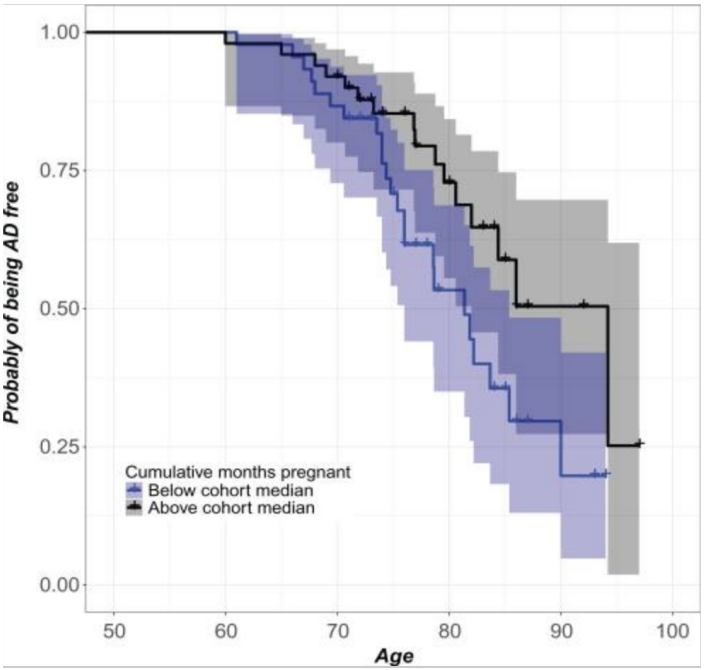

Figure 4. Higher cumulative months pregnant and probability of developing AD

More cumulative months pregnant is associated with a lower risk of developing AD. A Cox regression reported that women who were above the cohort median exhibited a 37.01% lower risk of developing AD when compared to women below the median (ß=-.99. exp(ß)=.40, P=.01, (5% CI =.17-.81) (Fox et al., 2018).

Immune response in Alzheimer's disease

Recently, inflammation has emerged as a vital player in the progression of AD with microglial activation acting as a key element (Kinney et al., 2018). Specifically, neuroinflammation refers to the inflammatory response within the CNS that is used by various pathological insults like infection, trauma, and toxins among others (Kinney et al., 2018). In neurodegeneration, this initially protective response can become unregulated and lead to neurodegeneration (Heneka et al., 2015) (Figure 6). Inflammation in the brain is typically measured via pro-inflammatory cytokines (IL-1ß, IL-6, IL-18), tumor necrosis factor (TNF) and reactive oxygen species by innate immune cells (Leng & Edison, 2021) (Figure 7). The release of these pro-inflammatory molecules is associated with synaptic dysfunction, neuronal death, and the reduction of neurogenesis (Leng & Edison, 2021). This response is followed by the activation of microglia to their phagocytic state, which if left unregulated, can result in the early pruning of synapses (Leng & Edison, 2021). On the other hand, there are also anti-inflammatory cytokines which regulate this inflammatory response (Kwon & Koh, 2020). Post-mortem AD brains confirm the chronic inflammatory response with the appearance of microglial connections to activated plaques in immunohistochemical analysis (Kwon & Koh, 2020).

The role of microglia: Healthy versus disease

Microglia are considered the resident immune cells of the CNS and hold an extensive role from birth to death (Borst et al., 2021). Accounting for 10% of the CNS cell population, they play a vital role in processes such as neurogenesis, neuronal plasticity, and regeneration, and are at the front line of the immune defense response for a variety of injuries (Calsolaro & Edison, 2016). The many functions microglia carry can be attributed to their variety in morphology (Vidal-Itriago et al., 2022). To study the diverse microglia morphology, research has distinguished microglia based on their morphology, density, and electrophysiological properties. Importantly, there is a lack of understanding in how the structure and function of microglia manifests (Vidal-Itriago et al., 2022). Furthermore, differences in microglia between females and males adds to the complexity of microglia structure and function. Specifically, steroid sex hormones, like estradiol, which exist differently in females and males can reduce inflammatory potential (Villa et al., 2016).

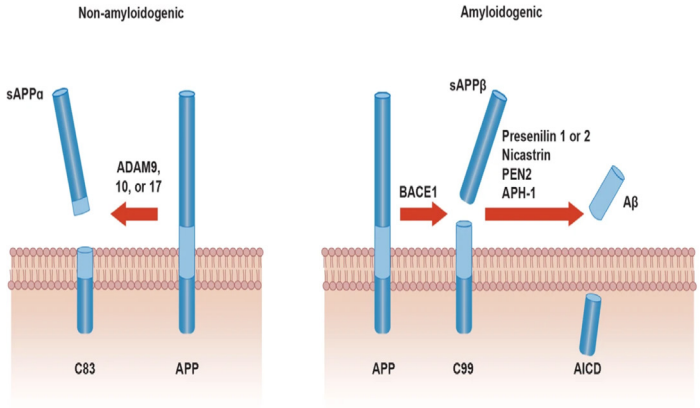

Figure 5. Amyloid precursor protein (APP) secretase pathways (Hampel et al., 2021)

APP cleavage through alpha (ADAM9, 10, or 17) secretase (left) or beta (BACE1) secretases (right).

With regard to structure, microglia exhibit diverse morphologies that can provide insight into the physiological role they play (Vidal-Itriago et al., 2022). The different microglial states include a ramified, hyper-ramified, and amoeboid among others (Vidal-Itriago et al., 2022). Usually, a ramified state is considered the 'resting' state of microglia. This state is characterized by a high number of branching with primary and secondary branching and aid to help surveil nearby areas. The hyper-ramified state has extensive branching and is typically seen in acute and chronic stress models. The amoeboid microglia have a rounded morphology which is correlated to its high phagocytic and migratory capacity. Research to quantify the different microglia morphologies indicate that microglia morphology is affected by different environments (Martinez et al., 2022). Here, neuron-glia cultures were treated with a control substance and N-methyl-D-aspartate (NMDA) treatment to mimic cell death like that seen in ischemia (Martinez et al., 2022). The researchers found that in NMDA treated cultures had significantly more cell debris, an indicator of cell death. These NMDA treated groups also exhibited a significantly greater number of hypertrophic morphology when compared to the control group. With the addition of lipopolysaccharide (LPS) to trigger inflammation, the cell cultures had significantly less hypertrophic morphology. Furthermore, microglia are classified into a classical (M1) or alternative (M2) state (Guo et al., 2022). The M1 pathway is associated with proinflammatory responses which include the release of several cytokines that are neurotoxic (Guo et al., 2022). The M2 pathway then regulates M1 through the release of anti-inflammatory cytokines and growth factors which help in regulating synaptic strength and plasticity (Guo et al., 2022). Interestingly, during disease, microglia can hold both M1 and M2 characteristics.

The aging and diseased brain can provide insight into the structure and function of microglia (Tremblay et al., 2012). Research has shown that the aging brain is characterized by cellular changes to neurons and glial

cells (Tremblay et al., 2012). Interestingly with age, microglia increase in number, become irregularly distributed, and exhibit variable cell bodies, branching, and morphologies (Tremblay et al., 2012). Specifically, Iba1-labeled microglia changed in density, distribution, and morphology during aging. Microglia in older mice decrease in process arborization, between 12 months and 24 months of age. Furthermore, research using social isolation as a stress model notes that microglial branching could provide insight into the functional role of microglia based on morphological change (Ferrara et al., 2022). Here, isolation appeared to increase microglia skeleton complexity in adolescent rats while decreasing in adult rats, with similar findings in total process length in adolescent and adult rats, respectively. Although stress has been shown to impact microglia morphology, there is a lack of research investigating the impact of pregnancy on microglial activation in mothers (Ferrara et al., 2022). Therefore, understanding how microglia morphology changes throughout disease progression in parous female rodents could provide meaningful insight into microglia function in the brain.

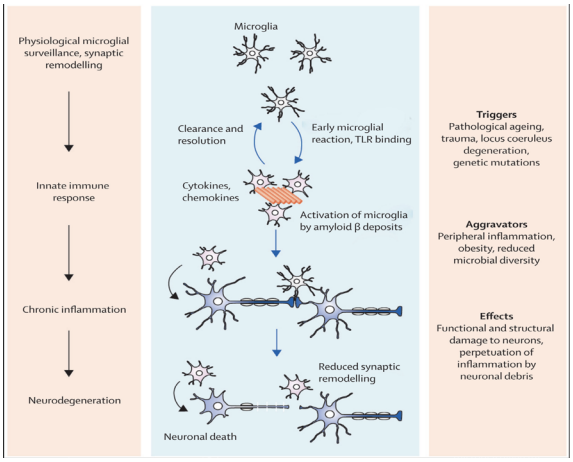

Figure 6. Microglial activation in the presence of plaque pathology (Heneka et al., 2015)

In summary, pregnancy and postpartum are noted to act as a protective barrier against AD. Despite the neuroprotective impact, the underlying pathogenic mechanism which disproportionately impacts more women than men is not well understood (Kommaddi et al., 2021). Additionally, the interaction between prolonged estrogen levels after pregnancy, its neuroprotective factors on plasticity and inflammation in the hippocampal DG provide a crucial opportunity to further the understanding of the impact of pregnancy on AD pathogenesis (Kommaddi et al., 2021). In this study, we hypothesize that pregnancy will protect against anxiety-like behaviors, cognitive decline, and amyloid pathology, while also reducing overall microglia number but increasing branch length.

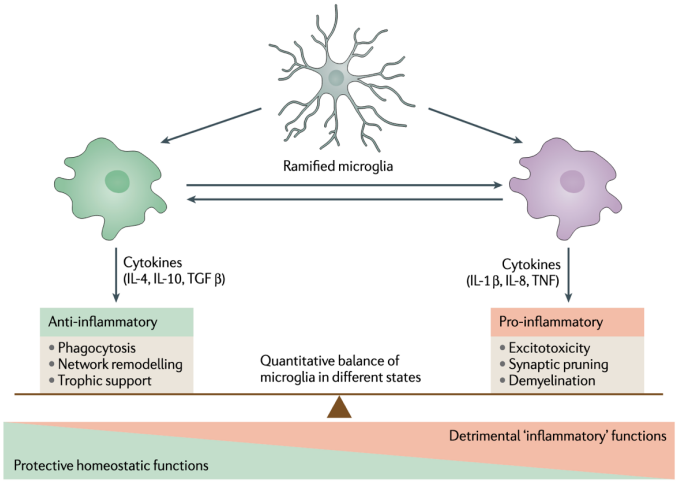

Figure 7. Microglial dual activation, from healthy to disease (Leng & Edison, 2021)

Nonsuicidal Self-Injury in the Bisexual+ Community: *Associations with Identity-Related Stress and Coping Styles*

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Research indicates that rates of nonsuicidal self-injury (NSSI) are high among bisexual, pansexual, queer, and fluid (bi+) individuals (Liu et al., 2019). Minority stress theory has been used to explain these disparities and identify risk and protective factors, such as identity-related stress and coping (Feinstein & Dyar, 2017). However, little research has focused on adapting and applying minority stress theory to understanding NSSI among bisexual individuals, through validated, bi-specific measures of identity stress. I examine associations between bi+ identity-related variables and NSSI and whether these associations are moderated by coping styles in a sample of bi+ individuals (N = 497) collected as part of longitudinal study at Rosalind Franklin University. My research found gender was consistently a robust predictor of NSSI, while associations between bi+ variables and NSSI were less robust or not statistically significant. This work encourages further research with a variety of samples.

Introduction

Non-suicidal self-injury (NSSI) is defined as "the direct, deliberate destruction of one's own body in the absence of intent to die" (Nock, 2009). NSSI has been described as a harmful coping mechanism "associated with a broad array of self-reported functions, including emotion-regulation, self-punishment or communication of distress" (Edmondson et al., 2016; Klonsky, 2007b). Some common methods include cutting, scratching, hitting, and burning oneself (Muehlenkamp & Gutierrez, 2004). There is some evidence that rates of NSSI may be increasing. A meta-analysis found NSSI prevalence rates were at 22% at lifetime, specifically higher among adolescents (Xiao et al., 2022).

Certain demographics, largely marginalized groups, have a higher risk of NSSI and suicidal ideation, likely due at least in part to minority stressors. Unlike general stressors, minority stressors stem from prejudice against gender and sexual minority individuals (Frost & Meyer, 2024). As seen in Meyer's (2003) Minority Stress Model, minority stressors may include both external stressors (distal minority processes) and internal stressors (proximal minority processes). According to Meyer, distal minority processes refer to external pressures, such as discriminatory actions (Meyer, 2003). Conversely, proximal minority processes refer to the marginalized individual's reactions to distal minority processes. Proximal minority processes include the concealment of identity and internalized stigma (Meyer, 2003). Minority stressors have high associations with a decrease in mental and physical health outcomes (Meyer, 2003), including depressive symptoms, anxiety, negative affect, and low self-esteem (Lick et al., 2013).

Meyer's (2003) model also suggests that associations between these stressors and health outcomes are moderated by coping, among other factors. Coping refers to "thoughts and behaviors mobilized to manage internal and external stressful situations" (Folkman & Moskowitz, 2004). Research says that there's two types of coping styles: engagement, where one actively addresses the stimuli, and disengagement, where one actively avoids the stimuli (Carver & Connor-Smith, 2010). There is a considerable body of empirical work that is consistent with Meyer's model. Coping has also been understood as a moderator in relationships between stress and its outcome, where an individual's coping style influences associations between stress and health (Ngamake et al., 2016). Similarly, Thomassin and colleagues (2017) found that positive reframing, an example of engaging coping, was shown to reduce the association between poor emotion expression and NSSI.

*This author wrote this paper as a senior thesis under the direction of Dr. Benjamin Swerdlow

Of relevance to the current study, bisexual individuals (bi+), or those who are attracted to people of more than one gender (Nelson, 2024), are more likely to report NSSI than gay and lesbian individuals (Liu et al, 2019). One potential reason for this difference is that bisexual individuals experience high levels of stigma both outside and inside the LGBTQ+ community (Liu et al., 2019). Indeed, negative attitudes toward bisexuality are present in heterosexual and homosexual individuals (Eliason, 1997). In other words, bi+ people experience extensive biphobia or otherwise known as negative attitudes towards bisexual people (Nelson, 2024). For example, in a sample of heterosexual undergraduate students, 50% rated bisexual women as "unacceptable," while 61% rated bisexual men as "unacceptable," compared to rating lesbian (38%) and gay men (43%) as "unacceptable" (Eliason, 1997). Specifically, it was common for men to be more accepting of bisexual women (Eliason, 1997). These results align with prior research suggesting acceptance towards bisexual women is more common due to heterosexual male sexual attitudes (Genter, 1987). Eliason's (1997) sample of heterosexual undergraduate students emphasizes the prevalence of biphobia. As opinions regarding gay and lesbian individuals become more positive, opinions on bisexual individuals remain in "the middle of the road," meaning there are still disagreements on bisexuality (Dodge et al., 2016). Research has also highlighted the exclusion of bisexual individuals in measurement development, data collection, and data analysis. Ross and colleagues (2017) note that bisexual individuals are often either consolidated with homoerotic identities (gay or lesbian identities, put into sexual orientation groups based on their partners) or completely left out of studies. Consequently, bisexual identities are overlooked, which the potential that this may further maintain or exacerbate stigma.

Some recent work, though, has begun focusing more specifically on bisexual individuals. For example, Feinstein and Dyar (2017) reviewed evidence that bisexual individuals have an increased risk for mental health and substance use problems compared to their homosexual counterparts. Research has also increasingly emphasized the impact of internal minority stressors on marginalized identities. Some common internal minority stressors are anticipated negativity, anticipated negative reaction towards their identity (Quinn et al., 2014); internalized negativity, the internalized belief that their identity is unnatural (Pollitt & Roberts, 2022); identity uncertainty, how uncertain one is about their identity; and identity affirmation, feeling of pride about their identity (Paul et al., 2014). Research has focused on looking at associations between bisexual individuals and internal minority stressors. Specifically, Pollitt and Roberts (2022) focused on associations between bisexual participants' level of internalized binegativity and their connection to the queer community. Compared to men, bisexual women reported higher levels of internalized binegativity, and that connectedness to the LGBTQ community was a protective factor against internalized binegativity (Pollitt & Roberts, 2021). Results proved that internalized stressors potentially play a crucial role in mental health disparities for bisexual people. Another example is provided by Dunlop and colleagues (2021). They found that thwarted belongingness, or "the unmet need to belong in a group" (Van Orden et al., 2012), may have a dynamic relationship with NSSI endorsement in a bisexual sample. Collectively, all of this research emphasizes that internalized identity-related stressors are important risk factors for NSSI.

Studies done by Dumas and Pepper (2023) use validated measured of bi-specific identity. Using the Minority Stress Theory, they explored how bi-specific stress contributed to NSSI with measures specified towards bisexual identity. Despite this, more research needs to be done on NSSI within the bisexual population and its connection to Meyer's (2003) Minority Stress Model, specifically focusing on internal stressors. Considering this, the main objective of this study was to investigate the main and interactive associations of bi+ identity-related variables with NSSI. More specifically, I conducted a secondary data analysis of the Bisexual+ Identity, Stress, Trauma, and Resilience study (BI-STAR), which is being conducted by Dr. Brian Feinstein and colleagues. BI-STAR is an 18-month-long longitudinal study aimed at understanding "how stress and trauma influence mental health, substance use, and relationship functioning among bi+ people." In particular, I focused on the following variables as potential correlates or predictors of NSSI in this study: bi+

identity stressors (anticipated binegativity, internalized binegativity, identity uncertainty, identity affirmation). Beyond identity-related variables, I also considered whether associations of these variables with NSSI might be moderated by coping strategies (disengaging and engaging coping styles).

In planning and conducting these analyses, I was guided by several specific research questions: 1) Which of the bi+ identity-related internalized stress variables (i.e., higher anticipated binegativity, higher internalized binegativity, lower identity affirmation, and higher identity uncertainty), if any, would be associated with or predict endorsement or frequency of NSSI, either when considered separately or conjointly? 2) Would associations between internalized bi+ stress and NSSI be moderated by coping styles (engaging and/or disengaging)?

I preregistered these questions and associated hypotheses on the Open Science Foundation. My hypotheses were as follows:

H1) Higher levels of internalized binegativity will be associated with higher rates of NSSI at baseline (i.e., endorsement of lifetime NSSI, endorsement of past 6-months NSSI, frequency of past 1-month NSSI), even when adjusting for anticipated binegativity, identity affirmation, identity uncertainty, and gender. Higher levels of anticipated binegativity will be associated with higher rates of NSSI, even when adjusting for internalized binegativity, identity affirmation, identity uncertainty and gender. Lower levels of identity affirmation will be associated with higher rates of NSSI, even when adjusting for anticipated binegativity, internalized binegativity, identity uncertainty, and gender. Higher levels of identity uncertainty will be associated with higher rates of NSSI, even when adjusting for anticipated binegativity, internalized binegativity, identity affirmation, and gender. H2) The positive associations between binegativity (internalized or anticipated) and NSSI will be moderated by engaging coping, such that the rates of NSSI will be higher when binegativity is higher and engagement is lower. The positive associations between binegativity (internalized or anticipated) and NSSI will be moderated by disengaging coping, such that the rates of NSSI will be higher when binegativity is higher and disengagement is higher.

Method

Participants and Procedures

The current study utilized the Bisexual+ Identity, Stress, Trauma, and Resilience (BI-STAR) dataset, a recently completed longitudinal study, provided by Dr. Brian Feinstein and colleagues. The overarching goal of BI-STAR is to understand "how stress and trauma influence mental health, substance use, and relationship functioning among bi+ people" (B. Feinstein, personal communication, 2024).

Participants were recruited via advertisements on social media and were assessed for the following inclusion/exclusion criteria: 18 years of age and over; reported a sexual identity of bisexual, pansexual, queer, or fluid; reported attractions to more than one gender or regardless of gender; living in the United States; able to read English; had access to the internet; and provided a phone number and email address. For sample demographic characteristics, see Table 1.

Before participating in the main study, participants completed an online eligibility screening. Once they completed all criteria and passed checks for inattentive/careless responses, they received a link to the consent form for the main study. Additionally, participants had to complete a questionnaire to ensure they understood the consent form; if they answered five or more questions incorrectly, participants had to retake the questionnaire. Those who passed the questionnaire and consented to the study received the baseline survey. Participants were asked to complete a baseline survey (T1) and follow-up surveys at 6 months (T2), 12 months (T3), and 18 months (T4). Originally, there were 513 participants, but 16 were excluded due to data quality concerns (e.g., multiple indicators of carelessness or inattentive responding), which brought the dataset to 502 participants. Participants received a $25 Amazon gift card for every survey they completed and a raffle ticket for a chance to win an additional $100 Amazon gift card. All surveys were programmed and completed via Qualtrics (Smith et al., 2005).

The Institutional Review Board at Rosalind Franklin University approved the BI-STAR study before any participant recruitment or data collection. For the purpose of our study, we focused on the following measures: the Bisexual Identity Inventory (Paul et al., 2014), the Lesbian, Gay, and Bisexual Identity Scale (Mohr & Kendra, 2011; adapted towards bi+ identity), the Self-Injurious Thoughts and Behavior-R (measured at both T1 and T2; Fox et al., 2020), and the Coping Strategies Inventory Short-Form (Clifton et al., 2007). Participants in the study completed several other measures at each time point, but these are beyond the scope of the current study and so will not be discussed further.

Measures

The Bisexual Identity Inventory, or BII, (Paul et al., 2014) is a self-report questionnaire that seeks to measure several aspects of bisexual identity, including the extent to which respondents anticipate that they will encounter binegativity, have internalized binegnativity, and affirm their bisexual identity. The BII is notable in that it was developed specifically with the bi+ context in mind and addresses stereotypes and stigmas that are specific to bi+ individuals, rather than lumping bi+ individuals with gay or lesbian individuals. The BII contains 46 self-reported items on a 7-point Likert scale from 1 = *strongly disagree* to 1 = *strongly agree.* There are three subscales: anticipated binegativity (five items, e.g; "When I talk about being bi+, I get nervous"), internalized binegativity (4 items, e.g., "My life would be better if I was not bi+"), and identity affirmation (six items, e.g., "Being bi+ is rewarding to me.). Several adaptations were made to the BII for the BI-STAR study, those being: reducing survey length, edits to clarify sexual orientation (e.g., "bisexual" was changed to "bi+), gender edits for inclusivity, and briefly edited response options. Prior research shows that BII is validated in bi+ individuals. Feinstein and colleagues (2024) have previously used BII when examining disclosure, experiences of minority stress, and mental health outcomes among bi+ adults. In their study, BII was validated, finding results such as internalized binegativity was higher among bi+ cisgender women, which is consistent with prior research (Dorell et al., 2024).

The Lesbian, Gay, and Bisexual Identity Scale or LGBIS (Mohr & Kendra, 2011; adapted towards bi+ identity) measures identity centrality and uncertainty. The LGBIS is a revised and extended version of the Lesbian and Gay Identity Scale (Mohr & Kendra, 2008), containing a new Likert scale with no neutral option. The revised LGBIS incorporates more inclusive language and less stigmatizing language. The LGBIS contains 27 self-reported items on a Likert scale from 1= *disagree strongly* and 6= *agree strongly.* BI-STAR study uses nine items out of the 27. Items are designed to assess respondents' feelings about their sexual identity; specifically, how uncertain they feel about their sexual identity (four items, e.g., I can't decide whether I am bi+ or gay/lesbian) and how central their sexual identity is to their overall identity (five items, e.g., Being bi+ is a very important aspect of my life). For the purposes of the BI-STAR study, several adaptations were made to the LGBIS: all instances of "LGB" were changed to "bi+", and the order of the two items was flipped for clarity. Prior research used the Lesbian and Gay Identity Scale (2008) to measure concerns about being stigmatized (Timmins et al., 2019), with successful results indicating that there are indirect negative effects of outness on the expectation of rejection.

The Self-injurious Thoughts and Behavior-Revised Questionnaire, or SITB-R (Fox et al., 2020), accounts for self-harm, suicidal ideation, suicidal planning, suicide attempts, and proximity to suicide. For the purposes of these analyses, I focused only on the items related to NSSI. More specifically, I focused on the items that assessed lifetime engagement in NSSI (a dummy-coded dichotomous variable measured only at T1; "In your lifetime, have you ever purposefully hurt yourself?"; 0 = *no,* 1 = *yes*), engagement in NSSI in the past six months (a dummy-coded dichotomous variable measured at T1 and T2; "In the past 6 months, have you ever purposefully hurt yourself?"; 0 = *no,* 1 = *yes*), and frequency of NSSI in the past month (a count variable measured at T1 and T2; "In the past month [30 days], on how many days did you hurt yourself without wanting to die?;

possible range of 0-30). Adaptions included: Consolidated individuals into groups based on relationships (e.g., brother, sister, mother, father = family member), replaced gendered relationships with gender-neutral language, added religious or spiritual leaders to disclosure measures, and added examples to crisis phone lines and medical professionals. Chang and colleagues (2024) used SITB-R to assess NSSI and STB (suicide ideation, planning, etc). Regarding gender, they found similar results to prior research: trans and gender-diverse individuals had the highest risk of NSSI, while cis men had the lowest (Chang et al., 2024).

The Coping Strategies Inventory Short-Form, or CSI-SF (Clifton et al., 2007), accounts for problem-focused engagement, problem-focused disengagement, emotion-focused engagement, and emotion-focused disengagement. The CSI-SF is a 16-item self-report measure, which is developed from the 78-item Coping Strategies Inventory, or CSI (Tobin et al., 1989). The 16 items measure how people handle stress, specifically, the strategies that people say they tend to use. Responses are provided on a 5-point Likert scale from 1 = *never,* 2 = *seldom,* 3 = *sometimes*, 4 = *often*, and 5 = *almost always* (Addison, Jenkins, & White, 2024). Problem-focused engagement include items like, "I make a plan of action and follow it." Emotion-focused engagement include items like, "I try to talk about it with a friend or family." Problem-focused disengagement include items like, "I try to put the problem out of my mind." Emotion-focused disengagement include items like "I try to spend time alone." For the purpose of this study, we focused on two superordinate factors: engaging coping and disengaging coping. Adaptations included: "I try to talk about it with a friend, or family" changed to "I try to talk about it with friends or family." In a study of nursing students' coping strategies, CSI-SF was validated through confirmation that measurement levels correlated with social support and anxiety in an adverse environment (Lainsamputty & Gerungan, 2024).

Data Analysis Plan

I preregistered my hypotheses and data analysis plan on the Open Science Framework (OSF). For the analyses, I used data from the baseline (T1) and 6-month follow-up (T2) time points. Before testing the hypotheses, I calculated univariate statistics (e.g., mean, standard deviation, frequency, skewness, kurtosis) for all key variables. Considered covariates were age and gender.To test hypotheses, I visualized bivariate associations and calculated bivariate statistics. I used Pearson R correlations for pairings of continuous variables with dichotomous variables and pairings of dichotomous variables. To test hypothesis one and hypothesis two, I computed parallel multiple regression models where I entered gender, internalized binegativity, identity affirmation, anticipated binegativity, and identity uncertainty as regressors and the binary NSSI variables as the outcomes (e.g., endorsement of lifetime NSSI at baseline, endorsement of past 6-month NSSI at baseline, and endorsement of past 6-month NSSI at the 6-month follow-up). In these models, I computed odds ratios for each regressor, as well as an overall coefficient of determination (Tjur's *d*). Regarding the count of past-month NSSI (at baseline and again at the 6-month follow-up), I again computed parallel generalized linear models. The model specification was based on the extent to which the outcome variables were dispersed relative to a Poisson distribution, which was assessed with the check_overdispersion function in the performance package in R. For the count of past-month NSSI variables that were significantly over dispersed, I used a negative binomial regression. If they were not over dispersed, I used Poisson regression. I computed incidence rate ratios for each regressor. All of these steps were repeated for Hypothesis Two but with the relevant moderators and moderation terms added (e.g.; coping styles). I used the approach described by McCabe et al. (2020, 2021) with the modlgm package in R.

All analyses were carried out in R v. 024.12.0.467 (R Core Team, 2024), including the following R packages: Questionr (Barnier et al., 2023), Hmisc (Harell, 2025), Modglm (McCabe, 2025), Performance (Lüdecke et al., 2021), Psych (Revelle, 2025), sjPlot (Lüdecke, 2024), and MASS (Venables and Ripley, 2002).

Heterologous Expression of Mycobacterium tuberculosis Virulence Factor, Mtb-BrkB, in *Escherichia coli*

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Tuberculosis (TB) infection is caused by bacterial pathogen *Mycobacterium tuberculosis* (Mtb). While treatments for TB exist, it remains the world's deadliest infectious disease, killing more than 1 million people a year. Although antibiotic treatments for tuberculosis exist, their longevity and inaccessibility, as well as the rise of drug-resistant tuberculosis (DRTB) means that antibiotic treatment alone are not widely effective. Therefore, new avenues of combatting TB are required by targeting specific, understudied virulence factors in Mtb. Mtb-BrkB is a 35kDa protein in *Mycobacterium tuberculosis* which has been shown to promote virulence. When Mmar-BrkB, the *Mycobacterium marinum* ortholog of Mtb-BrkB, is mutated in *Mycobacterium marinum*, infection in zebrafish is attenuated. The goal of this project is to express and isolate Mtb-BrkB for the purpose of downstream structural and functional analysis. The Mtb-BrkB-6XHis protein was recombinantly expressed in *E. coli*. The cells were subsequently lysed and Ni-NTA resin affinity chromatography was used to isolate the protein. Analysis of presence and purity of protein was determined by SDS-PAGE and Western Blot using mouse monoclonal anti-6XHis antibodies. The future directions of this research include a scaled-up production of Mtb-BrkB, a multimerization assay to determine the quaternary structure of Mtb-BrkB, and structural analysis by cryo-electron microscopy.

Introduction

Bacteria are plentiful in the soil, water, air, and even the human body. Many of these bacteria do humans no harm and can even help with bodily processes such as digestion. However, some bacteria have specially evolved to invade, live, and multiply within human hosts. These bacteria are pathogenic, or disease causing. While human pathogens, including viruses, fungi, bacteria, and others, are relatively small in number, with only 1513 reported species of bacteria which can infect humans (Bartlett et al., 2022) out of a predicted total of between 10^{11}-10^{12} microbial species on earth (Locey & Lennon, 2016), their effect on global health can be immense. In 2019, 7.7 million deaths were associated with bacterial infection (GBD 2019 Antimicrobial Resistance Collaborators, 2022). Bacteria which are pathogenic rely on virulence factors, cellular machinery, molecules, and regulatory factors which allow pathogens to invade, survive, and evade immune response in a host.

One such pathogenic bacteria is *Mycobacterium tuberculosis*, discovered in 1882 by Robert Koch to be the bacterium responsible for tuberculosis infection (Koch et al., 1882). *M. tuberculosis* is an obligate pathogen, meaning it cannot survive outside of a host. Within a host, *M. tuberculosis* takes advantage of the hosts immune response to survive and multiply. While the bacterial cause of tuberculosis was only discovered in 1882, it is an ancient disease that has long plagued humanity, with the earliest written record of tuberculosis infection dating back 3300 years (Barberis et al., 2017). Despite an available vaccine and antibiotic treatment, tuberculosis continues to infect people around the world, causing more deaths than any other infectious disease. By studying how tuberculosis so effectively infects humans, we can continue to strive towards new strategies to combat and eradicate tuberculosis. In order to target *mycobacterium tuberculosis*, it is important to understand the etiology, the global burden of disease, the characteristics of the disease-causing bacterium, and mechanisms of disease transmission in order to move forward with new solutions.

Tuberculosis: The Disease

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (Koch, 1882). The most prevalent type of TB is pulmonary TB, which infects the lungs and is spread primarily by an infected individual coughing the bacterium through the air. In 2024, 80% of all TB cases were pulmonary TB, compared to just 19% of cases were extrapulmonary (VidyaRaj, C.K., 2025). Extrapulmonary TB infects cells outside of the lungs such as in the blood stream, causing miliary TB, the kidneys, causing genitourinary TB, the lymph nodes causing tuberculosis lymphadenitis, etc. Pulmonary TB symptoms include a cough which lasts 3 weeks or longer, chest pain, coughing blood or phlegm, fever, fatigue, and weight loss, which all worsen with time. Risk factors of TB include HIV/AIDS, diabetes, malnutrition, chemotherapy, severe kidney disease, misuse of alcohol, and use of steroids or tobacco products (Mayo Clinic 2023).

While TB is treatable by antibiotics, inaccessibility and drug-resistance contribute the continued prevalence of the disease. Current TB treatment includes a drug regimen of 4-9 months (CDC 2020). The first line drugs used for drug-responsive TB include Isoniazid, Rifampicin, Pyrazinamide, and Ethambutol. The second-line TB drugs for drug resistant TB include Bedaquiline, Cycloserine, and Kanamycin. While drugs show cure rates as high as 95% in clinical trials, in actual treatment programs their performance is much worse due to high patient dropout rates (Bendre et. al. 2021). Current TB drug regimens have a very long course of treatment, which is often inaccessible to patients leading to the early termination of drug regimens. Incomplete courses of antibiotics can increase the risk of drug-resistance and relapse.

The prevalence of drug-resistant TB is a growing issue. Drug resistance is caused by the acquisition of mutations which allow for antibiotic resistance. This can be caused by incomplete or inadequate antibiotic treatment, and then spread by normal mechanisms of transmission. There are two classes of drug resistant TB: multi-drug resistant TB (MDR-TB) which is resistant to first line drugs such as isoniazid and rifampicin and extensively drug resistant TB (XDR-TB) which are resistant to first and some second line drugs (Gandhi et al., 2010). The global rate of MDR-TB is 11.6% of all TB cases (Salari et al., 2023). Drug resistant TB is a major global health problem because it is more difficult to treat that drug responsive TB, and existing TB solutions and antibiotics are ineffective against it. The course of antibiotics to treat MDR-TB can take between 18-24 months (Gandhi et al., 2010). Therefore, new solutions including increased accessibility of treatment and new chemotherapies for tuberculosis are necessary in the face of rising drug-resistance.

The Bacille Calmette-Guérin vaccine (BCG) is a prophylaxis for TB. It was developed in 1921 from Mycobacterium bovis, a bovine strain of tuberculosis, by Albert Calmette and Camille Guérin. The French scientists cultured a virulent strain of M. bovis on potatoes until virulence was lost or attenuated (Luca & Mihaescu, 2013). The BCG vaccine is still administered in countries with high rates of TB; however, its efficacy is variable. While it is effective at preventing extrapulmonary TB in children under 5, it is less effective at protecting against pulmonary TB in adults (Bendre et al., 2021). Because most TB cases are pulmonary TB, this vaccine is not adequate as the sole protection against TB.

The Global Health Burden of TB

Globally, 10.8 million people got tuberculosis in 2023, and 1.25 million died from the disease (WHO 2024). Despite available treatment options, TB continues to be the deadliest infectious disease worldwide, with the exception of the years 2020-2022 when COVID-19 surpassed TB in the number of deaths. Additionally, the global burden of TB is not being shouldered equally. TB is not geographically neutral; most of the TB incidences are in South-East Asia, Africa, and the Western Pacific. The 30 highest TB burdened countries shoulder 87% of the worldwide TB burden in 2023, with just 8 of these countries, India, Indonesia, China, the Philippines, Pakistan, Nigeria, Bangladesh, and the Democratic Republic of Congo, accounting for over 60% of the worldwide TB incidence (WHO

*This author wrote this paper as a senior thesis under the direction of Dr. William H. Conrad.

2024). In 2023, the United States reported only 9,633 cases of TB, which is 0.08% of the global total, however, incidence rose 15.6% from 2022 (CDC, 2024). Therefore, while TB incidence is low in the US, it is still prevalent and the deadliest infectious disease in the world. Moreover, the disease burden of TB is shouldered disproportionately by the Global South and countries that may struggle to afford the high cost of lengthy TB treatment.

High burden countries face higher rates of TB incidence due to the high rates of poverty which lead to undernutrition, increased transmission due to overcrowding, and inability to pay the cost of treatment for TB. There is an inverse correlation between a country's GDP and TB incidence per 100,000 people. One of the highest risk factors for TB is undernutrition, which accounted for about 1 million new cases of TB in 2023 (WHO 2024). As well, overcrowding, poor ventilation, and in house TB contact are risk factors for TB and lead to disease transmission (Lee, J. et. al., 2022). This suggest that poverty is a risk factor for tuberculosis, and TB disproportionately affects those who are impoverished and undernourished.

Low-income countries, which are more likely to have new cases of TB, also are less likely to be able to shoulder the cost of treatment. In India, which accounts for 26% of TB cases (WHO 2024), the burden of cost shouldered by patients can be extremely high. Between 2000 and 2018 in India, catastrophic costs, defined as costs over 20% of total annual household income, affected between 7% and 32.4% of patients with drug-responsive TB and 68% of patients with drug-resistant TB (Chandra et al., 2020). These costs only plunge families struggling with tuberculosis further into poverty. Current TB treatment is often long, inaccessible and complex for low-income countries which are most effected by TB.

Moreover, low-income countries face greater difficulties in providing timely treatment for TB. The delayed diagnosis of TB can lead to delayed treatment and further transmission of the disease. In low-income and low-middle-income countries the median patient delays due to financial, physical and social difficulties of seeking treatment was 28 days. The median healthcare delay, which is caused by complicated administrative processes, referral systems, and delay in diagnosis, in low-income and low-middle-income countries was 14 days. The median treatment delay in low-income and low-middle income countries was found to be 14 days. This brings the total delay to about 56 days compared to a total delay of about 14 days in upper-middle income countries (Teo et al., 2021). This lengthy delay from onset of symptoms until the beginning of treatment shows disparities of treatment in low-income countries and may allow for the continued transmission of TB prior to treatment. More work needs to be done in order to make TB treatment accessible and reduce delays.

Tuberculosis: The Bacterium

Mycobacterium tuberculosis (M.tb) is a pathogenic bacterium discovered in 1882 by Robert Koch. It is a bacillus or rod-shaped bacterium of the *Mycobactericeae* family. It has a very unique cell wall structure containing mycolic acids, which give the *Mycobactericeae* family its name. Typically, bacteria fall into either the gram-positive or gram-negative cell wall category. The basic structure of the cell wall of a gram-negative bacterium includes an inner cell membrane, an outer membrane, and peptidoglycan or other lipoproteins or glycoproteins within the periplasmic space between the membranes. The basic structure of a gram-positive bacterium includes an inner membrane and a thick layer of peptidoglycan surrounding the cell. The mycobacterial cell envelope is extremely complex (Figure 1). It contains an inner plasma membrane and a rigid peptidoglycan layer, between which sits the periplasmic space. The plasma membrane functions to regulate cellular intake of nutrients and it is the location of cell wall synthesis (Lee et al., 1996). The plasma membrane is made up of a typical phospholipid bilayer, but also contains phosphatidylinositol mannosides (PIMs), and lipomannans (LMs). PIMs may cause the mycobacterial membrane to be less permeable, decreasing susceptibility to antibiotics (Dulberger et al., 2020). Peptidoglycan maintains cell structure and shape. Arabinogalactan is a branched sugar molecule that forms the link between the mycomembrane and peptidoglycan. Lipoaribomannans (LAMs) are anchored in the plasma membrane and covalently attached to the arabinogalactan sugars, and they are an important part of the mycobacterial envelope's structural integrity. The mycolic acids which give mycobacteria their name form the outer membrane of the mycobacterial cell envelope, and they also help to make the mycobacterial cell wall less permeable to antibiotics. The mycomembrane is 100-1000 times less permeable to b-lactam antibiotics than a gram negative envelope (Dulberger et al., 2020). Therefore, the thickness and complexity of the mycobacterial cell envelope plays a role in *M. tuberculosis* survival and impermeability to antibiotics. The mycomembrane is composed of the inner leaflet of mycolic acids covalently attached to the arabinogalactan sugars, and an outer leaflet which is attached to trehalose monomycolate (TMM) and trehalose dimycolate (TDM) sugars which are on the surface of the mycomembrane (Dulberger et al., 2020). As well the outer mycobacterial membrane contains phthiocerol dimycocerosates (PDIMs) on the surface. PDIMs may interfere with immune recognition of pathogen associated molecular patterns (PAMPs), inhibiting detection of *Mycobacteria* and immune response (Cambier, Takaki, et al., 2014). Therefore, the mycobacterial cell membrane is not only structurally important but also helps *M. tuberculosis* survive within the host.

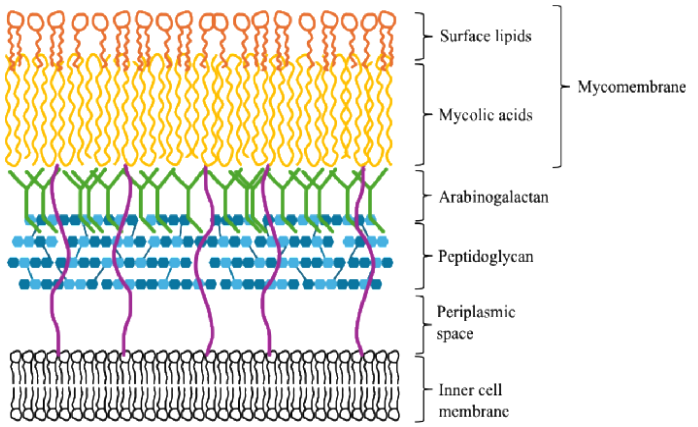

Figure 1. The mycobacterial cell envelope.
The mycobacterial cell envelope has a unique structure that contains an inner cell membrane (black), peptidoglycan (blue), lipoaribomannan (purple) arabinogalactan (green), and the mycolic acids (yellow) and surface lipids (orange) which are characteristic of the *Mycobactericeae* family.

Mycobacterial cell growth depends on cell wall synthesis. Mtb has two different growth stages. The first is rapid growth which occurs when there is little stress and excess nutrients. The second is growth stasis in which *M. tuberculosis* replicates slowly and experiences stress. Most of the cell envelope lipid synthesis is upregulated during rapid growth and downregulated during growth stasis phases including PIMs which make up the plasma membrane and the mycolic acids and trehalose sugars which make up the mycomembrane (Dulberger et al., 2020). However, LAM and LM synthesis is upregulated during starvation (Betts et al., 2002), indicating that they are more abundant in slow growth phases, and may have a role in protecting mycobacteria in the stressful intracellular growth environment. Many antibiotics which target *mycobacterium tuberculosis* target different parts of the cell wall synthesis. Isoniazid targets mycolic acid synthesis, and ethambutol target arabinogalactan synthesis (Dulberger et al., 2020). Since the cell wall synthesis is necessary for Mtb to replicate, these antibiotics are bacteriostatic or stop bacterial growth.

Tuberculosis Model of Pathogenesis and Transmission

Tuberculosis continues to infect and kill millions of people globally not only because of global health initiative shortcomings, but also because tuberculosis is a skilled pathogen. It is able to survive many different intracellular and extracellular environments within the host, evade immune response, and recruit immune cells in order to create a survival niche. The path of TB transmission and infection typically follows the steps: 1) entry into a new host through the lungs, 2) infection of a macrophage, 3) formation of a granuloma and intracellular replication, 4) granuloma maturation, and

5) granuloma necrosis and extracellular replication. The cycle complete with transmission of bacterium from infected host to a new host (Figure 2).

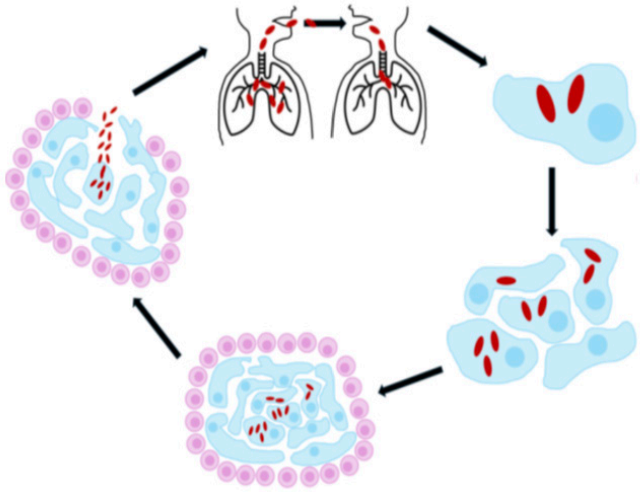

Figure 2. Mycobacterium tuberculosis path of infection and transmission.
Mycobacterium transmission is initiated by the transfer of airborne droplets from an infected individual's lungs to an uninfected individual's lungs. The bacteria are then engulfed by a macrophage, in which they can divide. More macrophages are recruited to the infection and the granuloma begins to form. A mature granuloma recruits lymphocytes which form an epithelial-like layer around a necrotic center. Finally, the granuloma bursts and releases the mycobacteria tuberculosis bacteria into the extracellular space of the infected individual's lungs.

TB is an airborne pathogen which is spread through tiny droplets containing 1-3 bacterium through coughing from the source infection to a new host. When the new host inhales the droplets, they may become infected with tuberculosis. Infection is initiated in the lower lung, which provides the host niche that tuberculosis needs to survive. The droplet size of tuberculosis infectious is inversely correlated with infection (Ratcliffe & Wells, 1948). Therefore, a larger bacterial burden in a larger droplet is less successful because it cannot reach the lower alveolar space.

Once it has entered the lungs, TB avoids detection by the host immune response by secreting a surface lipid, phthiocerol dimycoceroserate (PDIM), that masks pathogen associated molecular patterns (PAMPs) or markers which the immune system recognizes as pathogenic and activates immune response. Simultaneously, TB recruits' macrophages that are growth-permissive by secreting phenolic glycolipid (PGL) which activates macrophage chemokine *CCL2* which recruits macrophages (Cambier, Falkow, et al., 2014). This strategy at once hides from the immune response and recruits macrophages which are growth-permissive works well in the lower lung where there is relatively low microbial and immune club. Therefore, mycobacterium tuberculosis requires surface lipids in order to evade host immune response and infiltrate host macrophages.

Tuberculosis must then survive and replicate within the macrophages. The Mycobacterium tuberculosis is phagocytosed by the macrophage. The phagosome is a vesicle that digests pathogens and foreign particles. Therefore, TB must be able to survive or avoid acidification by the phagosome. TB escapes the phagosome into the cytosol of the macrophage and replicates within this intracellular environment.

Mycobacterium tuberculosis then recruits other macrophages to form a granuloma, an ability that is predicated on the ESX-1 efflux pump. A granuloma is a cluster of immune cells that forms an epithelial-like layer around the infected core. This protective layer was thought to contain tuberculosis to stop its spread, but it also acts as a protected environmental niche in which tuberculosis can multiply. Granulomas form when the infected macrophages undergo apoptosis, or cell death, and recruit more macrophages to engulf the dying cell and bacteria. This process repeats to form a mass of macrophage cells that expands the niche of the Mtb, allowing it to divide rapidly in early infection. The internal environment of macrophages is toxic to Mtb. One reason Mtb is able to survive in the intracellular environment of the macrophages is the virulence factor, ESX-1. As well, Mtb has the unique ability to synthesize tryptophan.

Once, a granuloma has formed it shifts from apoptotic cell death of the macrophages into necrosis of the macrophages. Apoptosis keeps to cell membrane of the macrophages intact, keeping Mtb encased within the cell, while necrosis leads to the lysis or breaking up of the cell. This creates a granuloma with a necrotic core, which provides a good extracellular environment for Mtb to replicate. The mechanism of macrophage necrosis may be triggered by host dysregulation of TNF, or tumor necrosis factor. Because of the necrosis of the granuloma, and Mtb's rapid replication, Mtb is able to escape the granuloma into the lungs. There the bacteria are aerosolized into droplets and are able to be transmitted into a new host through coughing or sneezing.

Targeting TB Requires New Solutions

While the mechanism of TB infection are well-studied and antibiotic treatments exist, TB remains a global health issue because current therapies, prevention, and public health strategies have not overcome the burden of poverty. TB prevalence is highly associated with poverty and social determinants of health. Therefore, new solutions to address TB globally must address alleviating poverty and providing direct assistance to countries and individuals with high TB burdens. Providing a basic income to patients affected by TB can improve health outcomes and reduce the risk of incurring catastrophic treatment costs. In Peru, TB-affected household which received intervention in the form of monthly cash transfers (an average of US$137 per household over the total course of treatment) in addition to traditional TB treatment were less likely to have catastrophic costs over the course of TB treatment than those without intervention (Wingfield et al., 2016). Therefore, providing even small monthly payments of financial aid to TB-affected household can help alleviate costs of treatment and prevent catastrophic financial hardship. As well, this economic intervention improved health outcomes. In the intervention group, treatment was successful for 64% of patients compared to 53% of patients in the non-intervention or control group (Wingfield et al., 2017). This suggests that alleviating poverty and the barrier of cost of treatment improves health outcomes in patients with TB.

Malnutrition is one of the prominent risk factors associated with TB

Malnutrition is also associated with poverty and access to food. In Brazil, patients who were given health care coupled with monthly food vouchers to had 13% greater rate of being cured compared to patients who only received traditional TB treatment (Reis-Santos et al., 2022). This suggests that providing adequate nutrition to TB patients who might not otherwise have access to it improves health outcomes. Therefore, socioeconomic interventions have a positive effect on the effectiveness of TB treatment.

Directly Observed Therapy Short course (DOTS) is a strategy for TB treatment developed by the World Health Organization that involves political commitment, timely diagnosis through sputum microscopy, a six-to-eight-month standardized treatment regimen with directly observed treatment for at least two months, a regular supply of anti-TB drugs, and a standardized recording and reporting system to track patient progress. DOTS has been shown to improve cure rates of TB and decrease failure rates due to drug resistance in China ("Results of Directly Observed Short-Course Chemotherapy in 112,842 Chinese Patients with Smear-Positive Tuberculosis. China Tuberculosis Control Collaboration," 1996). The DOTS strategy has received some controversy for the direct observation of treatment parameter, which requires a healthcare worker to directly observe that anti-TB drugs have been taken by the patient. This method is considered paternalistic and may not be effective. In Pakistan, patients who received treatment according to WHO guidelines with either healthcare worker observation family

member observation, or no observation did not show significant difference in cure rates (Walley et al., 2001). Therefore, the success of the DOTS strategy is most likely due to the earlier diagnosis and strengthened and standardized level of care rather than the direct observation of treatment.

While addressing social determinants of health can greatly improve treatment outcomes, another route to eradicating TB is prophylaxis or preventative treatment and vaccines. The Bacille Calmette-Guerin vaccine is currently the only vaccine for tuberculosis, and as previously discussed, it is largely ineffective at treating pulmonary TB in adults. While about 16 other vaccine options are under different phases of clinical trials, none have been officially licensed. The rBCG or AERAS-422 vaccine trails were terminated despite showing increased anti-mycobacterial activity because of association with varicella zoster virus, which can cause shingles in adults, in two cases after administration (Hoft et al., 2016). Another vaccine candidate, which entered phase two trials, the MVA85A which expresses the *M. tuberculosis* antigen 85A was found to be safe and well tolerated by patients but did not increase efficacy of protection compared to the BCG vaccine (Ndiaye et al., 2015). A promising vaccine candidate is the MIP vaccine which was originally developed in India for leprosy, but has shown to increase immune response taken in conjunction with the BCG vaccine in comparison to only BCG in mice (Saqib et al., 2016). However, these effects have not yet been studied in human trials. Therefore, new vaccine options must continue to be explored if an effective prophylaxis for pulmonary tuberculosis is to be found.

As well, in order to address the rising problem of drug-resistant TB, new chemotherapeutic solutions are needed. In order to develop new antibiotics, new drug target candidates must be identified and examined. These drug targets are virulence factors, or molecules that enable pathogens to invade the host, evade the immune system, and replicate within the host. Some new drug targets have been identified including GyrA, a DNA gyrase which catalyzes DNA supercoiling, DnaN, a subnit of DNA polymerase III, Pks13, which is essential for mycolic acid synthesis, and others (Huszár et al., 2020). These novel drug targets are promising avenues to new drug candidates; however, the process of drug discovery is complex and it is important to continue to search for novel drug targets. Because *M. tuberculosis* has a complex mechanism of pathogenesis and survives in multiple environmental niches, an ideal drug candidate would target a virulence factor that contributes to both intracellular and extracellular survival of Mtb in the host.

Mtb-BrkB: A Possible Virulence Factor

Mtb-BrkB is an Mtb putative transporter protein also known as Rv2707, according to the H37Rv Mtb reference genome. Its function is currently unknown; however, it is a protein of interest because it was found to promote Mtb growth in mice in a transposon screen. The Rv2707 transposon mutant Mtb growth was attenuated over 4-fold in mice (Sassetti & Rubin, 2003) (Fig. 3). Because a disruption in Rv2707 or Mtb-BrkB causes attenuated infection in mice, Mtb-BrkB is likely to play a role in the in vivo survival of Mtb and its virulence. Mtb-BrkB is so called because it contains the *Bordetella* resistance to complement-mediated killing (BrkB) domain. The *Bordetella pertussis* BrkB (Bp-BrkB) is a virulence factor which was found to be necessary for serum resistance in *B. pertussis* and has paralogs in *E. coli* (yhjD), M. tuberculosis and M. leprae (Fernandez & Weiss, 1994). The BrkB domain is highly conserved among both pathogenic and nonpathogenic mycobacteria, indicating that it may have a function that is crucial to survival, but has been altered to promote virulence in pathogenic bacteria. *Mycobacterium leprae*, which causes leprosy and cannot be grown in vitro due to the small size of its genome (1,604 protein-coding genes) contains an ortholog of Mtb-BrkB. Since the *M. leprae* genome is so parsed down to only genes which are necessary for its survival within a host, the presence of a BrkB ortholog suggests that BrkB plays an important role in host infection, *Mycobacterium smegmatis* (Msmeg) and *Mycobacterium marinum* (Mm), which are both commonly used model organisms to study Mtb, also both contain an ortholog of Mtb-BrkB. Msmeg is a non-pathogenic bacterium that shares >2,800 orthologs with >50% identity with Mtb (Sparks et al., 2023).

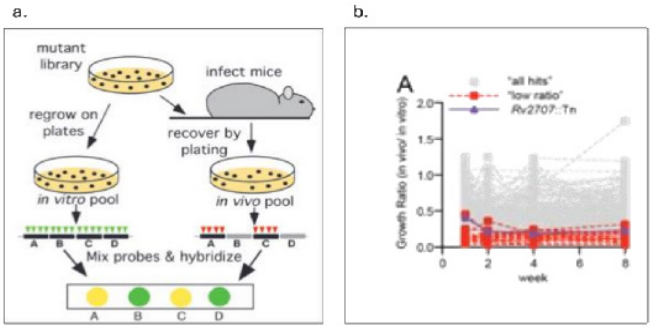

Figure 3. Mtb-BrkB transposon mutation attenuates infection in mice (Sassetti & Rubin, 2003).
(a) Sassetti and Rubin created a transposon mutant library and compared growth of transposon mutants *in vitro* and *in vivo*. (b) The growth ratio of *in vivo* to *in vitro* growth was plotted over time of infection in weeks and low ratio (<0.5) hits are highlighted in red. Mtb-BrkB transposon mutant indicated by blue line.

M. marinum shares >3,000 orthologs with >85% identity with Mtb, and infects zebrafish with a TB-like illness, making it a useful model for studying TB (Stinear et al., 2008). Previous research in the lab has shown that the Mm ortholog of Rv2707, MMAR_2006 or Mm-BrkB, is required for Mm intracellular and extracellular growth in zebrafish. A transposon mutant library of Mm mutants was constructed including a mutant with a transposon insertion in Mm-BrkB. Mm wildtype (WT), the transposon mutant Mm (MMAR_2006::Tn), and the transposon mutant with heterologously expressed Mtb-BrkB (Mtb complement) were used to infect zebrafish larvae. At five days post infection, zebrafish infected with MMAR_2006::Tn showed a lower bacterial burden compared to WT. The bacterial burden by Mtb complement infection showed no difference to WT (Fig. 4). This indicates that Mm-BrkB is required for Mm infection in zebrafish, and that Mtb-BrkB rescues infection in Mm-BrkB transposon insertion mutants, indicating Mtb-brkB plays a role in infection. As well, intramacrophage growth in zebrafish larvae was determined 2 days post infection. The average proportion of macrophages infected with ³5 bacterium after 2 days was significantly lower for MMAR_2006::Tn than WT. This indicates that the role of Mm-BrkB may be related to intramacrophage growth. Finally, MMAr_2006::Tn grew less than WT in the presence and absence of macrophages, indicating that Mm-BrkB's role in infection may have an extracellular component as well as an intramacrophage component. The behavior of the Mm ortholog of Mtb-BrkB in Mm infection of zebrafish can provide insights into the role of Mtb-BrkB in tuberculosis infection. Therefore, Mtb-BrkB is a possible virulence factor of interest, and may help Mtb survive in intracellular and extracellular environments in the host.

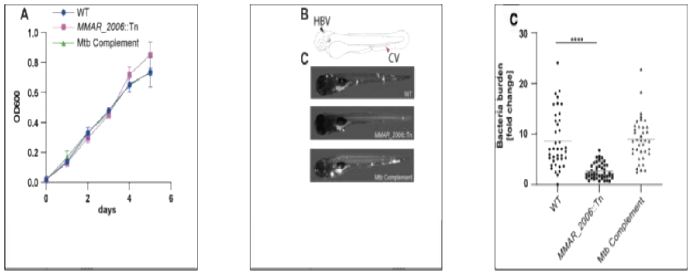

Figure 4. Mm-BrkB mutant shows attenuated growth in zebrafish, and Mtb-BrkB complement rescues infection.
(a) The optical density (OD600) of *in vitro* liquid culture of wild type *M. marinum* (WT), Mm-BrkB transposon insertion mutant (MMAR_2006::Tn), and Mm-BrkB mutant with Mtb-BrkB heterologously expressed (Mtb complement) was measured over time in days showing similar growth rate in vitro. (b) Zebrafish were infected via caudal vein (CV) and imaged five days post infection. (c) Bacterial burden in zebrafish infected with (MMAR_2006) Tn was significantly attenuated compared to WT, and Mtb complement rescued bacterial burden to WT level.

The structure of Mtb-BrkB is predicted to be a 6-transmembrane helix containing protein with cytosolic amino and carboxy termina by AlphaFold Server (Fig 5,6). Because Mtb-BrkB is a transmembrane protein of small size (~35kDa), this indicates that it may play a role in a larger protein complex that may be involved in transport across the inner membrane of Mtb.

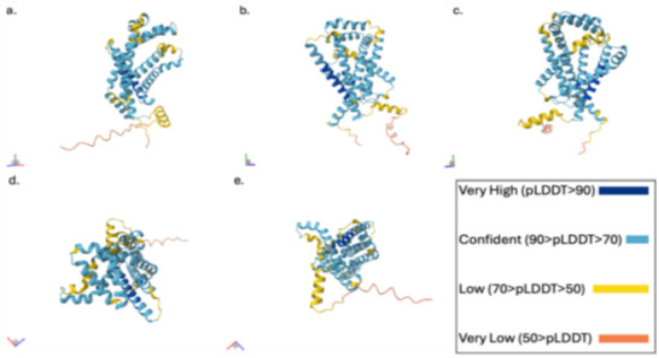

Figure 5. AlphaFold predicted structure of Mtb-BrkB from five perspectives.
The predicted structure of Mtb-BrkB is shown from front (a), turned 90° to the right (b), to the left (c), to the top (d), and to the bottom (e). the confidence of the prediction is given by the predicted local distance difference test (pLDDT) which measures the local confidence per residue.

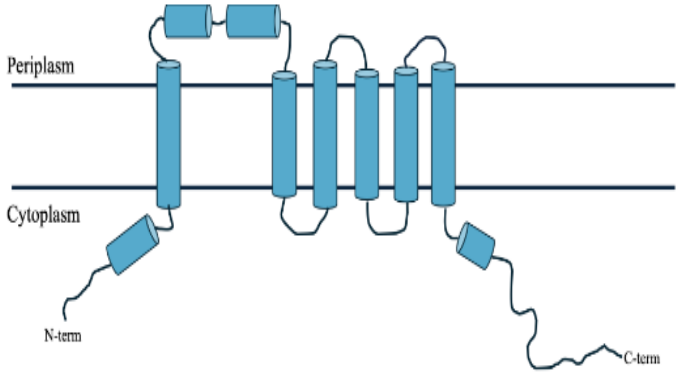

Figure 6. Simplified model of Mtb-BrkB.
Cylinders represent alpha-helices, and drawn lines are unstructured regions. The predicted protein structure has 6 transmembrane helices and 4 smaller helices, 2 cytoplasmic and 2 periplasmic. Both the N-terminal and the longer C-terminal tails are cytoplasmic.

Introduction to Thesis Research

The goals of this study are to heterologously express and isolate Mtb-BrkB from E. coli for downstream functional and structural analysis. In order to do so, the expression of Mtb-BrkB in E. coli must be tested and optimized for growth, lysis, and elution conditions to achieve high yield of protein. This study used E. coli as a model organism, and it was transformed with a Mtb-BrkB coding plasmid vector. Another mycobacterial protein, Mtb-MscL, a mechanosensitive ion-channel, was used as a positive control throughout the study because it has previously been expressed and purified from E. coli (Chang et al., 1998). Mtb-MscL is, like Mtb-BrkB a small membrane protein, making it a good positive control.

Escherichia coli as a Model Organism

Escherichia coli (E. coli) is a bacillus gram-negative bacterium. *E. coli* is often used as a model organism in the laboratory because it has a relatively simple and well-studied genome as well as a fast replication time of ~20 minutes. Comparatively the replication time of Mtb is 16-24 hours. This makes *E. coli* a good model organism because it shortens growth time significantly. The general model of heterologous expression in *E. coli* starts with the transformation of *E. coli* with a plasmid containing a target protein coding sequence, an induction system, and an antibiotic resistance gene. The successfully transformed *E. coli* can then be selected for with inhibitory concentrations of antibiotic in growth media. In this case, ampicillin was used. The heterologous expression of the target protein can be turned on or induced by an inducing agent, and the *E. coli* can by lysed to extract and purify the target protein form the lysate (Fig. 7).

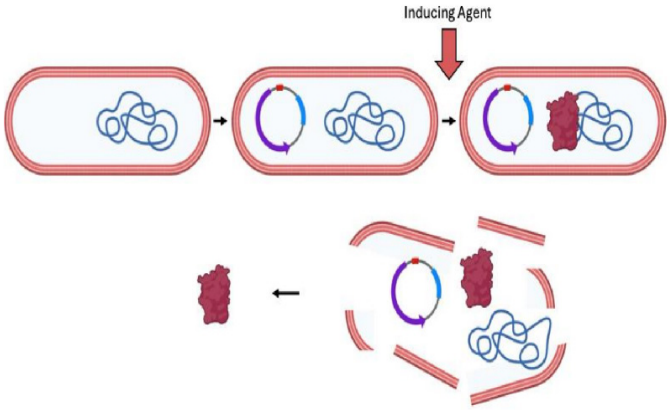

Figure 7. Overview of heterologous expression in E. coli.
Heterologous expression in E. coli is done by transforming E. coli with a protein-coding plasmid. E. coli will then express the target protein in the presence of an inducing agent (in this case, IPTG), and can by lysed. Target protein is then purified from crude lysate.

The strain of *E. coli* used to heterologous produce Mtb-BrkB was BL21(DE3) competent *E. coli*, which is a T7 expression strain of *E. coli*. Therefore, the BL21 *E. coli* produce the T7 RNA polymerase which will transcribe genes downstream of a T7 promoter, which is a common promoter on pET plasmid vectors. The T7 RNA polymerase gene is regulated by a *lacUV5* promoter which allows for expression of the T7 RNA polymerase in the presence of lactose or isopropyl β-D-1-thiogalactopyranoside (IPTG), allowing expression to be induced. It is also deficient in proteases Lon and OmpT, which prohibits the strain from degrading the heterologous produced proteins, leading to high protein yield (BL21(DE3) Competent *E. Coli* | NEB, n.d.).

However, there are some difficulties with using *E. coli* for the purpose of producing Mtb-BrkB. Membrane proteins are much harder to express in *E. coli* than cytosolic proteins, and membrane protein expression often leads to much lower yields. There are some strategies to overcome these difficulties including using *E. coli* strains optimized for protein expression such as BL21(DE3) E. coli, using solubilizing agents such as detergent or urea, and attaching solubility promoting tags to the target protein.

As well, *E. coli* inner membrane lipid composition is typically made up of about 75% phosphatidylethanolamines (PE), about 20% phosphatidylglycerols (PG), and about 5% cardiolipins (CL) (Rowlett et al., 2017). Comparatively, the inner membrane lipid composition of Mtb is much more complicated. It contains glycerophospholipids phosphatidylinositol (PI), phosphatidylglycerol, phosphatidylserine (PS), phosphatidylethanolamine (PE), cardiolipin (CL), and mannosylated forms of PI known collectively as PIMs. As well, lipoaribomannans (LAMs) are anchored in the inner membrane (Jackson, 2014). Therefore, if the association of Mtb-BrkB is dependent on interaction with lipids which are found in the Mtb membrane and not the *E. coli* membrane, expression of Mtb-BrkB in *E. coli* might lead to aggregation of proteins. Despite the difference in membrane composition between *E. coli* and Mtb, expressing Mtb-BrkB in *E. coli* may be possible as suggested by the presence of a natively occurring BrkB ortholog in *E. coli* called YihY which is a membrane protein of unknown function which contains the BrkB domain.

Designing Plasmid DNA Constructs

A plasmid is a circular piece of DNA, that can be introduced to a bacterium via the process of transformation. Plasmids are useful because they introduce genes into the bacteria which will be transcribed and translated into proteins using the bacteria's cellular machinery. Two plasmids were constructed and used for the expression in *E. coli*: one plasmid containing Mtb-BrkB, and one plasmid containing Mtb-MscL (Fig. 8). Mtb-MscL is used as a positive control for the experiments since it is a Mtb protein which associated with the membrane. Mtb-MscL has previously been shown to be a gated ion-channel that forms a homohexamer (Chang et al., 1998). Since it was previously heterologously expressed in E. coli, it is used as a positive control for Mtb-BrkB.

The plasmid construct used to express Mtb-BrkB in *E. coli* uses the pET-21a(+) backbone. It includes an origin of replication (Ori), ampicillin resistance gene (*AmpR*), the lac I protein and lac O operator, the T7 promoter, the ribosome binding site (RBS) the Mtb-BrkB genetic sequence with a 6x-histidine tag. The origin of replication allows the plasmid to be replicated by the *E. coli*. The ampicillin resistance gene allows inhibitory concentrations of ampicillin to be used in order to select for the plasmid carrying *E. coli*. The lac I gene and lac O operator work in tandem to allow protein expression to be regulated based on the presence of lactose or IPTG. The lac I gene codes for the lac repressor protein which binds to the lac O operator, prohibiting transcription of the downstream genes. When lactose or IPTG are present, they will bind to the lac repressor protein causing a conformational change which will decrease its binding affinity to the lac O operator sequence, allowing the transcription of downstream sequences. The T7 promoter sequence signals for the T7 RNA polymerase produced by BL21 de3 *E. coli* to attach to the plasmid and begin transcribing. The T7 terminator sequence signals for the RNA polymerase to end transcription. The ribosome binding site signals for ribosomes to bind and begin translation of the mRNA to protein. Therefore, the protein which will be produced will be the Mtb-BrkB protein with 6 histidine residues at the carboxy terminus. The plasmid construct used to express Mtb-MscL is similar but uses the pET19b(+) backbone which has a different backbone sequence and different restriction sites. As well, the coding sequence will code for the Mtb-MscL protein with 10 histidine residues at the carboxy terminus.

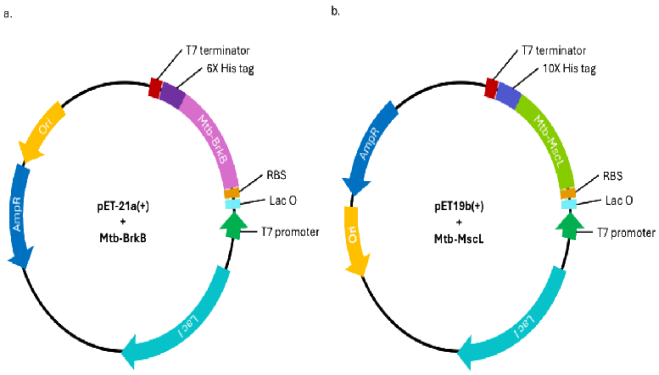

Figure 8. Design of Mtb-BrkB and Mtb-MscL plasmid vectors.
The design of the plasmid construct to express Mtb-BrkB (a) and Mtb-MscL (b) in BL21 E. coli both contain ampicillin resistance genes (AmpR), origins of replication (Ori), lac I repressor protein coding sequences (Lac I), lac O operators (lac O), ribosome binding sequences (RBS), multi-histidine tags (6X or 10X his tag), and T7 promoter and terminator sequences.

Nickel Affinity Chromatography

The inclusion of the histidine tag on the carboxy terminus of the target proteins is useful for purification and isolation of the protein from cell lysates. This takes advantage of the affinity binding between the histidine amino acid and nickel ions. Positively charged nickel ions (Ni^{2+}) form non-covalent bonds with the nitrogen of the histidine residue (Fig. 9). Therefore, in a technique called nickel affinity chromatography, nickel ions associated on agarose beads can be used to selectively bind to the histidine residues of a His-tag. The proteins containing His-tags will bind to the beads, while other proteins in the clarified *E. coli* lysate will not. Then, imidazole, which has the structure of the histidine side chain (Fig. 9) can be used to disrupt and replace this bond to elute the purified protein fraction from the nickel beads. Therefore, using Nickel affinity chromatography, the heterologously expressed Mtb proteins can be purified for further functional or structural examination.

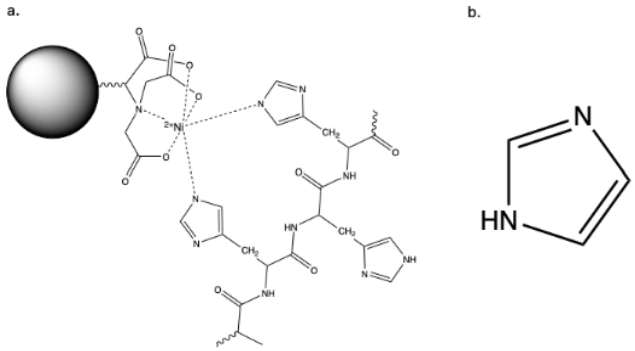

Figure 9. Poly-histidine tag affinity binding to Ni^{2+} beads compared to the structure of imidazole.
(a) The proton poor nitrogen of the histidine side chain will form non-covalent bonds with positively charged nickel ions which are coordinated to an agarose bead. (b) The structure of imidazole is the same as the side chain of a histidine residue, allowing for competitive elution.

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Behavioral Profiling of 5xFAD Mice: *Investigating β-Amyloid–Driven Deficits in Cognitive and Non-Cognitive Domains*

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As the quality of life improves and human lifespan extends, the prevalence of Alzheimer's disease (AD) continuously grows. AD is a devastating neurological condition affecting millions and driving extensive research to uncover its mechanisms. Because amyloid-β plaque deposition is the earliest pathological hallmark of AD, therapies aimed at slowing plaque formation are most effective when initiated at the very onset of disease—making sensitive, early behavioral biomarkers imperative. The 5xFAD mouse, which develops aggressive amyloid pathology, offers a powerful platform for uncovering early behavioral signatures. Here, I subjected 5xFAD and wild-type littermates to a comprehensive battery of assays spanning decision-making, attention, exploration, memory, and other non-cognitive domains. We hypothesized that plaque-driven behavioral deficits would surface as soon as plaques emerged. Although robust amyloid deposition was detected in the subiculum, its burden correlated only weakly and non-significantly with behavioral performance. These results underscore the need for larger sample sizes, plaque quantification in additional regions (e.g., cortex), and further refinements to assay sensitivity in order to pinpoint reliable behavioral indicators of early AD.

I. Introduction

Where did I leave my keys? Did I turn off the iron before I shut the door? Did I lock the door? Forgetting is a universal experience—something that happens to all of us, often daily. It usually brings a touch of irritation or frustration as we struggle to recall specific details and replay the particular moments in our minds. Now imagine waking up day after day with the same sense of frustration and inability to remember even the most basic things. You first forget where you usually keep your toothpaste. Then you can't recall where you live, and eventually, you no longer recognize your loved ones. Scary, isn't it? This is what daily life can feel like for a person with Alzheimer's disease. It is not just their memory that fades, but their very self—their identity, their life, and everything that once defined them—all slipping away.

1. Overview of Alzheimer's Disease

1.1 Alzheimer's Disease Facts and Figures

Alzheimer's disease (AD) is a neurodegenerative disease and the most common type of dementia, making up 60-70% of the cases (WHO, 2023). According to the Alzheimer's Association, there are approximately 6.9 million people living with AD in the USA and the numbers are expected to grow to 13.8 million by 2050. Age is the number one risk factor for developing AD, which explains the continually increasing prevalence of the disease as lifespans expand globally (Alzheimer's Disease Facts and Figures, 2024). The number of deaths due to AD was approximately 119,399 in 2021 worldwide (Alzheimer's Disease Facts and Figures, 2024). AD is not just a devastating condition for the patients and their families, but also a significant burden to the country's economy. In 2020, Americans spent 196 billion US dollars in direct medical costs and caregiver time equivalent to 254 billion dollars (Nandi et al., 2024). The National Institutes of Health (NIH) approved an additional fund of 100 million dollars aimed at AD research, making a total of 3.4 billion dollars in 2024 (Alzheimer's Association, 2024).

1.2 AD Pathology, Etiology, Symptoms, and Risk Factors

The two molecular hallmarks of AD are beta-amyloid (Aβ) plaques and tau tangles (Fig. 1A & B) that precede and cause physiological deteriorations to the brain. Such changes are atrophy defined by tissue and brain volume loss (enlarged ventricles and shrunken gyri) (Fig. 1C). Also, neuronal death and microglial inflammation in the hippocampus correlate with the progressive decline of cognition (Rao et al., 2022). There are two subtypes of AD: Late Onset (LOAD) and Early Onset (EOAD). The overwhelming majority of the patients (95%) have LOAD (after 65 years old), which is sporadic. The rest (5%) is EOAD (before 65 years old), which is determined by genetic predisposition and displays more aggressive progression (Mendez, 2019). Along with the pathological deterioration, there are also several stages of symptoms that AD patients go through as the disease progresses. The stages include preclinical (development of plaques and tau tangles with no pronounced behavioral deterioration), mild (repeating questions, memory loss, poor judgment), moderate (withdrawal from social activities, shortened attention span), and severe (inability to communicate, weight loss, loss of bladder and bowel control) (National Institute on Aging, n.d.).

In terms of Aβ plaque accumulation, a thorough plaque localization analysis showed that first deposits are seen in the neocortex, then spread into limbic regions including the hippocampus and entorhinal cortex, as well as the basal ganglia, and ultimately accumulate in the cerebellum. On the other hand, Braak and Braak showed that neurofibrillary tangles initially aggregate in the lower brainstem (locus coeruleus) and then spread into the hippocampus, eventually spreading into neocortical areas (Hampel et al., 2021) (Fig. 2). Autosomal dominant mutations in genes such as APP, PSEN1, and PSEN2 that alter Aβ precursor protein are related to the EOAD, whereas age-related changes and sporadic mutations that are more difficult to identify underlie LOAD (Uddin et al., 2021). Some of the LOAD related risk factors include mutations in APOE (APOE 4 isoform), BIN1, CLU, PTK2B, CR1, MS4A2, PICALM, IQCK, and TREM2 (Chen, Petty, Sha, et al., 20).

2. Disease Process of AD

2.1 Aβ Plaques Formation

The Aβ peptide plays a crucial role in AD pathology and is formed because of altered cleavage of Amyloid Precursor Protein (APP) by β and γ secretases. APP is located on chromosome 21 and belongs to the family of proteins that also includes APP-like proteins 1 (APL1) and 2 (APL2), all of which are type-I transmembrane proteins in mammals (Coulson et al., 2000; Wasco et al., 1992). The role of APP is still being explored, but most studies suggest that it may be responsible for neurite outgrowth (Bibel et al., 2004), synaptogenesis (Moya et al., 1994), cell adhesion (Soba et al.,2005; Wang et al., 2009), and other functions substantial for cell activity and proliferation (Zhang et al., 2011).

APP is found in several isoforms produced by alternative splicing of the exons (coding region of gene) 7, 8, and 15 (Menéndez-González et al., 2005). The most abundant form of APP in the brain is APP 695 — produced mainly by neurons and lacks Kunitz-type serine protease inhibitory (KPI) domain sequence (KPI-). Whereas APP 751 and 771 are predominantly expressed in glial cells and contain KPI (KPI+) sequence (Chen et al., 2017). The concentration of KPI+ APP mRNA significantly increases, whereas KPI- APP mRNA decreases in AD brain. KPI+ APP mRNA level is also positively related to Aβ production and accumulation (Menéndez-González et al., 2005). Considering the differences between the KPI- and KPI+ APP isoforms concentrations, it is crucial to understand the underlying molecular mechanism of the KPI. KPI is a ubiquitous protein that belongs to the protease inhibitor I2 family and is found across different species. Structurally, it is a relatively small 60-80 amino acid residue-protein (de Magalhães et al., 2018). KPI containing APP isoform has been shown to be prone to homodimerization with more molecular mechanisms to be discovered (Ben Khalifa et al., 2012; Byun et al., 2023). Evidence also suggests that KPI+ APP interacts with α-secretase, inhibiting its normal function. This inhibition might be key to a shift toward β-secretase cleavage, increasing Aβ production and contributing to AD pathology (Lesné et al., 2005).

*This author wrote this paper as a senior thesis under the direction of Dr. Eun Jung Hwang.

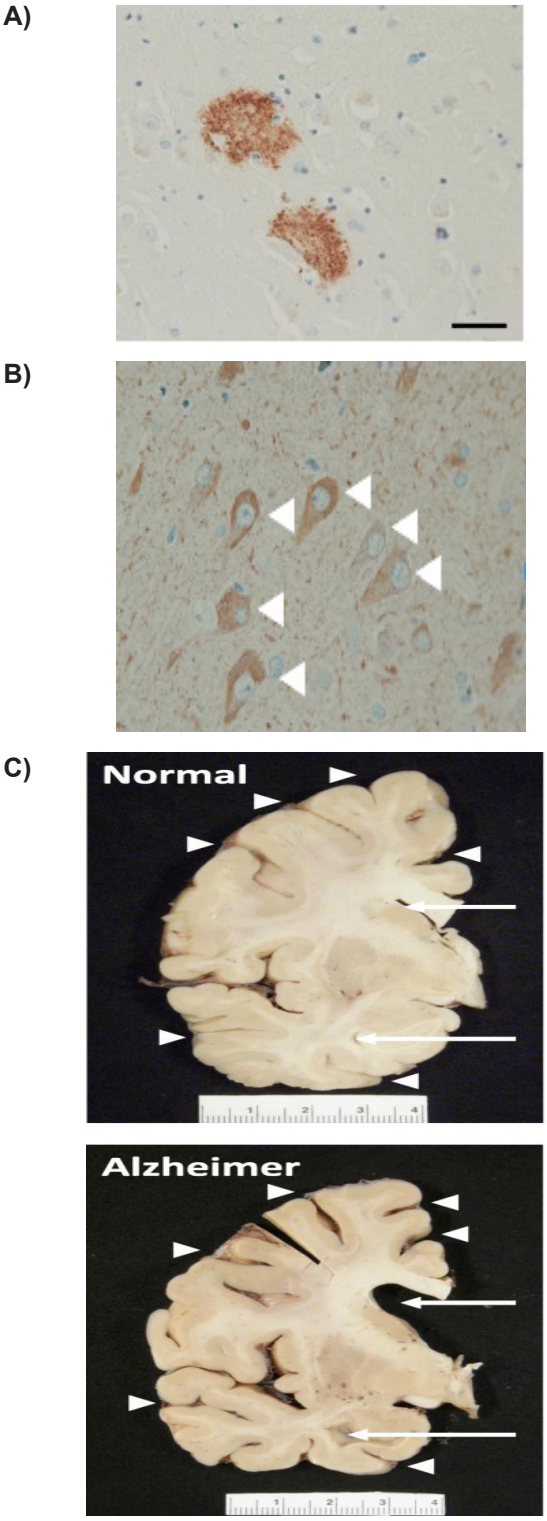

Fig. 1. **Micro- and Gross-level Pathological Changes in AD patients**. (**A**) Densely packed neuritic Aβ plaques in AD patient brain. (**B**) Matured neurofibrillary tangles (denoted by arrows) and pre-tangles (denoted by arrowheads). (**C**) Atrophy of the brain. On top is the normal/healthy brain section. On the bottom, AD brain shows marked atrophy, dilation of the lateral ventricle (top arrow), and a shrunken hippocampus (bottom arrow) and shrunken gyri (arrowheads). (DeTure & Dickson, 2019).

APP is synthesized in the endoplasmic reticulum and transported to the trans-Golgi network (TGN), where it undergoes sorting and modification. From the TGN, APP is directed to the plasma membrane, early endosomes, and other intracellular compartments. Its localization and trafficking are dynamic, with endosomes being a key site for its amyloidogenic processing into amyloid-beta peptides, which are implicated in AD pathogenesis (Jiang et al., 2014). Following prior cleavage by β-secretase, processing of APP by γ-secretase generates Aβ peptides of varying lengths.

Another key gene implicated in AD is PSEN1 that is known to be the most common genetic cause accounting for ~6% of EOAD cases. PSEN1 encodes presenilin-1 (PS1), which functions as the catalytic subunit of γ-secretase, an intramembranous protease that cleaves a variety of type 1 transmembrane proteins, most notably APP (Kelleher & Shen, 2017). Most PSEN1 mutations are heterozygous showing autosomal dominance heritance pattern. Two specific mutations, Asp40del (delGAC) and Ala79Val, show a stronger link to EOAD, compared to others. Both mutations lead to high production of Aβ42, but not Aβ40. In contrast, mutations such as Gly209Arg, Gly209Val, Leu235Pro, Cys410Tyr, Leu435Phe lead to decrease in Aβ40 production. Current data shows that loss of function in PS1 is the driving force of AD progression. Although not fully established, some evidence suggests that PSEN activity may result in abnormalities in synaptic functions, leading to neuronal loss, and tau hyperphosphorylation (Bagaria et al., 2022).

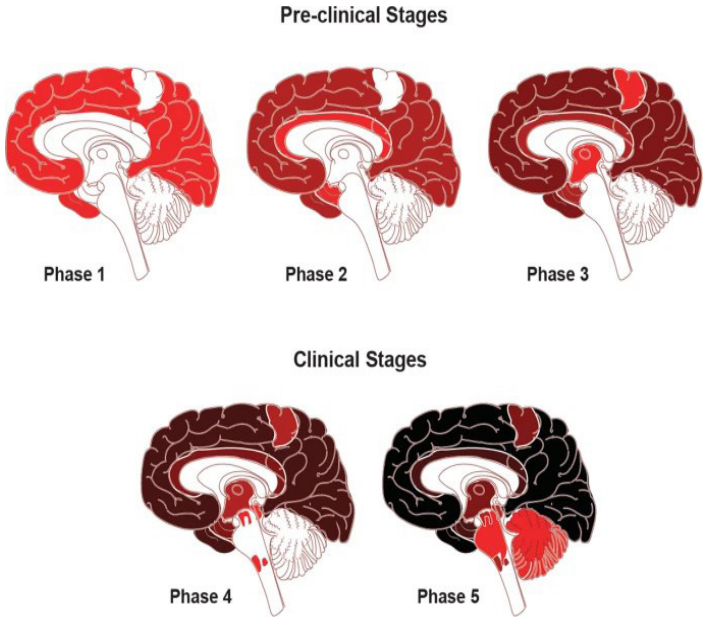

Fig. 2. **Amyloid Plaque Accumulation in Different Stages of AD.** Initial aggregations are depicted in bright red, with continued deposition in the same region represented by progressively darker shades, illustrating the progression from stage 1 to stage 5. Plaque deposition begins in the neocortex (stage 1) gradually spreading to limbic system (stage 2), thalamus (stage 3), pons (stage 4), eventually reaching the cerebellum in stage 5 of the disease. (Hampel et al., 2021).

In a healthy brain, APP is typically cleaved by α secretase that maintains its normal structure and function of APP. This cleavage produces sAPPα and C83. sAPPα is crucial for neuroplasticity and cell survival (Furukawa et al., 1996) and is protective against neurotoxicity (Han et al., 2005). sAPPα has also been positively linked with activation of muscarinic acetylcholine receptors (Haass et al., 1995). C83 is subsequently cleaved by γ secretase, forming p3 and APP intracellular domain (AICD) (Fig. 3). According to Kuhn et al (2020), there is no conclusive data on the role of the p3 peptide. Some researchers claim its neuroprotective role, while others highlight its neurotoxicity. Similarly, in AD brain, APP cleavage by β secretase produces sAPPβ and C99. C99 is further cleaved by γ secretase, producing Aβ and AICD. Considering that AICD is produced in both cases, there is no solid understanding of its contribution to the development of AD. Whether it is toxic to the brain or protective is an important topic of debate (Muller et al., 2008) (Fig. 3). Most relevant to AD are the two isoforms of Aβ, Aβ40 and Aβ42, which are both found in AD plaques. Those isoforms can form three types of aggregates: Aβ40, Aβ42, and Aβ42/Aβ40. Compared to Aβ42, Aβ40 takes a much longer time to

aggregate, and is therefore less likely to form insoluble deposits or drive pathological changes (McGowan, 2005). The third type of aggregates, Aβ42/Aβ40, displays a delay in accumulation similar to Aβ40. Only Aβ42 formation is a specific isoform that is specifically implicated in AD pathology and neurotoxicity (Gu & Guo, 2013; Kuperstein et al., 2010). Structurally, through nucleation, Aβ monomers aggregate into different types of assemblies: oligomers, protofibrils and amyloid fibrils. Aβ fibrils are larger and insoluble and are capable of forming amyloid plaques, whereas oligomers are soluble and can spread around the brain (Chen et al., 2017). The latter aggregates at the early stages of the disease and are considered to be highly toxic, driving the amyloidogenic pathway in AD (Sehar et al., 2022). Aggregated Aβ plaques can exist in both loosely and densely packed forms. Research indicates that densely packed plaques do not form spontaneously but rather through a process involving microglia. Microglia organizes the loosely packed Aβ oligomer structures into dense-core plaques, thereby potentially limiting their toxicity. In other words, microglia-mediated plaque compaction may reduce the harmful effects of Aβ accumulation on surrounding neurons (Huang et al., 2021).

2.2 Neurofibrillary Tangle Formation

Tau protein is encoded by MAPT gene located on chromosome 17. Exons 2, 3, and 10 are crucial for canonical functioning of tau. Exons 2 and 3 are translated to N1 and N2 aspects of N- terminal responsible for signal transduction and membrane interaction. Exon 10 on the other hand encodes the R2 region—the second repeat in the C-terminal microtubule-binding domain and is strongly associated with neurodegeneration. Alternative splicing of exon 10 results in either 3-repeat (3R) or 4-repeat (4R) tau isoforms, depending on whether the exon is excluded or included. Since the microtubule-binding domain of tau mediates its interaction with microtubules, it plays a key role in maintaining microtubule stability, regulating their dynamics, and supporting axonal transport (Park et al., 2016). The boundary between exon 10 and intron 10 contains an RNA sequence that forms a stem-loop structure through self-complementary base pairing. This stem-loop is a hot spot for *MAPT* gene mutations, as numerous pathogenic intronic variants are concentrated within this region. These mutations destabilize the stem- loop, leading to aberrant splicing of exon 10 by increasing its inclusion. The resulting alteration in RNA secondary structure enhances spliceosome accessibility, thereby promoting mis-splicing (Kar et al., 2011; McCarthy et al., 2015). In a healthy brain, the levels of 4-repeat (4R) and 3- repeat (3R) tau isoforms are typically balanced at an approximate 1:1 ratio. Disruption of this equilibrium has been implicated in the pathogenesis of various tauopathies. While no definitive association has been established between a specific tau isoform and AD, emerging evidence suggests that distinct subtypes of AD-related tauopathies may exist, potentially influenced by alterations in tau isoform expression or ratio (Liu & Gong, 2008).

Considering the role of tau in maintaining the stability and architecture of microtubules and subsequently axons, tau may play a crucial role in signal transduction and viability of the neuron (Wang & Liu, 2008). The accumulation of the tau protein starts at the brainstem and limbic systems, progressively spreading through the entire brain (Gabitto et al., 2024). Tau protein goes through multiple post-transcriptional modifications such as glycolysis, nitration, truncation, etc., however, phosphorylation is the most well-studied modification.

Phosphorylation is one of the most common causes of tau dysfunction which leads to tauopathies in AD (Avila et al., 2004). The role of Aβ plaques in tau tangle formation is undeniably important as Aβ plaques directly promote tau phosphorylation and thus its tangle formation. Other than phosphorylation, cleavage of tau can also contribute to tangle formation. Caspases - a family of 14 enzymes, cleave substrates at specific aspartic acid (Asp) residues.

Cleavage by caspase at tau's C-terminus or N-terminus leads to impairment in mitochondrial bioenergetics, weakening of axonal transportation, neuronal injury and cognitive decline.

Caspase-3 cleaves tau at the C-terminus tail at Asp421, which removes 20 amino acids, promoting rapid assembly of neurofibrillary filaments. This cleavage event contributes to neurite loss, mitochondrial fragmentation, and tangle aggregation, driving cognitive decline (Rizzi & Grinberg, 2024). Aβ can indirectly trigger caspase 3 (CASP3) to produce a self- aggregating and neurotoxic tau oligomer. (Zhang et al., 2023). More specifically, Aβ induces mitochondrial dysfunction, which leads to the release of cytochrome-c (a molecule released by mitochondria when cell receives apoptotic signal) and caspase-9 activation, activating downstream CASP3.

Although less studied, caspase-6 also plays role in AD pathology. The levels of caspase-6 were negatively related to cognition and the 14-441 fragment of Asp13 cleaved by this enzyme plays role in tangle maturation. Unlike caspase-3, its activation seems to be independent from Aβ and starts at an earlier stage of AD. Taken together, tau tangle formation is a complex process that involves several independent mechanisms and can vary depending on the stage of the disease. It can also be Aβ dependent, posing an important question about the interplay of those AD biomarkers.

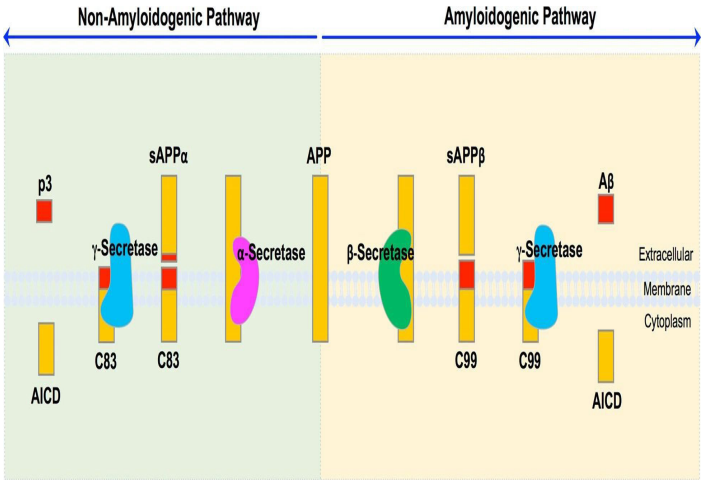

Fig. 3. **APP Healthy and Pathological Cleavage Mechanisms.**
The amyloidogenic pathway involves β-secretase and γ-secretase, while the non-amyloidogenic pathway involves α- secretase and γ-secretase. β-secretase cleaves APP, generating soluble APPβ (sAPPβ) and a membrane-bound fragment C99. γ-secretase then processes C99, producing amyloid intracellular domain (AICD) and amyloid-beta (Aβ) peptides, which can aggregate into toxic oligomers and fibrils. Non-Amyloidogenic Pathway: α-secretase cleaves APP within the Aβ region, preventing Aβ formation. This generates soluble APPα (sAPPα) and C83. γ-secretase then cleaves C83, producing AICD and a short, non-toxic peptide called p3. (Hur, 2022).

2.3 Aβ and Tau Synergy

Aβ and phosphorylated tau tangles have been considered the hallmarks of AD for a long time. Their relationship has been considered temporal rather than synergetic, but recent findings highlight their interdependence in disease pathology. Several in *vitro* experiments have shown consistent results supporting the interdependence of Aβ and tau tangles. Human neural stem- cell-derived 3D culture systems with overexpression of mutant APP and PSEN1 (with no tau mutation) induced both Aβ and tau aggregation, tau following accumulation of Aβ (Lee, et al., 2016). However, blocking Aβ in cultures through inhibition of either β- or γ-secretases prevented tau pathology, again demonstrating the necessary role of Aβ in the accumulation of tau tangles (Israel, et al., 2012). In the experiment where human brain-derived pathological tau (AD-tau) was injected in a wildtype mouse brain (no prior plaques), no AT8-positive DN was detected, suggesting the synergy between plaques and tangles for the formation of DN (He et al., 2018). Furthermore, study by Virginia Lee's group revealed that injection of AD-tau into 5xFAD mice leads to formation of AT8-positive dystrophic neurites (DN) around Aβ aggregates.

Another study showed that individuals experiencing progressive memory loss present with hypometabolism in the posterior cingulate

cortex which is an area strongly linked to tau and plaque interaction (Zhang et al., 2021). Additionally, findings from the Alzheimer's Disease Neuroimaging Initiative (ADNI)—a longitudinal study focused on identifying AD biomarkers— indicate that tau-related cortical thinning is exacerbated in the presence of Aβ (Fortea et al., 2014).

In *vivo* experiments, in which synthetic or brain-derived Aβ was injected into the cortex or hippocampus of P301L-mutant tau mice, showed accelerated tangle formation both at the injection site and synaptically connected areas (Bolmont et al., 2007). The same trend was observed when human brain-derived paired helical filaments (PHF major tangle component) were injected into the brains of APP/PSEN1 mice; enhanced tau propagation was observed compared to wild-type mice (Vergara et al., 2019). 3-Tg mouse model expresses both tau and plaques, but plaques form and aggregate before tangles. Antibodies directed against Aβ plaques reduced early-disease but not late-disease tau formation (Oddo, S. et al., 2003).

Overall, independent sets of *in vitro* and *in vivo* experiments have consistently confirmed that Aβ plaques are key to creating the environment that promotes tau aggregation leading to the disease progression.

Conversely, tau has been shown to contribute to enhanced toxicity of Aβ, leading to more rapid deterioration of cognitive and motor phenotypes. For instance, tau knockout mice showed rescued cognitive performance and reduction in plaque load by 50% (Leroy et al., 2012). On the other hand, reduction of the endogenous tau levels did not alter plaque load, yet it still reversed memory impairment and decreased mortality (Nisbet et al., 2015; Roberson et al., 2007). Mechanistically Aβ and Tau can both interact with key proteins such as Fyn and NMDA receptor leading to Ca^{2+} influx and subsequent neuronal death. Aβ toxic oligomers bind to cellular prion protein (PrP) which further activates Fyn kinase and phosphorylates tau via NMDAR GluN2B subunit causing Tau aggregation. Additionally, Tau showed to independently influence NMDAR through binding to Fyn and allowing its interaction with postsynaptic density protein (PSD) forming a complex that exacerbates Aβ initiated cytotoxicity. Further supporting the influence of tau on Aβ, absence of tau decreases Aβ induced toxicity through Tua-Fyn-PSD mentioned mechanism (Zhang et al., 2023).

Taken together, Aβ appears to initiate a pathological loop in which tau amplifies neurodegeneration. This cycle ultimately accelerates disease progression, as tau becomes a key mediator of Aβ-induced toxicity. Therefore, while Aβ plaques facilitate tau aggregation; tau aggregation could also promote Aβ formation, further accelerating disease progression.

2.4 Microglia Involvement in Alzheimer's Disease Pathology

Microglia are myeloid cells that coordinate immune responses in the brain, playing a crucial role in maintaining brain health. Two broad categories of microglia are classical (M1) and alternative (M2) types where the former provokes inflammation and neurotoxicity, while the latter drives anti-inflammatory and reparative responses (Tang and Le, 2016; Colonna and Butovsky, 2017). The recently discovered subpopulation of microglia is disease-associated microglia (DAM) that surrounds the plaques in AD and other neurodegenerative brain diseases. Although the role of microglia in AD has not been fully established, some findings suggest that it might play a role in compacting plaques when surrounding neuritic Aβ plaques. An important aspect of the microglia-plaque interaction is the mechanism through which microglia detect Aβ plaques. Two TAM receptor tyrosine kinases, Axl and Mer, have been identified as key players in the identification of Aβ plaques, leading to their subsequent organization and phagocytosis.

Specifically, Axl mRNA has been shown to be overexpressed in disease-associated microglia (DAM) during the later stages of AD. Studies using double knockout ($Axl^{-/-}$ and $Mer^{-/-}$) mice demonstrated a reduced level of dense-core plaques and impaired Aβ plaque detection and organization (Huang et al., 2021). This supports the notion that microglia are primarily focused on protecting the brain from the toxic effects of Aβ plaque deposition, by actively engaging in the clearance and compaction of Aβ plaques and thus limiting the harmful impact of plaques on neurons.

Triggering receptor expressed on myeloid cells 2 (TREM2) is one of the risk factors for developing sporadic AD also known as LOAD, emphasizing the importance of microglial response in AD brains. TREM2 controls key microglial roles, such as phagocytosis, migration, lysosomal degradation and metabolism. In the context of AD, it is required for Aβ plaque compaction and neuronal health, highlighting microglia-neuron interaction and microglia-driven response to plaque accumulation (Van Lengerich et al., 2023). Additionally, this receptor is involved in switching microglia cells from the basal homeostatic to a disease-associated state.

TREM2 deficiency results in lower expression of several genes responsible for microglial activation, including inflammatory cytokines, trophic factors, and proteins related to phagocytosis (Czapski & Strosznajder, 2021).

Specifically, DAM in AD has shown to undertake two-stage transformation. Initially, homeostatic microglia shifts to Stage 1 DAM (intermediate stage) due to neurodegeneration- associated molecular patterns (NAMPs). Then, Stage 1 to Stage 2 DAM transition is modulated by TREM2 upregulation (Fig. 4). The gene suspected in maintaining the microglia in Stage 2 is Apolipoprotein A (APOE) that is involved in the autocrine or paracrine loop and upregulated in AD brains (Samant, Standaert, & Harms, 2024). This TREM2-APOE pathway seems to play a critical role in the transition and maintenance of DAM. Reduction in TREM2 results in decrease of apoptotic neuron clearance (Takahashi et al., 2005). Complementary reduction or deletion of APOE leads to reduced DAM signature in the disease brain (Song and Colonna, 2018). Hence, DAM might be potentially involved in plaque-debris clearance, however their role does not seem to be uniform, but instead AD stage dependent.

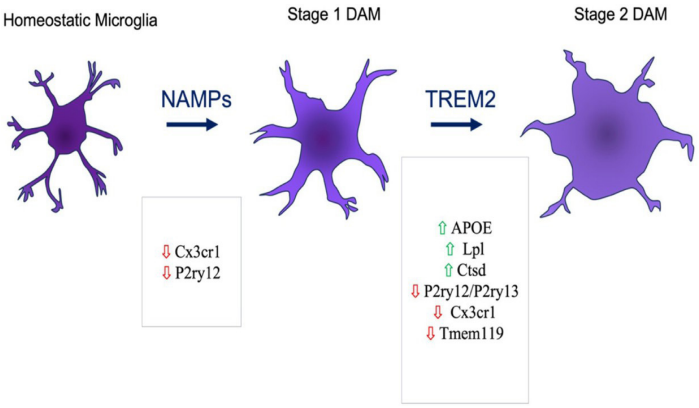

Fig. 4. **Microglial Transition into Disease Associated Microglia (DAM).** NAMPs regulate the first stage transformation through downregulation of homeostatic genes. The second stage transformation is caused by TREM2 signaling being a key component for final change. This process is accompanied by upregulation of some and downregulation of other homeostasis related genes. (Samant, Standaert, & Harms, 2024).

3. Behavioral Symptoms

Annually, 10-15% of adults (> 65 years old) diagnosed with Mild Cognitive Impairment (MCI) develop AD (Alzheimer's Association. n.d.). MCI is defined as an in-between stage between healthy aging and dementia characterized by memory, language, and other cognitive impairments with no adverse effects on daily life activities (National Institute on Aging, n.d.). Hence, it is crucial to study the contributing factors and characteristics of each stage of AD progression including MCI for timely pharmacological and behavioral interventions. It is worth noting that MCI has shown to be reversible when adopting a healthy lifestyle such as performing cognitively challenging tasks (Gates et al., 2010), eating healthy food (Lee et al., 2013), and exercising (Geda et al., 2010). The reversibility once again highlights the importance of understanding the timeline of the disease progression and its triggering factors.

3.1 Cognitive Behaviors

Since memory loss is a hallmark of AD-related cognitive deterioration, investigating its role in both pathological and normal aging is essential. By comparing memory function in healthy aging and AD, researchers can distinguish cognitive decline caused by the disease from age- related changes, helping to uncover underlying mechanisms and identify potential intervention targets.

Working Memory (WM) is key to maintaining and manipulating short term memories, contributing to decision-making and general cognition. The brain areas involved in formation of working memory—identified using positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) scans—include prefrontal cortex (PFC), parietal regions, cingulate gyrus and hippocampus (Kirova, Bays, & Lagalwar, 2015), all highly affected areas in AD patients. It is worth noting that it is natural to observe deterioration of WM and other cognitive functions associated with weakened connectivity among the mentioned areas in aging brain. A study comparing brain activity in young and older individuals performing the same cognitive task found an age-related increase in the recruitment of brain areas that are less task specific to account for weakened brain connectivity (Martins, Joanette, & Monchi, 2015; Poirier et al., 2021).

However, this altered brain activity pattern becomes even more pronounced when comparing individuals with mild cognitive impairment (MCI) and early-stage AD. A recent study shows a strong positive relationship between functional connectivity of the brain and AD progression marking the important effect of disease on brain connectivity (Carrasco-Gómez et al., 2024). Additionally, evidence provides that along with brain changes there are several aspects of cognition showing strong deterioration. Specifically, divided attention tasks reveal key differences between these groups showing episodic memory deficits in addition to WM in the early stages of AD. As the disease progresses, individuals exhibit worsening manipulation skills (memory recollection), failures in inhibiting irrelevant stimuli, and declines in selective attention. Since cognitive impairment is one of the first preclinical signs of AD, individuals with the poorest cognitive performance at the MCI stage are more likely to develop AD, while others have a greater chance of recovery (Kirova et al., 2015).

While decision-making in AD is relatively understudied, it remains a critical aspect of the disease alongside memory decline. It is central to the question of whether AD patients can actively participate in their treatment planning and make independent decisions. Santos et al. (2022) define decision-making as a complex mental process consisting of four components: 1) the ability to store, recall, and understand the meaning of information; 2) the ability to apply information in a relevant context; 3) logical thinking and mental comparison; and 4) the expression of choice and the maintenance of that choice until completion. This paper evaluates decision-making ability in relation to cognition and clinical factors (such as quality of life and awareness about the disease) in AD patients. The results suggest that while cognition is the major contributing factor, it is not the sole determinant, emphasizing the importance of using various measures to assess decision-making ability in AD patients (Santos et al., 2022).

Furthermore, curiosity is a basic cognitive factor encoded in our daily behaviors and conserved across species. There are numerous benefits to displaying curiosity, which drives learning and motivation—both of which are key to survival. A diminished sense of curiosity or information- seeking behavior is often linked to depression and apathy, common traits in AD patients (Kidd & Hayden, 2015). Due to the vagueness of the term, there is no specific definition of curiosity, and the subject of focus varies across labs and studies. For instance, curiosity can range from a “desire to respond to trivia questions” to the “strategic deployment of gaze in free viewing” (Gottlieb et al., 2013).

Most studies on human curiosity operationally define it as a preference for directing gaze toward novel, unfamiliar, or irregular objects. The available data provides evidence for diminished exploratory behaviors in AD patients in the later stages of the disease, as measured by exploratory eye movement (Daffner et al., 1992). In contrast, healthy subjects tend to devote more attention and time to watching novel or unusual objects, displaying signs of curiosity and exploratory behavior (Daffner et al., 1994). More recent studies have shown an age dependent decrease in exploratory behavior using various more sensitive measures and in various tasks (Mata et al., 2013). Hence, exploratory behavior appears to be dynamic throughout human life, suggesting AD may have an additive effect on exploratory behavior deterioration in aged individuals.

Similar to humans, a pronounced effect of age on exploratory behavior was observed in mice. Studies on aging mice reveal a tendency to repeat choices and limit exploration in various decision-making tasks (Hwang et al., 2023). For instance, when given multiple options, older mice exhibit a strong preference for sticking with prior choices, even in the absence of a reward. This highlights a shift in decision-making strategies with age. There is no conclusive data in AD mice, except for one recent study that investigated exploratory behaviors in mice by measuring active whisking behavior—a sign of healthy curiosity in rodents. Surprisingly, the results showed no genotype effect on exploration when comparing 5xFAD to control mice at 6- 7 months of age (Grant et al., 2020).

3.2 Non-Cognitive Behaviors

Motor impairment is a non-cognitive aging phenotype reliably associated with AD development (Buchman et al., 2020). Recent studies showed that motor dysfunction precedes MCI by several years (Yu et al., 2019) potentially being a clinical marker for MCI and AD. Beeri et al. (2021)conducted a nested substudy of 1,160 aging individuals from three longitudinal studies, assessing baseline motor activity and tracking changes over a seven-year period with annual cognitive check-ups. The results showed that better motor performance at a baseline (hand dexterity, hand strength, gait function) correlated with a reduced risk of developing MCI, and hand strength was also independently related to AD.

Gait impairment provides insight into interplay of the cognitive and motor components in AD patients enabling a better understanding of complex behaviors. A study by Kim et al. (2025) on gait impairment offers a novel, more in depth analysis of this specific motor function in relation to cognition and its role in tracking AD development. This study suggests that gait is an example of goal-oriented behavior that depends on various cognitive functions for its effective execution. In support of the positive relationship between gait and AD progression, Aβ plaque deposition has been shown to contribute to the deterioration of gait in AD patients (Del Campo et al., 2015). Additionally, this study identified the correlation between gait velocity and cortical atrophy in two major brain networks, each associated with distinct cognitive functions: default mode (DMN) and salience (SN) network in AD patients. According to a comprehensive review of DMN research by Menon, V. (2023), DMN is a collection of various regions that are active during the resting state to consolidate and process information. It is also involved in internally focused thought processes (self-reflection, daydreaming, mind wandering, recall of personal experiences) which constitute semantic and episodic types of memories. SN on the other hand is active when choosing which external stimulus needs to be attended, acting as a switch between DMN and other brain networks required for performing a specific task (Schimmelpfennig, 2023) and plays major role in working memory (Fox et al., 2005). Given these functional roles of DMN and SN, atrophies in those two networks in AD may underlie a large range of altered behavior spanning cognition, decision-making and motor skills in AD patients.

4. Current Therapies and Therapeutic Approaches

There are limited treatment options for AD patients, urging the development of more efficient therapeutics that can at least slow down the disease progression in patients. The currently available drugs focus on the Aβ plaque clearance after cleavage or regulating acetylcholine and glutamate levels in the brain at different stages of the disease.

Lecanemab (Lequembi) and Donanemab (Kisunla) are the two currently available treatments for Aβ clearance in the brain of MCI presenting AD patients (needs to be diagnosed in early stages). Some of the side effects are changes in vision, confusion, dizziness, headache, nausea, or seizures. People with moderate symptoms of AD are usually prescribed with Galantamine (Razadyne), Rivastigmine (Exelon), and Donepezil (Aricept) which inhibit enzymes breaking down acetylcholine, increasing the level of this neurotransmitter improving cognition and memory. These drugs are usually well tolerated, and the side effects range from nausea, vomiting, loss of appetite, and increased frequency of bowel movements. Lastly, Memantine (Namenda) is a drug prescribed for individuals with moderate to severe AD. It blocks NMDA receptors in order to prevent glutamate-induced cytotoxicity in the hyperexcited cells observed in AD patients. Here are some of the well-known side effects: headache, constipation, confusion, and dizziness (Alzheimer's Association, n.d.).

Alternative non-drug approaches for slowing down disease progression have been recently developed. Sovrea et al. (2025) provides a comprehensive review of the currently used non- drug treatment including focused ultrasound (FUS) and transcranial pulse stimulation (TPS). Both of which are non-invasive: TPS sends ultrashort ultrasound pulses, also known as shockwaves, to targeted small brain regions (Cont et al., 2022) whereas FUS uses acoustic waves. The primary limitation of FUS is the brain depth it can reach, and it needs to be paired with MRI for precise targeting (Krishna,Sammartino,Rezai, 2018). Microglial activation, synaptic plasticity and other physiological changes are amongst some of the FUS benefits (Fig. 5A). TPS has shown significant improvement in cognition through morphological and functional changes in the brain (Fig. 5B) (Sovrea et al., 2025). A study by Nazarian, Yashin, & Kulminski (2019) showed that repeated TPS sessions lead to sustained cognitive benefits in memory retention and executive function. Although these methods are promising, there is still a need for further research into their long-term effectiveness.

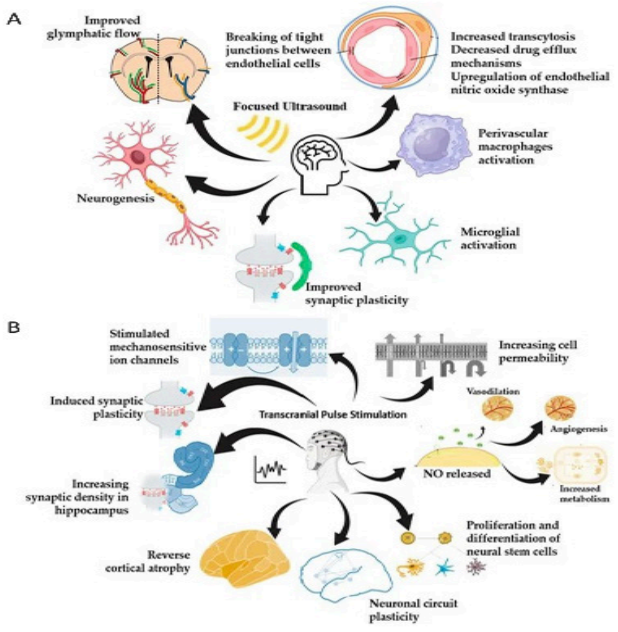

Fig. 5. **Benefits of Focused Ultrasound (FUS) and Transcranial Pulse Stimulation (TPS).**

(**A**) The benefits of FUS as a non-drug, non-invasive therapy for AD Some of the key benefits include microglial activation, leading to an increased rate of amyloid plaque clearance, neurogenesis to compensate for extensive neuronal loss, enhanced blood-brain barrier (BBB) permeability, and modulation of neuronal activity to restore disrupted circuits.(**B**) The benefits of TPS as a non-drug, non-invasive, and novel therapy for AD. Some of the key benefits include increased synaptic density in the hippocampus, leading to improved hippocampal function, reversal of cortical atrophy, aiding in the restoration of damaged brain regions, and enhanced neuroplasticity, potentially slowing disease progression. (Sovrea et al., 2025).

5. Animal Model Research

Alzheimer's disease is a complex condition with unclear, multifactorial causes limiting our ability to effectively prevent its development. One of the advantages of in vivo research over in vitro in the context of AD is the ability to study the complex interplay of various factors involved in the development of pathology. *In vitro* studies, using isolated cells, offer a more simplified view of the disease process, often disregarding the intricate interactions between different cell types, and brain regions that occur in the human brain. In contrast, *in vivo* models allow for a more holistic approach, capturing the dynamic interactions and systemic changes that are crucial for understanding AD pathology (Drummond & Wisniewski, 2017).

After the discovery and establishment of the "amyloid cascade hypothesis" stating that Aβ is the central component of plaque in AD in 1984 (Glenner & Wong, 1984), the challenge was to develop an effective model to study the hypothesis further. The first major milestone in developing the AD model was made in 1995 when PDAPP was developed. This model expressed one APP mutation accompanied by memory loss at the age of 3 months, and plaque accumulation by the age of 7-8 months (Yokoyama et al., 2022). This was a breakthrough for *in vivo* AD research, leading to the development of the current 50 mouse models for AD (*MODEL- AD*, 2023).

Currently, most AD research focuses on identifying clinical biomarkers for effectively detecting and predicting the disease progressions on one end and developing efficient model systems for uncovering its underlying mechanisms on the other end. Animal models are essential for the latter effort, as they provide a complete system where intricate disease processes involving organically connected cell types, brain regions, and organs can be investigated. Many AD animal models have been developed to replicate some pathological physiology and behavioral symptoms observed in human AD patients, but no single model recapitulates the comprehensive and heterogeneous nature of human AD. Thus, it is important to determine which aspects of human AD can be properly investigated in each model.

On the other hand, 6xTg can be used to test therapies involving tau related gene inhibition to observe its effect on Aβ plaque production and vice versa. 5xFAD is frequently used to study the distribution and accumulation of amyloid plaques. This model provides insights into the mechanisms underlying plaque pathology, one of the hallmark signs of AD, often in the absence of tau pathology. Alternatively, models like 3xTg can be used to study secondary AD symptoms involving loss of olfaction, which is one of the initial preclinical symptoms in AD patients.

Behavior is another aspect crucial to evaluate in mouse models as AD patients typically exhibit non-cognitive symptoms before cognitive impairments emerge, making it crucial for understanding early-stage AD. While all the models mentioned are used for behavioral evaluations, the current lack of consistent results highlights the urgent need for more effective and replicable methods of behavior and cognition assessment in AD research.

5.1 Mouse Model: 5xFAD

5xFAD mice express five familial mutations in APP (Swedish K670N, M671L), (Florida I716V), (London V717I), and PSEN1(M146L, L286V) genes, but no neurofibrillary tangles are observed in this model (Oblak et al., 2021). Plaque accumulation starts at the age of 2 months and continues with age mimicking human pathology. The transgenes were implemented under the control of the Thy1 promoter (sensitive to progesterone) to ensure the expression specifically in neurons to mimic the localization observed in humans (Jankowsky et al., 2017). Based on these findings, we expect female mice to have a more deteriorated cognition and behavior as well as more plaque accumulation compared to the age-matched male mice. Visual (Wang et al., 2017) and olfactory (O'Leary et al., 2020; Lenoir et al., 2019) functions have been examined across different studies but have not shown any consistent results. Substantial research has also been performed in spatial memory of 5xFAD mice using Morris Water Maze (MWM) Test that showed significant learning impairment and

latent escape as early as 5 months of age (Tang et al., 2016). O'Leary, et al. (2020), on the other hand, did not see memory impairment till 12-15 months confounded by motor impairment. One of the major gaps in research using 5xFAD mice is the lack of robust cognitive characterization which is key in understanding biological processes underlying behavioral deficits and thus critical for evaluating the effectiveness of potential therapeutics (Padua et al., 2024). Inconsistent results across studies can be potentially introduced through differences in housing, food consumed, or types of tests used to measure those factors. Besides inconsistent results in cognitive and behavioral deficits, the limitations of 5xFAD model include the lack of other pronounced physiological parameters such as tau tangle formation. Nevertheless, this model is attractive due to the strongly expressed plaques in the brain as well as the simplicity of breeding.

5.2 Mouse Model: APP/PS1 KI

APP/PS1 KI model was developed by inducing several point mutations in APP and PS1 genes, namely: M233T and L235P in PS1 and the London (V717I) and Swedish (K670N/M671L) in APP under the control of the Thy1 promoter (*APP751SL/PS1 KI | ALZFORUM*, n.d.). APP/PS1 Ki is a rare type of transgenic mouse model for studying AD that expresses neuronal loss along with amyloidosis which are not as common in other models. The neuronal loss starts at the age of 6 months and worsens gradually with age (Faure et al., 2011). Despite the pronounced pathological changes in the brain, there are conflicting ideas regarding the non-cognitive and cognitive changes in this mouse model. Initially, cognition was found to deteriorate at the ages 7-8 months with no pronounced non-cognitive changes (Serneels et al., 2009, Radde et al., 2006). Later findings stated that APP/PS1 KI demonstrates memory impairment as early as 6 months (Faure et al., 2011), while the most recent data shows those changes in age of 11 months (Webster et al., 2013). A paper evaluating the effect of aerobic exercises on cognition of 4-month-old APP/PS1 KI mice has showed decline in spatial memory at the baseline and significant improvement because of consistent exercising (Wang et al., 2024). Anxiety on the other hand did not show significant differences compared to the wildtype littermates (Webster et al., 2013). All in all, this mouse model shows latent cognitive and almost no non-cognitive changes compared to other AD models. However, it is very effective in studying neuronal loss and plaque accumulation following AD pathology development. Due to the limited intrinsic behavioral changes, this model might provide insight into the effect of external factors such as stress induced behavior shift in the presence of AD markers.

5.3 Mouse Model: 6xTg

To address the challenges of accurately representing AD in mouse models and exploring the interplay between tau and amyloid plaques, researchers have developed models expressing both plaques and tangles. These dual-model systems will potentially provide a more comprehensive understanding of the pathological, cognitive, and behavioral changes associated with AD, offering insights closer to the human condition.

This is a relatively new model for studying (developed in 2021) AD that aims at expressing both plaques and tau tangles by crossbreeding 5xFAD and JNLP3 (overexpresses MAPT mutation inducing aggressive tangle production) (Uras et al., 2023). This model effectively expresses various pathological AD features including plaque formation, abnormal tau phosphorylation, neuronal loss and astrocyte activation (Tag et al., 2022). Behaviorally, this model shows heightened anxiety and depression like state as well as hyperlocomotion at ages 9-11 months. Memory impairment is observed at around the same age (Tag et al., 2022), which contradicts earlier findings claiming memory decline to happen at 2 months of age (Kang et al., 2021).

Although deemed effective, this model has very limited information available on the non- cognitive and cognitive changes. Only a few studies provide insights into the onset of non- cognitive impairments, and thus a comprehensive understanding of their progression remains lacking. Moreover, conflicting reports on the timing of cognitive decline emphasize the need for further studies to establish a clearer timeline of behavioral and functional impairments in this model.

5.4 Model: 3xTg

The 3xTg mouse is characterized by mutation of three genes APP, PSEN1, and Tau, and therefore expresses both amyloid beta and tau tangles. This model is useful for studying the correlation between amyloid beta and tau tangles and it also effectively shows cognitive decline that starts at the age of 6 months and progressively worsens with age (Belfiore et al.,2019). 3xTg mice express mild cognitive deterioration with the Barnes Maze being the most sensitive measure of cognition (Kurt et al., 2015). One of the preclinical symptoms of AD is a loss of olfaction, which can be measured in this task. According to the buried food test findings, female mice spent a significantly longer time looking for food compared to the male, the latency to find the food also deteriorated as the mice aged, showing age-dependent loss of olfaction (Mitrano et al., 2021). The strength of this model is the expression of both hallmark signs of AD that allows us to study the relationship between Aβ and tau tangles. Additionally, mild cognitive decline is observed at 6 months of age making it an effective model to study cognition and pathology interaction in AD. The major disadvantages of the model, however, are the lack of neuronal loss regardless of plaque and tangles expression and variability among the colonies (Zhong et al., 2024). The last was likely caused by genetic drift that took place in this model population introducing phenotypic heterogeneities.

6. Assessment of Behavioral Symptoms in AD Mice

Studying AD phenotypes in mice requires a variety of behavioral and histological assessments. The collection of behavioral data is crucial because histological analysis is only obtained after sacrificing mice. Additionally, human AD patients start demonstrating behavioral and motor deficits before cognitive symptoms become apparent. Non-cognitive symptoms referred to as Behavioral and Psychological Symptoms of Dementia (BPSD) highlight affective dysregulation, with apathy and depression being the most prevalent symptoms (Selles et al., 2018). Similarly, a study conducted using Alzheimer's Disease Assessment Scale (ADAS-Noncog) identified tremor, depression, psychotic symptoms as the most prominent non-cognitive symptoms in AD patients (Fernández et al., 2010). Additionally, anxiety is commonly seen in individuals with MCI before the start of AD as well as considered a contributing factor to a more rapid transition from MCI to AD (Mendez, 2021). Detection and treatment of early symptoms might be an effective strategy to slow down disease progression. Hence, a thorough characterization of behavioral phenotypes—such as speed of mouse movement, gait, and freezing behavior—is essential for evaluating the effectiveness of mouse models in replicating non-cognitive aspects of AD in humans, uncovering the underlying pathophysiology of behavioral deficits in AD, and assessing the efficacy of therapeutic interventions.

6.1 Open Field Test

The open-field test (OPT) is a widely used assay that examines the animal's free locomotor activity and exploratory behavior. During an open-field test, mice are placed in the center of the open-field arena and left to roam freely for 5-10 minutes while the video is being recorded with an overhead camera. The usual kinematic variables extracted from the video include velocity, total distance, and time spent in the center compared to the time spent in the periphery of the arena.

In addition to video recording, an infrared (IR) beam brake system may be utilized for monitoring voluntary locomotor activity 24/7 mostly used for home cages under different light brightness (Klein et al., 2022). It is an effective tool to measure behaviors such as rearing and climbing; it can also be used to measure trajectory, distance traveled, and position distribution. However, it is not effective in studying social interactions involving multiple mice in the same cage. Although both tracking methods are effective, video tracking allows analysis of more complex behaviors and can be used for a wider variety of analyses compared to the IR beam. The open field test is also used for measuring anxiety in mice. Healthy mice tend to acclimate to the environment and as the level of anxiety decreases, they

spend more time in the center compared to the periphery (Carter & Shieh, 2015). However, it is not the most effective measure because due to the rodents' innate fear of predation, mice naturally spend less time in the open space of the arena corresponding to its center (Pentkowski et al., 2021).

A paper characterizing 5xFAD mouse model report that the distance traveled as well as velocity shows significant decline at the age of 18 months. Younger mice of 8 months exhibit a greater preference for the center of the arena compared to the control group, indicating reduced anxiety-like behavior (Forner et al., 2021). A significant reduction in locomotor activity has also been observed between 4-month-old and 6-month-old ages in 5xFAD mice, compared to control in both males and females (Poon et al., 2023). Taken together, OFT revealed alterations in some non-cognitive functions in 5xFAD mice.

6.2 Morris Water Maze Test

The Morris water maze (MWM) test is one of the most commonly used methods for evaluating short-term and long-term spatial memory in mice. Most papers in the field use the MWM results as a way to measure memory, learning, and motor activity while swimming. 5xFAD mice regardless of their sex show deterioration in learning at age 6-9 months and only worsened in older mice, with co-occurrence of locomotor dysfunction starting at the age of 9 months.

Memory deterioration however did not occur until age 12-15 months where females showed inconsistent performance between ages 9-12 months providing inconsistent evidence (O'Leary & Brown, 2022). Another paper analyzing the data from TG-2576 (AD model) mice discusses the appearance of cognitive deficit between 12-18 months requiring a large sample size, suggestive of a small effect size (Choi et al., 2023). As such, despite its wide popularity, MWM test shows inconsistencies in determining the age of onset for cognitive and behavioral deficits. Other disadvantages of this test include induced stress in mice reflected in high cortisol levels, general unwillingness to be in water, and spatial learning variability (Othman, Hassan, & Has, 2022).

These may interfere with memory consolidation and overall performance of the mice, a confounding effect between cognition and emotion. Recently published papers have offered to optimize MWM for a more reliable evaluation of discussed behaviors (Bailoo et al., 2024). To sum up, MWM is an important method for evaluating an array of cognitive and behavioral deficits but requires some revisions to improve the consistency and robustness of results.

6.3 Elevated Plus Maze Test

The Elevated plus maze (EPM) test is used to measure the level of anxiety in AD mice depending on the time they spend in an open arm versus a closed arm. Naturally, closed arms are associated with safety, and open arms tend to induce anxiety. Therefore, the more time a mouse spends in the open arms, the lower its anxiety is considered to be. However, EPM studies show inconsistent results on 5xFAD mice. Some papers claim 5xFAD mice to have decreased anxiety (Forner et al., 2021), whereas the majority of the papers indicate no changes (Flanigan et al., 2014). Making it further confusing, a minority of studies found increased anxiety (Dong et al., 2020, Locci et al., 2021). The widely different EPM results might reflect confounding effects of AD mice's increased sensitivity in their vibrissa (Grant et al. 2020). Because of the increased sensitivity in their vibrissa, AD mice engage in barbering behavior less often than control and would not permit control mice to barber for them. Furthermore, AD mice, when their whiskers were trimmed, showed an increased frequency of entries to closed arms of the EPM (Flanigan et al., 2014). Conversely, 5xFAD mice, with their intact whiskers, visit the closed arms less frequently because they avoid stimulating their over-sensitive vibrissa from touching the walls of the closed arms rather than they are less anxious. Therefore, EPM is an unreliable measure of anxiety in 5xFAD mice.

7. Major Knowledge Gap in AD Research

Despite decades of extensive research, significant gaps remain in our understanding and treatment of AD. One of the most urgent challenges is identifying the early mechanisms that trigger disease onset and their associated behavioral changes. Current therapies largely target downstream pathological features—such as amyloid plaques and tau tangles—often after irreversible brain damage has occurred. To enable earlier intervention and meaningful prevention, it is critical to uncover the upstream molecular and cellular events that initiate the disease process and their correlated behavioral diagnostic markers.

While substantial progress has been made in characterizing molecular pathology, relatively little is known about how these changes disrupt neural circuits and large-scale brain networks. A deeper understanding of how AD alters information processing at the systems level—using techniques such as in vivo electrophysiology, calcium imaging, and functional connectivity analysis—is essential for linking cellular pathology to behavioral and cognitive decline. Additionally, increasing evidence highlights the critical role of non-neuronal cells—such as microglia, astrocytes, and peripheral immune cells—in modulating disease progression.

However, the precise contributions of these cell types remain poorly understood. Detailed mapping of glia-neuron and immune-brain interactions throughout disease progression may uncover novel targets, particularly within the context of neuroinflammation.

Another unresolved question involves the mechanisms through which it contributes to functional neural disruption and behavioral symptoms remain poorly understood. While soluble Aβ oligomers are believed to impair synaptic function and alter network activity, it is still unclear how these molecular changes translate into specific cognitive and non-cognitive deficits. Moreover, the relative contributions of soluble versus insoluble Aβ species to disease progression are still debated.

Additionally, the mechanism of amyloid pathology interaction with other key factors—such as tau accumulation, glial activation, and immune responses—to drive the full clinical presentation of AD is still elusive. These uncertainties highlight a critical need to better define how amyloidopathy affects neural circuits and behavior, particularly in the early stages of the disease, in order to improve the translational relevance of animal models and guide the development of more effective therapeutic strategies.

Animal models are indispensable for tackling these challenges and advancing the development of preventive and disease-modifying therapies. However, a persistent issue is the disconnect between preclinical success and clinical efficacy. Many treatments that show promise in animal models ultimately fail in human trials. This discrepancy may stem from the heterogeneity and multifactorial nature of AD—both in its pathology and clinical presentation. Animal models often capture only limited aspects of the disease and may not reflect the complex interplay of risk factors and compensatory mechanisms present in human populations. As a result, therapeutics that target isolated features may fall short in treating the broader, more variable AD spectrum.

Bridging the gap between preclinical predictions and clinical outcomes requires a clearer understanding of the strengths and limitations of each animal model. Yet, behavioral characterization across AD models remains incomplete and inconsistent, making it difficult to relate specific pathological mechanisms to functional outcomes.

To address these gaps, a multidisciplinary approach that integrates molecular biology, systems neuroscience, immunology, and behavioral analysis is essential. Toward this goal, my thesis focuses on a detailed characterization of both cognitive and non-cognitive behaviors in the 5xFAD mouse –known to be a reliable model for plaque accumulation— and how these behavioral changes correlate with the plaque burden.

8. Present Study

Goals

A key unmet need in AD research is the ability to detect the illness at its very onset. Currently available therapies aim to slow amyloid-β plaque buildup—the first pathological event that ultimately drives widespread neurodegeneration—so their success depends on intervening before irreversible damage occurs. Unfortunately, no reliable behavioral readouts exist for these earliest stages, either in people or in animal models. This dissertation addresses this critical gap by developing behavioral biomarkers sensitive to the initial emergence of amyloid pathology. Using the 5xFAD mouse—an established model that rapidly and faithfully recapitulates plaque formation—we will comprehensively profile cognitive, affective, and motor functions across early to late phases of deposition. Unveiling how plaques influence both cognitive and non-cognitive domains could provide a foundation for early disease diagnostics biomarkers, improving the effects of the currently available therapeutics, and developing new disease-modifying interventions. The specific aims of this study are: (1) Establish a clear timeline for cognitive and behavioral changes associated with AD progression in 5xFAD mice, (2) Explore the relationship between amyloid plaque burden and cognitive decline, (3) Investigate how the spatial distribution of plaque deposition related to specific behavioral deficits, such as impairment in motor coordination and anxiety-like behavior, (4) Assess how plaque location may contribute to disruption in neural network function, particularly those involved in decision-making and memory

Hypothesis

If amyloid plaques are sufficient to drive alternations in neuronal circuits and promote neurodegeneration, then 5xFAD mice should exhibit early onset of cognitive impairment, particularly in tasks that demand integration of multiple brain functions, including sensory processing, motor coordination, strategic action planning, and learning. To test this hypothesis, I will compare cognitive and non-cognitive task performance between 5xFAD mice and age- matched wild-type controls and investigate how performance metrics align with plaque pathology.

The Effects of Periodical Cicada Emergence on Ant Foraging Behaviors

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Periodical cicada emergences produce variety of noticeable, short-lived effects on their ecosystem, however their effects on ants are poorly understood. To quantify the foraging response of ants to periodical cicada emergences, the nutritional preferences and foraging rates of five ant communities in Lake County were measured during the 2024 periodical cicada emergence. Carbohydrates were significantly preferred over lipids, protein, salt, and water. Additionally, foraging rates were found to be significantly higher in the weeks after the periodical cicada emergence than during the emergence. Conclusions as to the extent of the impact of periodic cicada emergences will require subsequent summers of sampling, though data suggests predator satiation and nutritional compensatory behaviors were observed.

Literature Review

Pulse Ecology

Overview of Resource Pulses

A resource pulse is defined as a temporary availability of a resource which occurs relatively rarely within the ecosystem, or a brief period of abundance followed by a longer period of scarcity (Yang et al. 2010; Ostfeld & Keesing 2007). These pulses must have a comparatively greater magnitude of resource availability than non-pulse periods (Ostfeld & Keesing 2007). The effects of resource pulses can be localized to the area of the pulse, but still produce significant changes in the flow of energy, predation and herbivory, and can vary in their duration depending on the conditions of the pulse (Ostfeld & Keesing 2007). Resource pulses can be differentiated according to their duration and regularity, and the spatial scale over which they have an effect. When it comes to duration, pulses vary in how long the resources are present and accessible to consumers: resources may exist for a short time well within their consumers' lifespans before being consumed, or they may be able to persist beyond the initial pulse, such as in the case of some desert-dwelling seed crops that are dropped synchronously and can exist for years before being consumed or germinating (Ostfeld & Keesing 2007). Pulse regularity may vary as well, from being seasonal events to being governed by conditions that do not occur with any regularity and thus being unpredictable, especially on timescales of shorter-lived organisms (Ostfeld & Keesing 2007). Those that occur regularly may be cyclical on timescales too great for some consumers' lifespans and for these organisms the pulses may as well be randomly occurring, multiple generations may pass without ever encountering a resource pulse and learning to respond to it (Ostfeld & Keesing 2007). Lifespans of arthropods, small mammals or birds may be shorter than the intervals between resource pulses that can occur in their environments, such as periodical cicada emergences. Other resource pulses may be random and irregular: conditions that influence the pulses do not occur seasonally or annually, such as rainfall in otherwise arid environments (Chesson et al. 2004).

Effects of Resource Pulses

Resource pulses are categorized as a temporary abundance of a given resource, creating a wave of responses in the short- and long-term after the resource becomes available (Holt 2008). Resource pulses vary in their regularity and predictability with respect to other organisms affected by the pulses, and provide direct bottom-up controls and indirect top-down controls on the energy flowing within ecosystems (Ostfeld & Keesing 2007). The abundance of a new food resource will directly affect the organisms that use this resource, and the response of these organisms will have indirect impacts on other trophic levels through their increased abundance for predators. In other words, the decrease in predation pressure for organisms that would otherwise be eaten in non-resource pulse years. That predation pressure is released when consumers primarily exploit the resources from the pulse (McCary et al. 2021). Organisms may be specialized to exploit resource pulses, with their populations following that resource abundance very closely, booming and crashing along with the resource, such as weevils that feed on acorns from masting events (Ostfeld & Keesing 2007). Other organisms are generalists and will take advantage of this novel resource by adapting their feeding habits, such as birds consuming *Magicicada* instead of caterpillars (Getman-Pickering et al. 2023) or bears and foxes exhibiting more digging behaviors and changing foraging ranges to access *Lyristes* cicadas (Tomita 2021). Pulses can lead to an abundance of consumers exploiting them, in turn supporting a temporary boom in the population of consumers at higher trophic levels (Ostfeld & Keesing 2007). Terrestrial resource pulses may also provide nutrients that enrich the populations of plants, fungi, and bacteria through the decomposition of the resource and consumption by detritivores (Yang et al. 2006; Yang et al. 2008; Menninger et al. 2008). Nutrient resource pulses in marine ecosystems can support blooms of plankton, which in turn can drastically increase the productivity of the system. Marine food webs in polar waters are supported in part by resource pulses caused by seasonal upwelling of nitrates (NO_3-) (Varela & Harrison 1999), which contribute to highly productive systems that support highly concentrated populations of organisms such as fish, pinnipeds, and cetaceans (Beltran et al. 2021).

Insects whose life stages are synchronized, and whose populations occur in sufficient numbers can act as a resource pulse if they enter an ecosystem synchronously: cicadas are one such example. The periodical cicadas can emerge in localized areas leading to a high density of biomass available to consumers (Yang 2004). Their periodicity can vary, from the 4-year cycles in *Chremistica ribhoi* of northeastern India to the 13-17 year cycles of periodical cicadas in Northeastern North America (Hajong & Yaakop 2013; Getman-Pickering et al. 2023). These periodical cicadas synchronize their emergence from the soil where they spend the first 12 or 16 summers of their lives burrowed in, and the adults will persist for approximately a month, calling for mates, feeding, and laying eggs before dying (Ito et al. 2014). There may be up to several hundred cicadas emerging from a single square meter of soil, across areas as large as 105 km^2 (Yang 2004). This density of individuals means periodical cicadas can function as a resource pulse. The synchronicity and density of their emergence sets them apart from other co-occurring cicada species, in that their abundance will be far greater in synchronized broods than they will be for annual cicadas, whose life cycles are staggered such that they emerge every year, in lower densities than periodicals (Chiavacci et al. 2014). Annual cicada emergence densities can be closer to 18,000-26,000 cicadas per acre rather than the 1-2 million estimates for periodical cicadas (Dybas & Lloyd 1966). The pulse of resources is far greater for periodical cicada emergences than for annual cicadas, and thus likely has a far greater impact.

In terms of periodical cicadas' effects on their communities as a resource pulse, one documented response to their abundance is the decrease in predation by avians on other insects (Getman-Pickering et al. 2023). Insectivorous birds that would otherwise feed on seasonal caterpillars, had a temporary dietary change wherein they would decrease their caterpillar predation as they fed more on the abundant cicadas instead (Getman-Pickering et al. 2023). This left more caterpillars uneaten and able to feed on plants which otherwise would have been left unconsumed during a non-emergence year (Getman-Pickering et al. 2023). Indeed, the abundance of caterpillars coincided with a greater level of herbivory on white oak trees than in non-cicada years, as a likely indirect result of the periodical cicadas' emergence (Getman-Pickering et al. 2023). Avians are not the only predators of periodical cicadas, small mammals are also known to eat the insects (Krohne et al. 1991), and detritivores will consume the carcasses that accumulate over the course of the emergence (Yang 2005) which can lead to nutritional enrichment of soil ecosystems (Setlälä et al. 2022). A study performed in 2016 found that soil-dwelling arthropods and nematodes increased in abundance in plots treated with added periodical cicada carcasses, with nematode community composition increasing in

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diversity compared to nontreated plots (Setälä et al. 2022). This increase in diversity and abundance suggests the productivity of soil increases during cicada emergences, enriching fungal and bacterial-based soil food webs (Setälä et al. 2022). Thus, periodical cicada can provide additional nutritional input to their environment; plant-available N in the form of NH_4+ and NO_3- and P in the form of PO_4- been observed to be more abundant in soil enriched with periodical cicada carcasses (Setlälä et al. 2022).

Spatial Scale of Resource Pulses

Periodical Cicada emergences have effects over a relatively large area, with broods being relatively localized to areas wherein previous emergences have occurred. Their range of emergences can be measured in hectares, affecting large regions through their emergence (Williams et al. 1993). The input of food and nutrients thus affects organisms across a vast area. These patches are not evenly distributed, however, and they follow the fragmentation of North American forest habitats, contributing to an uneven distribution of cicadas over the regions in which they occur (Yang 2004). Cicadas in a given area that are synchronized are classified as broods, with cicadas of the same brood emerging on the same 13- or 17-year cycles(Cooley et al. 2009). These broods' spatial ranges can range across hundreds of square miles, and within these regions, there can be further variation in the concentration of emerging cicadas (Cooley et al. 2009). One of the largest broods, Brood XIV, for example, ranges across the states of Kentucky, Tennessee, Pennsylvania, Maryland, and Massachusetts, and thus ecosystems across these regions can all experience the effects of the cicada resource pulses (Cooley et al. 2011).

Predator Satiation Effects

The number of cicadas emerging at once during an emergence can lead to an overwhelming amount of food: the density of the broods has been estimated to be around 133,000 nymphs in an acre (Dybas & Davis 1962), to several million (Cooley et al. 2004). Cicada population density increases independent of predation. However, predation rates have been observed to plateau before the cicada emergence reaches its maximum population, which could be explained by the effect of predator satiation hypothesis (Karban 1982). Predator satiation occurs when the frequency of predation of a prey organism does not increase as the density of the prey increases, which means their appetite for this prey is fully met and these predators cannot or will not consume any more of that prey (Williams et al. 1993). The proportion of the cicada population that is preyed upon has been observed to decrease at the peak of the cicada emergence as well, further supporting this hypothesis (Williams et al. 1993). Around the initiation of the emergence, a greater proportion of the cicada population will be eaten by predators than during the peak of the emergence when the greatest number of cicadas will be above ground and active (Williams et al. 1993). During the peak of the emergence, predation rates do not increase in proportion to the number of cicadas present, suggesting they cannot consume any more cicadas (Karban 1982). Predators that consume cicadas may even primarily consume cicadas during an emergence, eschewing other food sources in favor of the abundant ephemera (Getman-Pickering et al. 2023).

Ant Roles in Ecosystems

Overview

Ants are ubiquitous arthropods in terrestrial ecosystems, with over 15,700 named species and more still yet to be described (Schultheiss et al. 2021). They are found on all continents with the exception of Antarctica, and range from temperate to tropical environments, being most abundant and diverse in the tropics, but still significant in non-tropical ecosystems (Schultheiss et al. 2021). With their foraging, nestbuilding, and insect-tending behaviors, ants serve as highly influential organisms within their habitats. Ant nestbuilding, foraging, and scavenging behaviors contribute to the nutrient cycling of their environment, returning nutrients to soil and facilitating nutrient reintegration (Finér et al. 2013), as well as aerating and transporting the soil (Schultheiss et al. 2021). This nutrient cycling service means more nutrients are available for uptake by other organisms in the ants' ecosystem. Many ants will include carrion in their diets, participating in detritivorous food web, and thus aiding further in nutrient cycling within their ecosystems (Fellers & Fellers982). They can also participate in mutualistic interactions by protecting Hemipterans that feed on plant material and receive sugar-rich honeydew (Ivens 2015). Furthermore, ants can engage in mutualisms with plants themselves through seed dispersal as ants transport elaiosome-bearing seeds to their nests and discard the seeds (Karnish 2024). Their foraging behaviors are subject to the influence of environmental factors such as temperature and humidity, as well as the nutrition and availability of food in their environment (Bezděčková et al. 2024; Schafer et al. 2006; Dusstour & Simpson 2009). For ant communities that share ranges with periodical cicadas in the Northeastern and Midwestern United States, little is understood about their response to the resource pulse provided by the cicadas' emergence. It could be expected there could be a behavioral response to the change in food availability and nutritional balance with the influx of cicada prey and carrion. Periodical cicadas might present a novel food source for ants, both as live prey and as carrion, and their large numbers may lead to a predator satiation effect among ants.

Nutrient Cycling

The foraging and nest-building behaviors of ant colonies mean that ants participate in the nutrient cycling of organic matter in their environments. Nest-building leads to the accumulation of nutrients in the soil around the nest through larvae, ant bodies, food, and waste (Finér e. al 2012). This leads to ant nests concentrating the cycling of nutrients and can present a hotspot of nutrients that can be exploited by plants (Finér et al. 2012), (Fischer et al. 2003). The refuse piles that ant nests produce provide nutrients for plants that live in or around the nests which can be highly beneficial for these plants, especially in areas of low nutrient availability (Farji-Brener & Werenkraut 2017). Ant nests can serve as islands of fertility, supporting more plants in and around them than in soil without ant nests (Farji-Brener & Werenkraut 2017). Their presence can contribute to increased plant species richness within their ecosystems and can be integral to the plant community survival in desert biomes that may have limited water or nutrients available to plants (Farji-Brener & Werenkraut 2017). Additionally, ant nests provide habitats for other microfauna and can host distinct microclimates with different temperatures and moisture levels from the soil outside their nests. (Laakso & Setälä 1998). The excavation of nests contributes to an increase in soil invertebrate diversity, such as earthworms, nematodes and microbial colonies, some of which are specialized for the environmental conditions of ant nests (Laakso & Setälä 1998). Micro- and macroinvertebrates's roles in soil are not fully quantified, but their roles as decomposers, soil aerators, and microbial predators mean that they are highly important for the exchange of nutrients between macro- and microfaunal communities, and for the nutrient and oxygen availability within soil (Briones 2014).

Ants as Predators

Ants are highly efficient predators and can exert top-down controls within their ecosystem on the organisms they prey upon (Wills et al. 2019). For example, through a common garden experiment in Midwestern United States grasslands, it was found that predation rates decreased when ant populations were suppressed through ant-targeting poisons (Wills et al. 2019). Ants are predators of the larvae and eggs of many larger insect species, controlling and limiting the populations of these insects through their activity (López & Potter 2000). These larvae can include agricultural pests such as cutworms (*Noctuidae*) and Japanese beetles (*Popillia japonica*) (López & Potter 2000). Thus, their consumption of small prey means that ants can play an integral role in influencing the abundances of other arthropods within their ecosystem through top-down control. In their absence, a release of this control on their prey can come, which may lead to changes in their population sizes (Wills et al. 2019). Ants' eusociality additionally enables them to subdue prey larger than themselves with the aid of nestmates, extending their top-down predation effects to a diverse group of other arthropods (Cerdá & Dejean 2011).

Ants as Scavangers

The detritivorous diets of many ant species, including those represented in the Lake County ant communities, mean that ants play

a role in the decomposition of organic matter. They have been found to be some of the dominant scavenging arthropods when experimentally supplied with insect carcasses, as they would be the first to locate and utilize these resources (Faller 1982). Ants are considered to scavenge readily, upon decaying prey both small and large, and the majority of ants will exhibit this behavior as part of their foraging behaviors (Holway & Cameron 2021). Their scavenging behavior is expected to be a key factor in how ants interact with cicada pulses, as the carcasses of the abundant cicadas will provide another source of carrion in the landscape of decaying matter that ants may utilize when foraging. For example, in previous scavenging rate studies in the context of cicada emergence, ants were found to respond with greater activity within one week of carcasses being available to them (Yang 2006). This suggests ants are affected by the presence of cicadas when scavenging, though whether their scavenging rates of other carrion is affected is yet to be determined.

Interspecies Symbiotic Interactions:

Ants participate in a variety of mutualisms, particularly in ones that affect plants: ants feed at nectaries, transport seeds to feed on elaiosomes and tend herbivorous insects for food. A source of carbohydrates for many ant species is the honeydew produced by herbivorous insects that feed on plant phloem: Hemipterans that produce this honeydew can often be seen tended by ants, being guarded from predation, or even moved to new locations to forage (Ivens 2015). This means that ants will have an indirect interaction with plants by way of the herbivorous insects they protect and interact with. The species that display this behavior frequently belong to the subfamilies Dolichoderinae, Formicinae, and Myrmicinae, whose ranges can overlap with that of periodical cicadas (Ivens 2015). Ants can access the nectar produced by flowers and extrafloral nectaries when interacting with plants, which can lead to some pollination occurring as the ants access carbohydrates from the nectar they consume (Fagundes et al. 2015). Ants that harvest and consume seeds form relationships with the seedbearing plants they feed on: protein and lipid-rich elaiosome structures on the seeds can mimic the smell of rotting prey, and this incites foragers to return these seeds to their nests, often bringing the seeds with them, dispersing them further than the plant could unaided. Plants that produce elaiosome-bearing seeds benefit from having highly nutritious elaiosomes, as these seeds are more likely to be taken back to the nest or moved away from their parent plant when the foragers bring this highly valuable food source to their colony (Fischer et al. 2008). Nutrition of these elaiosomes are good sources of amino acids and simple sugars that are easily digestible by the colony members (Fischer et al. 2008). Elaiosomes produce chemicals that simulate the scent of decaying insects, which further appeals to foraging ants that consume these food resources (Fischer et al. 2008). These relationships, while not directly related to the predatory and detritivorous interactions ants may have with periodical cicadas, do describe the primary carbohydrate sources for ant foragers, which is a key part of their nutritional regulation. It is from these nectar and honeydew sources that ants would likely seek out additional carbohydrates when balancing their nutritional intake. Additionally, these interactions demonstrate other impactful roles ants have within their ecosystem.

Ant Foraging Strategies

Seasonal and Temperature Effects on Foraging

Factors that affect ant foraging include the nutrient requirements of the colony, as well as the environmental conditions to which the colony is exposed. Foraging activity is often restricted to specific temperature ranges within the ant foragers critical thermal maximums and minimums (Jayatilaka et al. 2011). Yearly, ant communities tend to increase their foraging activity in summer months, as colonies grow and require more nutrients (Bezděčková et al. 2024). As colonies begin to grow, with populations of larvae increasing within the nest, colonies will require a greater ratio of protein to carbohydrates than when the colony is more fully established and increasing at a slower rate later in the active season, wherein the carbohydrate requirements increase (Bezděčková et al. 2024).

Optimal foraging conditions can vary across different ant species, even within the same community. Species with a greater tolerance for high temperatures will have greater foraging presence at these temperatures than those with lower thermal tolerance maximums (Stuble et al. 2013). These thermal tolerances can lead to niche partitioning within ant communities, reducing competition by decreasing the overlap between optimal foraging temperatures of different species (Lessard et al. 2009). Warm temperatures tend to encourage foraging and other activities, as these allow ants to move faster due to their ectothermic body temperature regulation (Tizón et al. 2014). Most ants will be active within the range of 10 – 40 ˚C, except for some species that are specialized for hot, arid conditions (Tizón et al. 2014). Late spring to early fall, with their relatively warm temperatures thus increase ant foraging activity, especially in temperate regions where activity may slow or stop altogether in the cold of winter, early spring, and late fall (Win et al. 2018).

There is evidence of niche partitioning via thermal tolerances for ant species: ants will forage at different times during the day, with temperature as a contributing factor involved with that behavioral partitioning (Jayatilaka et al. 2011). For example, the diurnal *Myrmica croslandi* foraging starts at higher soil temperatures than the nocturnal *Myrmica pyriformis*, with *M. croslandi* exhibiting experimental thermal limits greater than *M. pyriformis* (Jayatilaka et al. 2011). Ant species that are more behaviorally dominant within their community are more likely to be active at a narrower range of temperatures than subordinate species that cannot compete as successfully with the more dominant species (Fellers 1989). Subordinate species may be active at wider ranges of environmental conditions, advantageous for reducing the chance of encountering more dominant species and engaging in interspecific competition or conflict, which can be harmful to the individuals involved (Fellers 1989).

Search Behavior and Foraging Success

Foraging success can depend on the behavioral and physical traits of the ants involved, especially when in competition with other species in their community. For instance, the size of an ant's legs, and the directionality of the paths they take to forage are factors in the way that they approach foraging (Pearce-Duvet et al. 2011). Ants that possess long legs in comparison to their body length are able to discover food sources more quickly, by moving more efficiently to allow them to travel farther (Pearce-Duvet et al. 2011). Species that travel more linearly will be able to run across more new food sources (Pearce-Duvet et al. 2011). In contrast, smaller species and those that do not have these linear search paths will be less likely to encounter novel food sources (Pearce-Duvet et al. 2011). The rate of successful foragers returning to the nest may be a factor in encouraging more foragers to be sent out of the nest. For example, in desert-dwelling *Pogonomyrmex*, the rate of foragers returning to their nest with food was correlated with the stimulation of foragers still in the nest to leave (Schafer et al. 2006). This suggests a mechanism for a proportional foraging response to the amount of food available in the environment (Schafer et al. 2006). When a forager returns after locating a food source, they can enable recruitment through laying pheromone trails as they hunt down food and subsequent foragers are then able to follow that trail to the food source (Lixiang et al. 2014). A positive feedback loop can occur, wherein more heavily-traveled pheromone trails will be supplemented with pheromones by the additional foragers, enticing more foragers to recruit to that food source (Lixiang et al. 2014). Ants can additionally perform tandem-running to show new foragers to a novel food source, where the ant that originally found the food leads another to the food's location (Franklin & Franks 2012). This allows ants to more quickly disseminate information through their colony (Franklin & Franks 2012).

Interspecies Competition While Foraging

There are three distinct roles of ants when it comes to interspecies foraging competition: opportunistic foragers, extirpators, and insinuators. Ants that are opportunistic are the first to arrive to food sources but are usually unable to defend it from competitors for very long and are timid around other ant species (Lach et al. 2010). Extirpator ants, on the other hand, do not find food sources first but are able to force other ants from the resource via aggressive recruitment (Lach et al. 2010). Finally, insinuating

ants avoid the recruiting and aggressive extirpators via their small size and limited recruitment to baits. (Lach et al. 2010). This can be described as the "discovery-dominance tradeoff", wherein ants that are adapted to efficiently discover food have an inversely proportional ability to defend that food, and ants that are highly successful at defending food sources from others are less successful in locating new food sources (Fellers 1989). Some environmental factors may additionally affect the amount of competitive behaviors beyond the different competitive roles ants may fill in their environment. Ants can exhibit reduced competitive interactions when encountering abundant resources when compared with more limited resources, suggesting the magnitude of competitive interactions may be responsive to the availability of resources such as extrafloral nectaries (Fagundes et al. 2016).

Nutritional Requirements of Ants

Nutrient Prioritization and Regulation

Ants primarily require carbohydrates in their diet as adults as a source of energy to fuel their activity, with additional protein requirements to enable to growth of larval members of the colony (Dusstour & Simpson 2009). Ants will also consume lipids as well and will feed on salt baits when salt is a limiting resource in their environment (Renyard et al. 2024), though these are not as prominent in their diets as protein and carbohydrate macronutrients are (Reynard et al. 2024). Salts are an essential nutrient to ensure metabolic function, and ants must maintain a baseline level of the nutrient in their bodies (Kaspari et al. 2020). Lipids are also important for the growth of ants, and larvae supplied with lipid-rich diets can grow larger than those without (Bottcher & Oliveira 2014). Epigenetic and environmental factors may influence the eventual adult size of the larvae as well as their caste within the colony (Trible & Kronauer 2017), but lipids remain an important nutrient for supporting the growth of these larvae.

Ant colonies need to be able to adjust their foraging behavior to ensure they receive the proper ratios of macronutrients to ensure survival and colony growth, and they are capable of regulating this in response to different nutrient availability in their environment by controlling their individual intake of a food resource, and through changing foraging behaviors (Dusstour & Simpson 2009). A key part of this nutritional regulation is the presence of larvae in this colony, as larvae are capable of digesting proteins that adult ants cannot and will regurgitate these proteins as compounds that the adult members of their colony can successfully digest (Dussutour & Simpson 2008, 2009). Excess carbohydrates will be digested by foraging adults, and excess proteins may be digested by larvae to respond to nutritionally imbalanced diets that colonies may encounter or regurgitated by larvae and removed by adult nestmates (Dussutour & Simpson 2008; Dussutour & Simpson 2006). The dynamic between foraging adult ants, and the larvae they feed and received food from can be termed as a "social stomach", with foragers acquiring food and beginning to digest it, and the larvae able to consume and digest proteins and larger prey items (Dussutour & Simpson 2008; Dussutour & Simpson 2006). When there is an overabundance of one macronutrient, ants will adjust their foraging behaviors to address this nutritional imbalance, such as consuming more salt in response to an abundance of insect prey in their diet (Kaspari et al. 2020) or consuming more of a diluted carbohydrate source to receive the proper carbohydrate intake (Dussutour & Simpson 2008). The prioritization of carbohydrates in adult foragers' diets is due to this nutrient being used for energy, essential for any organism's survival, and because protein is less essential for their survival as they no longer are growing and producing more tissue as larve are (Dussutour & Simpson 2009).

Ants Responding to Cicada Resource Pulses

There are gaps within our understanding of ant responses to periodical cicada emergences. The limited study of ant responses to cicada emergences, including periodical cicadas, means little is known about the nutritional needs of ants experiencing these events. Whether they adjust the balance of nutrients in their diets in any way in response to the cicada emergence is not fully known. However it can be inferred that if cicadas change the nutritional intake of the colony due to their abundance, there would be a corresponding behavioral change in the ants foraging to ensure optimal nutritional intake. Regarding foraging, according to available literature, there is evidence cicada emergences can affect detritivore behavior. Across different families of detritivorous arthropods, there were varied responses to periodical cicada abundance in their environment (Yang 2005). Ants were included among the detritivores studied and showed a weak response to cicadas supplied in the experiment (Yang 2005). Regarding other recorded predation and consumption of cicadas by ants, Harvester ants (*Pogonomyrmex rugosus*) in the Chihuahuan desert, showing an increase of predation upon the annual cicadas within their ecosystem (Whitford & Jackson 2007). While annual cicadas do not emerge with the same abundance as periodical cicadas, they still represent a resource pulse in their ecosystem (Whitford & Jackson 2007). For example, the Pogonomyrmex predation on cicadas could suggest other ant species are also capable of predation upon periodical cicadas and a possible prioritization of this food resource by foragers.

Introduction

The periodical cicadas (*Magicicada* spp.) of North America emerge every 13 or 17 years, in broods that can number between tens of thousands to two million individuals per hectare (Lloyd & Dybas 1966). At the peak of their emergence, the cicadas present a virtually inexhaustible food source for the rest of their ecosystem, temporarily restructuring the food webs in their communities (Getman-Pickering et al. 2023) and providing a novel food source for small mammals (Krohne et al. 1991) and avians (Getman-Pickering et al. 2023) and enriching detrital food webs as their bodies are decomposed (Setälä et al. 2022). Ants consume carrion and hunt live prey like arthropods, likely being directly affected by the periodical cicadas that will present a novel and abundant food source, though this relationship has not been extensively quantified.

The effect of the periodical cicadas on their ecosystems indicates their role as a resource pulse, an "ephemeral event of resource superabundance" (Yang 2004). Their synchronized emergence and relative inaccessibility after the adults die and nymphs hatch mean that they present a highly abundant resource that is present for a short time before a period of scarcity (Ostfeld & Keesing 2007). Resource pulses' may affect consumers directly via the resources' consumption, (Ostfeld & Keesing 2007), or through indirect effects caused by changes in diet or behavior of the consumers that affect their other interations within the ecosystem. (Getman-Pickering et al. 2023). These effects may be top-down, such as in the release of predation pressure on caterpillars when birds begin to consume cicadas instead (Getman-Pickering et al. 2023), or bottom-up, with decomposing matter from the cicada pulse adding additional nutrients into soil food webs (Setälä et al. 2022).

Due to ant's influential roles within their ecosystems, determining their response to periodical cicada emergences on their foraging behavior will provide a more complete understanding of the effects of the emergence as a resource pulse. Ants are understood to be ecosystem engineers due to their nestbuilding behaviors, which change soil texture, nutrient content, and microbial communities as they excavate soil and move food and waste products through their nests (Cerdá & Dejean 2011). They also prey upon other arthropods and act as scavengers, meaning they not only exert top-down predation pressure on other arthropods in their ecosystem, but also participate in detrital food webs that can contribute to the cycling of nutrients within their environment. The ecological roles ants serve may be affected by the cicada emergence due to the large availability of high-quality food from the cicada bodies: there may be changes in predatory and scavenging relationships between ants and their prey.

As ants are both active predators and scavengers (Makino et al. 2024; Williams et al. 1993), it is likely that ant communities will be directly affected by cicadas that will represent a new source of prey and carrion. However, little has been documented about the effects of this resource pulse on the foraging behavior of ants. What is more understood is that ants can adjust their foraging behaviors in response to the resources available to them while foraging, adjusting ratios of protein and carbohydrates to ensure nutrition is received by the colony for growth and

survival (Dussutour & Simpson 2009). Ants tend to require a balanced or slightly carbohydrate-biased diet (Cook et al. 2011) and require an input of protein for the growth of the colony, particularly the development of larvae (Dussutour & Simpson 2006). Larvae within a colony play a key role in the regulation of nutritional intake, as colonies with larvae can regulate their nutritional intake to get a more balanced ratio of carbohydrates and proteins than colonies without larvae (Dussutour & Simpson 2006). Additionally, ants will modulate their foraging to the quality and abundance of food in their environment. Ants recruit other workers to valuable food sources when returning to their nest from successful foraging (Schafer et al. 2006; Dussutour & Simpson 2009). Successful foragers can stimulate ants still in the nest to venture out and forage as well, while unsuccessful foragers will not stimulate this foraging activity, leading to proportional foraging responses to food availability (Schafer et al. 2006). The ability of ants to regulate their foraging rates, and nutritional intake suggests that they will exhibit a response to the resource pulse of periodical cicadas.

This study aims to elucidate the foraging responses of temperate forest and grassland ant communities in Lake County, Illinois, over the course of a periodical cicada emergence. We studied the impact of the periodical cicada emergence through measuring the foraging rates of ants, as well as the nutritional demand of the ant communities. If ants are exposed to the resource pulse conditions of a periodical cicada emergence, we expected to see a greater demand for carbohydrates than other macronutrients given the choice between carbohydrates, lipids, protein, salt, and water. This response was predicted based on the nutrient regulatory behaviors of ants, wherein ants tend to balance the intake of protein and carbohydrates (Dussutour & Simpson 2006). The ant community will be presented with a highly-protein based food source from cicadas and it's expected this intake will elicit a proportional intake of carbohydrates. Additionally, when foraging ants are active during a periodical cicada emergence, we expect to see a reduction in foraging rates on other prey items. This predicted response was based on the predator satiation effects observed in other predators during periodical cicada emergences (Getford-Pickering et al. 2023).

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Investigating Neural Mechanisms of Altered Social Motivation Following Brief Social Satiation & Deprivation in Rats

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Social behaviors are an important marker for normal brain development in children and young adults. Several psychiatric disorders are characterized by their negative effects on social behaviors, such as autism spectrum disorder and schizophrenia. Social interactions are inherently rewarding, and any changes to social motivation may reflect alterations in how the brain encodes the value of social interactions. Although no statistically significant effects were found, female rats in the social deprivation condition showed a trend toward increased investigation and orientation towards a social stimulus, suggesting a potential increase in social drive following isolation. ZIF analysis indicated a negative correlation between ZIF expression in the basolateral amygdala (BLA) and nose-to-nose sniffing, suggesting that BLA activation may reflect stress and reduce prosocial behavior. Sex-specific effects and individual variability were observed, with females showing lower ZIF expression in the CG1 region compared to males.

1. INTRODUCTION

1.1 A Homeostatic Framework For Studying Social Behaviors

Social interactions include any form of communication and/or interaction between two or more individuals of the same species (Chen & Hong, 2018; Sato et al., 2023). While the terms "social behaviors" and "social interactions" are frequently used interchangeably throughout the literature (Chen & Hong, 2018; Lee et al., 2004; Modi & Sahin, 2019; Sato et al., 2023; Shankar, 2023; van Kerkhof et al., 2014; Wu & Hong, 2022), this thesis will make a distinction between the terms for the sake of clarity. The term "social behavior" will refer to the observable actions of an individual towards conspecifics, while "social interactions" will refer to the mutual exchange of social cues. Making this minor distinction between the terms is intended to highlight the feedback loop between an individual's unique neural mechanisms and their social environment. The goal of this thesis is to explore how the nervous system adapts to the social environment and drives social engagement as a dynamic physiological state.

The introduction will focus on exploring the neural substrates of social cognition and their adaptations to social need fulfillment. By reviewing the current literature on what is known regarding the physiological basis for social homeostasis, gaps in knowledge can be further explored and addressed.

1.2 Social Behaviors are Evolutionarily Conserved Mechanisms of Survival

Social interactions are vital for survival and reproductive success across species, making social behaviors an essential driver of evolutionary fitness (Sato et al., 2023). Various versions of social behaviors have been observed across species, with even the simplest species demonstrating a range of affiliative behaviors, such as the sharing of resources (Benabentos et al., 2009). For example, *Dictyostelium discoideum*, unicellular organisms known as "social amoebas", have been observed to aggregate upon starvation and decide to sacrifice themselves to support the survival of the others, if necessary (Benabentos et al., 2009). Despite knowing some of the other amoebas will "cheat" and refuse to sacrifice themselves, these social amoebas accept the potential risk and choose social cooperation above their self-interests (Benabentos et al., 2009). This example demonstrates how social behaviors have been observed in organisms without nervous systems, suggesting that they have been selected for in evolutionary history from a common ancestor due to their contributions to survival (Chen & Hong, 2018; Sato et al., 2023). These simple organisms employ an evolutionary strategy that considers the reciprocal nature of social interactions, where an individual's actions may not always elicit the desired reaction.

This concept of reciprocity within social interactions becomes more complex in species with nervous systems, as they enable more sophisticated forms of communication. Male *Drosophila melanogaster,* better known as fruit-flies, are known to perform various social behaviors to demonstrate their fitness as a potential mate for their preferred female. This includes singing to her by vibrating his wings, dancing in circles around her, and tapping her with his forelegs to activate mechanosensitive pheromone receptors (Sokolowski, 2010). If the female decides he is worthy, she will show she is receptive by presenting him with her vaginal plate, otherwise she kicks him away and leaves (Sokolowski, 2010). This example not only demonstrates that male fruit-flies are experts at handling social rejection, but it also illustrates the idea that as the nervous system becomes more complex, so too do social interactions. These fruit-flies, even with their simple nervous systems, remain capable of rich social interactions which utilize an amalgamation of chemosensory, mechanosensory, visual, and auditory cues from conspecifics (Benabentos et al., 2009). The sensory cues are reciprocal and dynamic, forming a feedback loop that gives the individual milliseconds to decide upon their next behavior and predict the response from their interaction partner (Chen & Hong, 2018).

Social behaviors become increasingly context dependent as the goals of the organism evolve past fulfilling their base survival and sexual needs (Sato et al., 2023; Wu & Hong, 2022). In mammals, there is a marked difference in the complexity of social behaviors compared to those of the social amoeba or fruit fly examples previously mentioned. It is hypothesized that the evolutionary origins of mammalian social behaviors are an adaptation of maternal behavior, with much of the same circuitry involved (Modi & Sahin, 2019). This is most evident in altricial species, such as humans, which are born with underdeveloped brains and are helpless without their caregivers to provide food, warmth, and protection (Sato et al., 2023; Wu & Hong, 2022). Mammals can be dependent on their caregivers for years at a time, thus a social bond between progeny and caregiver is required for survival across species. This social bond involves circuits that produce rewarding sensations to the caregiver and neuromodulators that promote feelings of attachment to make the bond lasting. The neural contributors to their sociality have thus evolved to enable the formation and maintenance of lasting social bonds (Sato et al., 2023; Wu & Hong, 2022).

1.3 The Social Environment Affects Brain Development

Beyond maternal social behaviors, the behaviors of the helpless infant are motivated by an evolutionary drive to form a social bond to their caregiver (Ferrara et al., 2023). Their social behaviors are centered on getting their caregiver to meet their needs, such as crying for their mother's attention. The neural circuits involved in promoting this social bond will continue developing throughout the lifespan and produce social behaviors gaining in complexity (Ferrara et al., 2023). The emerging social behavior changes that occur throughout development are dynamically influenced by the social environment of the developing individual (Figure 1). As the individual's social circle expands beyond their caretakers, such as entering school and meeting other developing minds, their brain will begin to develop essential circuits between the prefrontal cortex and the amygdala (Matthew & Tye, 2019).

Dopamine, the neurotransmitter of reward and motivation, modulates this circuit between the developing medial prefrontal cortex and the amygdala throughout adolescence, heightening social awareness and sensitivity to social reward and rejection (Matthew & Tye, 2019). The expanding social environment, coupled with the individual's growing independence from their caregiver introduces the importance of social inclusion versus social exclusion. Sensitivity to social rejection during adolescence is a mechanism of evolution, whereby the growing individual must seek out their new social contacts to ensure their survival (Matthew & Tye, 2019). If the developing individual can learn how to respond to the social behaviors of their peers, they will continue developing the social

*This author wrote this paper as a senior thesis under the direction of Dr. J Amiel Rosenkranz

skills necessary for the inherently dynamic nature of social interactions (Ferrara et al., 2023). For social animals, reacting to the social cues of others is instinctual, however, selecting the appropriate response is a learned skill (Eslinger et al., 2021). This skill of processing social information and using it to determine the best course of action is known as social cognition.

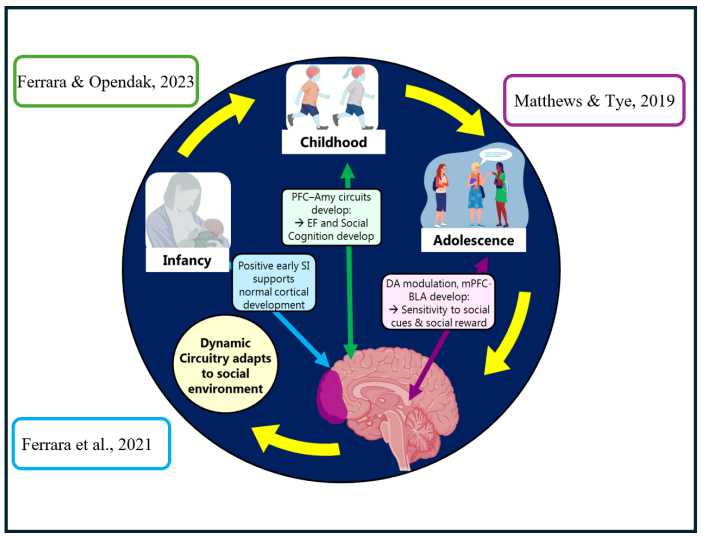

Figure 1. Social Interactions are guided by and influenced by brain development

The social environment forms a dynamic feedback loop with the nervous system, where healthy social interactions support typical prefrontal cortical-amygdala circuit development (Ferrara et al., 2021; Ferrara & Opendak, 2023). This circuit becomes refined with social experience, adapting to the more complex interactions in adolescence and becoming more sensitive to social cues with increased dopamine modulation driving the pursuit of social rewards (Matthew & Tye, 2019).

1.4 Social Cognition

Social cognition refers to the ability to decode the intentions of others from their social behaviors and then decide on the socially appropriate actions in response (Arioli et al., 2018). It reflects the integration of a wide variety of cognitive processes related to salience, reward-seeking, motivation, self-monitoring, and empathy (Bicks et al., 2015). Social cognition becomes refined throughout development as the individual learns how their behaviors influence others. Failure to develop the necessary skillset for engaging with others can reflect dysfunctional or underdeveloped circuits (Ferrara et al., 2023; Lee et al., 2004). Distinct facets of social cognition include social motivation, knowledge of one's own internal state, and knowledge of social cue interpretation (Bicks et al., 2015).

Significant deficits to any one of these facets of social cognition is often enough to warrant a psychiatric evaluation. Social dysfunctions are a fundamental dimension of many psychiatric disorders, which is why the Research Domain Criteria initiative put forward by the National Institute of Mental Health has asked researchers to focus their studies on social deficits (National Institute of Mental Health, 2024). The initiative is aimed at encouraging researchers to study mental health and psycoathology in the context of neurobehavioral functioning. They emphasize the need for researchers to ground their studies in understanding the neurobiology and physiological impact of psychiatric disorders because while the mechanisms remain poorly understood, so do the interventions to alleviate them. Additionally, social cognition is deeply intertwined with the ability to plan, remain focused, and to problem solve(Arioli et al., 2018). Neuroimaging studies have suggested that the prefrontal cortex and the amygdala both play a central role in facilitating the adaptive problem solving that takes place during social interactions (Lee et al., 2004).

1.5 Social Homeostasis: Loneliness is a Social Pain

Homeostasis refers to physiological processes that maintain stable states through compensatory mechanisms to meet physiological needs (Matthews & Tye, 2019). The state which functions as the "set point" is the state of homeostatic balance. Any deviations from homeostatic balance recruit coordinated responses to elicit interactions with the environment until the deviation is corrected (Matthews & Tye, 2019).

Deviations from homeostatic balance can also evoke motivated behaviors in response to unmet physiological needs that challenge the organism's survival. Natural behaviors, such as drinking or feeding, are initiated by motivated "drive" states which are regulated by neural circuits shaped by selective pressure (Betley et al., 2013). These motivated behaviors are negative "drive" states, where aversive conditions such as overheating, hunger, or thirst are actively avoided for the physical discomfort they cause (Matthews & Tye, 2019). Neural control of homeostatic balance requires flexible coordination of circuit components for these complex survival-oriented behaviors to be evoked (Betley et al., 2013; Matthews & Tye, 2019). Homeostasis has been classically understood as systems used to maintain thermoregulation, energy levels, and osmoregulation. One of the major goals of neurobiology has been to understand how neural adaptations are recruited and how they direct behavior. Social homeostasis has been proposed as a neuroscience model for understanding the neural adaptations that occur as adaptive functions to regulate behaviors in response to social interactions (Matthews & Tye, 2019).

Social homeostasis is the ability of individuals to compare the perceived quantity and quality of their social contacts and compare it to their established set-point. This comparison will adjust the amount of effort the individual is willing to expend to seek social engagement until they have reached their optimal set point (Lee et al., 2021). This optimal set point for social homeostasis is subjective and unique to the individual's perception. Within this model, loneliness functions as an aversive signal designed to promote motivated behaviors to seek social engagement (Matthews & Tye, 2019). Loneliness is defined as subjectively perceived social isolation, and it is unique to the individual (Hawkley, 2022). Functional MRI studies have noted that individual differences in perceived isolation predicted the brain's response to social information, showing more activation of the visual cortex when presented with unpleasant social images (Hawkley, 2022).

Much like hunger pains or extreme thirst, loneliness is the brain's painful reminder that social needs have not been met. The mechanism that drives social engagement is known as social motivation.

1.6 Social Motivation: Social Dysfunctions are Circuit Dysfunctions

Sensory perception systems play a vital role in social cognition. Social information is processed first through the dominant sensory modality of the organism. In humans, social information is processed first through visual perception. Recognition of a conspecific as familiar or unfamiliar is obtained through visual perception of their facial identity and facial expressions are used to infer their intentions (Babinet et al., 2021). In rodents, olfaction is the dominant sensory modality for processing social information. Rodents use their sense of smell to obtain social information, making olfactory cues the rat equivalent of social cues (Modi & Sahin, 2019).

Social sensory information is given greater attention than non-social stimuli in both humans and rodents, and this process requires a brain-wide effort to coordinate across multiple circuits and the recruitment of neuromodulators (Modi & Sahin, 2019). The prefrontal cortex plays a major role in attending to incoming social information, and one of the contributors to the heightened sensitivity and selective attention towards social stimuli is the amygdala.

1.6.1 The Almond at the Center of it All: The Amygdala

The amygdala is an almond-shaped cluster of nuclei located in the anterior part of the medial temporal region composed of grey matter (Watson et al., 2010). It is located anterior to the hippocampus and plays an essential role in emotional and affective processing (Watson

et al., 2010). The circuitry of the amygdala has been well-conserved across evolution, with mammals and non-mammals alike possessing an amygdala that performs analogous functions across species (see Figure 3) (Janak & Tye, 2015). Most research dedicated to the amygdala has focused on its role in fear and stress, however, the true role of the amygdala extends far beyond this simple characterization. While the amygdala does play an important role in the detection of threats, its true purpose is to promote recognition of information from the environment critical for survival (Labuschagne et al., 2024). This includes enhancing the salience of emotionally relevant stimuli (Janak & Tye, 2015). In simple terms, it helps the brain prioritize emotionally important stimuli by modulating attention towards it, influencing the memories that form around it, and affecting the behavioral responses evoked by the stimuli.

This places the amygdala at the core of the brain's emotion processing network (Balderston et al., 2015). The amygdala serves as a gateway in processing sensory information, sending and receiving projections across multiple brain regions to integrate sensory and cognitive inputs (Modi & Sahin, 2019; Watson et al., 2010). Several psychiatric disorders are affected by amygdala dysfunction, such as autism spectrum disorder, anxiety, and addiction (Huang et al., 2022). Neuroendocrine factors and neurotransmitters modulate the activity of the amygdala, influencing the neuronal activation patterns to evoke the socially appropriate response (Janak & Tye, 2015). Of these neurotransmitters, dopamine (DA) and oxytocin play an essential role in the amygdala's influence over establishing salience to social stimuli. The ventral tegmental area (VTA) is located in the midbrain and composed of ~60% dopaminergic (DA neurons) neurons, making this area an important source of DA in the mesocorticolimbic dopamine system, which our little amygdala is smack dab in the middle of (Cai & Tong, 2022).

The DA provided by the VTA is hypothesized to mediate the rewarding sensations associated with social interactions and social motivation overall (Modi & Sahin, 2019). The amygdala sends inputs back to the VTA, as well as the nucleus accumbens (NAc), another important dopamine heavy region that mediates reward in the striatum. By influencing the dopaminergic tone at multiple levels, the amygdala drives the DA needed to reinforce the valence of social stimuli (Modi & Sahin, 2019). At the same time this is occurring, oxytocin, a neuropeptide that influences social bond formation, is also increasing the salience of the social signals (Chu et al., 2012; Modi & Sahin, 2019; Sharp, 2017). When oxytocin is co-expressed with dopamine in medium spiny neurons found in the NAc, this enhances the rewarding sensations of social bonds (Modi & Sahin, 2019). The amygdala can be divided by the functional roles of its subnuclei, and the one most central to sociality is the basolateral amygdala. The basolateral amygdala receives projections from the VTA, is modulated by oxytocin, and it maintains reciprocal connections with the NAc and prefrontal cortex (Chu et al., 2012; Modi & Sahin, 2019; Sharp, 2017). This places the basolateral amygdala as an essential component of social motivation and social awareness.

1.6.2 The Basolateral Amygdala: Linking Sensory Input to Emotional Meaning

The basolateral amygdala (BLA) contributes to social perception by imparting emotional valence to potentially rewarding stimuli (Watson et al., 2010). This means that the BLA helps decide what the brain considers important enough to attend to, by weighing potential risks and rewards that the stimuli poses. Because of this, the BLA is central to decision-making, social perception, and emotional responses (Chang et al., 2015). The BLA receives input from sensory cortices and helps evaluate external cues for emotional or social significance, such as facial expressions or tone of voice. Meta-analysis of functional MRI studies has demonstrated that emotion-evoking stimuli, such as negative facial emotions (grimacing, frowning), altered the connectivity between the BLA and prefrontal cortex (Labuschagne et al., 2024). The impact of altered signaling in the BLA is often hyper-excitability, as the BLA requires tonic inhibition, meaning stable and constant inhibitory signaling for the stable regulation of emotional processing (Sharp, 2017). The tonic inhibition of the BLA is maintained by the glutamatergic (excitatory) cortical inputs it receives, which sounds counter intuitive to need excitation to downregulate the already hyperexcitable BLA. The inhibitory interneurons in the BLA, which mediate connections between other neurons, are activated when they receive excitatory cortical inputs, essentially turning the inhibition 'on' (Sharp, 2017). This is known as feed-forward inhibition and it is what controls the BLA's inherent excitability, as it ensures the BLA's principal glutamatergic neurons maintain more stable firing rates (Janak & Tye, 2015; Sharp, 2017). When this feedback inhibition is disrupted, the BLA's hyperexcitability is associated with disrupted motivation, anxiety, and emotional dysregulation (Janak & Tye, 2015; Sharp, 2017).

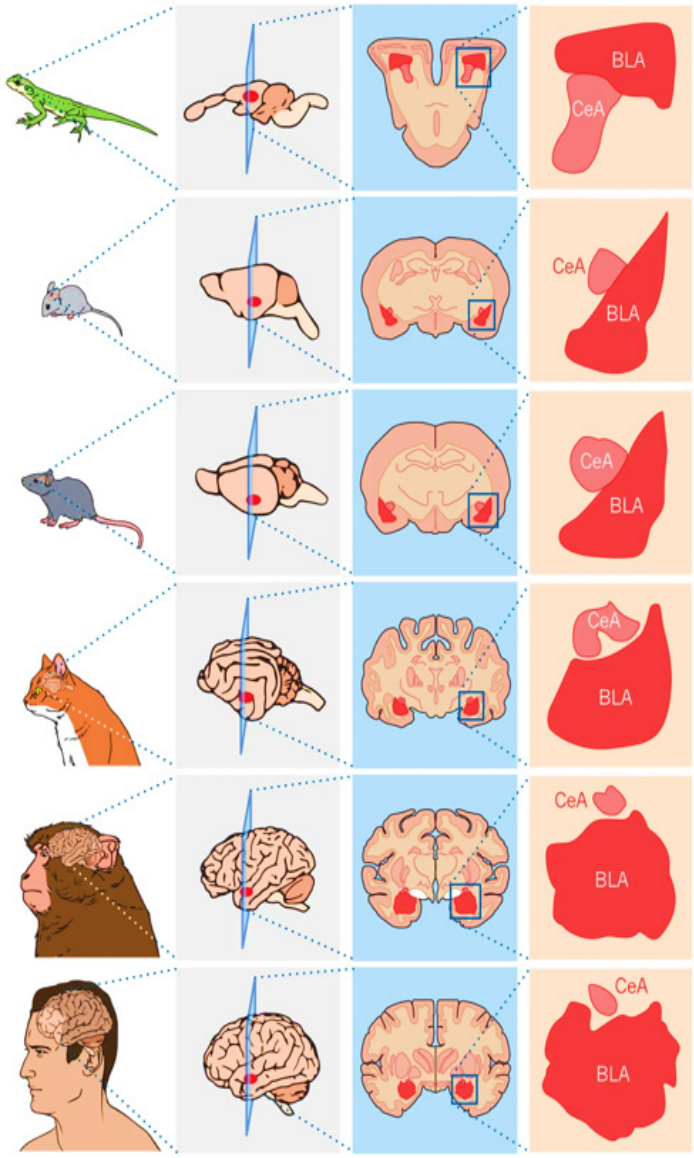

Figure 2. The Amygdala in All of Us
Amygdalar nuclei and its basic circuit connections and functions are conserved across species, the images shown are coronal sections from the brains of a lizard, mouse, rat, cat, monkey and human. This image is included to showcase how there is an amygdala in all of us, emphasizing the conserved nature of its contributions to sociality and survival. Image from Janak & Tye, 2015.

Along with these excitatory projections from multiple subcortical regions, membrane receptors throughout the BLA also work to modulate the activity of the BLA's GABAergic (inhibitory) interneurons (Janak & Tye, 2015; Sharp, 2017). Dopamine receptors expressed by BLA GABAergic interneurons inhibit GABA release when activated by projections from the VTA (Janak & Tye, 2015; Sharp, 2017). The inhibition of GABA release by

the VTA's projections suppresses the feed-forward inhibition of the BLA's principal neurons by GABA interneurons and increases overall BLA activity (Sharp, 2017). The BLA is part of a frontotemporal system that innervates several regions across the corticolimbic system, including the anterior cingulate cortex found in the medial prefrontal cortex (Huang et al., 2022).

1.6.3 Prefrontal Cortex

Along with the amygdala, another telencephalic powerhouse key to orchestrating higher order brain functions is the prefrontal cortex (PFC). The PFC refers to the region of the frontal cortex located anterior to the premotor cortex and supplementary motor area (Grossmann, 2013; Hathaway & Newton, 2025). The PFC came to the forefront of biomedical researchers' attention in 1848 when Phineas Gage miraculously survived having an iron tamping rod pierce through his frontal lobe (Harlow, 1868). Before his injury, Gage was reportedly a calm and dependable man who drastically changed after his accident, becoming short-tempered and disorganized (Harlow, 1868). His famous brain injury has been cited in every neuroscience and psychology textbook since then for revealing the PFC's functional connectivity as the neural substrate for personality and cognition (Anastasiades & Carter, 2021; Grossmann, 2013). Numerous neuropsychiatric disorders have been associated with neuromodulator dysregulations which impact the circuit level functions of the PFC (Gamo & Arnsten, 2011; Kas et al., 2014). These disorders include obsessive-compulsive disorder, attention deficit hyperactivity disorder, depression, schizophrenia, bipolar disorder, and autism spectrum disorder (Gamo & Arnsten, 2011). These disorders highlight the centrality of PFC functionality for its role in exerting cognitive control over thoughts and behaviors. Without the inhibitory control of the PFC to focus attention to relevant targets and filter out irrelevant stimuli, social and cognitive deficits can emerge (Chini & Hanganu-Opatz, 2021). Integration of external cues with internal emotional states underlies the PFC's ability to enable executive functions such as planning, attention, and decision-making to take place (Reppucci & Petrovich, 2016). Since Phineus Gage's accident, other lesion studies have demonstrated that functionally distinct subcomponents of the PFC work together to rapidly generate meaningful interpretations of incoming information (Kas et al., 2014; Szczepanski & Knight, 2014). Cytoarchitectonics, the study of the structural arrangement of cells in neural tissues, has been utilized to map out these divisions by their specific connectivity and contributions to functionality (Anastasiades & Carter, 2021; Kiwitz et al., 2020; Van De Werd et al., 2010). The cytoarchitectonic features of the PFC suggest that after it receives sensory input from sensory cortices, long-range afferents to its subdivisions from the ventral striatum, hypothalamus, and amygdala are integrated (Anastasiades & Carter, 2021; Kiwitz et al., 2020; Van De Werd et al., 2010). These connections have been implicated in priming the PFC for complex social cognitive processes by coordinating reciprocal loops with sites of sensory perception, motivation, and emotional valence (Kas et al., 2014; Modi & Sahin, 2019). The PFC subregion responsible for the integration of emotional and social information is the medial prefrontal cortex (mPFC) (Grossmann, 2013).

1.6.4 Medial Prefrontal Cortex

Located on the medial surface of the frontal lobe, the mPFC is composed of a complex network of interconnected regions thought to enable different forms of processing related to the expression of social behavior (Modi & Sahin, 2019). The afferent projections to the mPFC induce local circuit activity which facilitate higher sensory processing in response to social stimuli by the PFC (Chen et al., 2024). This increased sensory processing allows for heightened awareness of the environment and selective attention to potential threats (Capuzzo & Floresco, 2020). The mPFC is known as a key structure in the social circuit, where it mediates memory retrieval, cognitive flexibility, and inhibitory control to anticipate consequences (Chen et al., 2024). The mPFC is comprised of three main subregions in rats: the cingulate cortex the prelimbic cortex and the infralimbic cortex. The cingulate cortex (CG1) is involved in a wide range of functions, such as the regulation of emotion, decision-making, executive control, and prosocial behaviors (Simon IV & Rich, 2024). It is the rat equivalent of the anterior cingulate cortex (ACC) in humans, aiding in social processing which involves the intake of social cues to provide context and enable meaningful interpretations of them (Chen et al., 2024; Simon IV & Rich, 2024). The CG1 coordinates attentional processing to make judgements on social behavior, described as cost-benefit calculation that considers the entire context of a situation (Guo et al., 2019; Kitagawa et al., 2024). Several studies on observational fear have considered the CG1 as an empathy- promoting subregion, and the prelimbic cortex is more associated with fear expression (Chen et al., 2024). The connections it has to the hypothalamic-pituitary-adrenal (HPA) axis link its activation to stress and some studies have noted that prelimbic activity can reflect social motivation to dominance related decisions (Grossmann, 2013). For the infralimbic cortex, it is often considered to have a somewhat opposing role when compared the prelimbic, where the infralimbic cortex is associated in social buffering and stress recovery (Nett & LaLumiere, 2021). The infralimbic cortex (IL) is implicated in reward-seeking behaviors, but it can also regulate extinction, as some studies have observed the IL influence the inhibition of cocaine seeking (Nett & LaLumiere, 2021). The human equivalent of the infralimbic cortex is the ventromedial PFC, which mediates behavioral inhibition (Nett & LaLumiere, 2021).

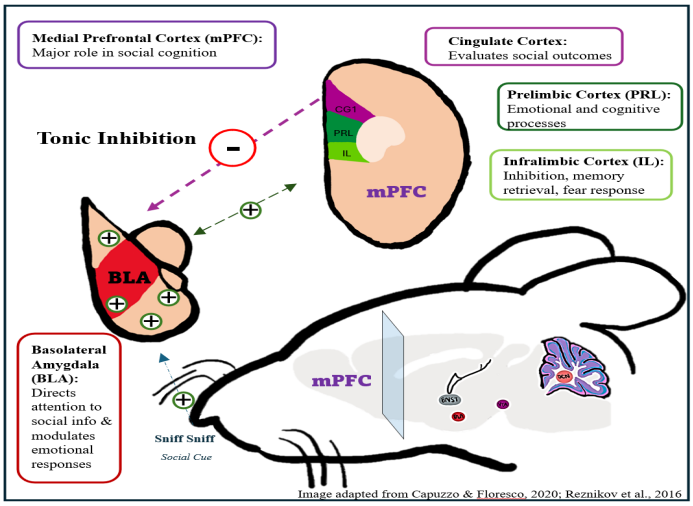

Figure 3: Prefrontal Cortex-Amygdala Circuit
Involved in social cognition, emotional regulation, and decision-making.

1.7 Social Deprivation vs Social Isolation: Historical Context, Ethical Delineation

In 1958, Harlow and Zimmerman infamously raised baby Rhesus monkeys in isolation chambers with either a "cloth mother" that provided comfort but no food, or a "wire mother" that provided food but no comfort. This study deprived the Rhesus monkeys of social touch, and the intense distress and permanent behavior deficits demonstrate the powerful role of social contact in shaping behavior and brain development. Their study established the importance of social and emotional bonds, as the monkeys would choose the comfort of touch over food. While their study is often cited as a foundational work, it is important to note that their study is now widely condemned for its extreme and unnecessary cruelty. It is now well established in the literature that long term social isolation produces detrimental effects on behavior and brain function, causing anxiety, depression-like symptoms and even self-harming. In contrast, the present study does not employ isolation, is not interested in replicating cruel studies, and did not revisit the questions we already have the answers to: long term social isolation is detrimental. Importantly, no rats were harmed in this study and they were housed in the same housing room as their former cage mates, leaving them with olfactory, auditory, and visual contact with conspecifics. Brief social deprivation (< 24 hours) was used as one of the conditions to mimic ethologically relevant reductions in social interaction to study how these brief periods of reduced social contact may shift motivational states and increase social drive.

1.7.1 Hypothesis

We hypothesized that (1) depriving rats of fulfilling their need for social interaction would increase ACC-BLA activity social drive, (2) sating those social needs would lower ACC-BLA activity and social drive, and (3) male and female neural activity would differ and manifest in distinct behavioral phenotypes.

Foucault Pendulum

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This project studies the Foucault pendulum, a free swinging 3-dimensional pendulum influenced by the rotation of the Earth. Three notable characteristics are derived: the pendulum's period, the impact of the Coriolis force on the bob, and apsidal precession due to ellipticity in the oscillations. Data from a magnetically driven Foucault pendulum taken during the summer of 2023 is compared with the predicted derivations for the Coriolis force. As there is a lack of data on the apsidal precession, the effect of elliptical oscillations is explored through a reference pendulum. We find that the Foucault pendulum, while a great tool for scientific demonstrations, can easily succumb to apsidal precession which may negate all effects of the Coriolis force. If a pendulum is to be constructed to demonstrate only the Coriolis force, great efforts must be undertaken to prevent elliptical oscillations.

Introduction

Léon Foucault was a 19th century French medical student, but his interest in early cameras and optics, as well as his dislike of blood, led him to pursuing physics[1]. He continued to pursue optics, making great strides and eventually getting nominated to be the *Académie des Sciences* reporter for the *Journal des Débats*, the most prestigious journal in France. There, he wrote on advancements in science covering all fields, from physics and chemistry to medicine and industry. He continued to work on his own optics projects, eventually earning a doctorate for his study of emission lines. However, he is not remembered for his work on emission lines, but rather his pendulum experiment done a couple years prior.

After a trip on rough seas, Foucault received inspiration upon seeing the spar of a ship still relative to the boat. He initially tried placing a vibrating steel rod in a rotating chuck, which would prove analogous to the eventual pendulum wire on a rotating Earth. The pendulum experiment started in Foucault's home with a 2-meter pendulum, but Foucault was not happy with all the interfering vibrations from the outside world. He ended up getting permission to put up an 11-meter pendulum in the Paris Observatory. This experiment proved so popular that an even larger demonstration was set up in the Panthéon of France. This monumental 67-meter setup was even more popular, inspiring similar demonstrations across Europe and the Americas.

This experiment has endured the test of time, and its impact on popular science is not to be discounted. However, the reception from physicists in Foucault's time was not so warm. At the time, the exact science behind the pendulum was not so well known, and many scientists proposed their own theories to explain it. Although modern physics has a better understanding of how the pendulum works, the Foucault pendulum is reserved for demonstration purposes without need for precise measurement. This thesis will describe the physics behind the Foucault pendulum and examine data taken from the summer of 2023 using a magnetically driven Foucault pendulum.

To view the remainder of this thesis, visit: https://www.lakeforest.edu/

*This author wrote this paper as a senior thesis under the direction of Dr. Michael Kash.

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