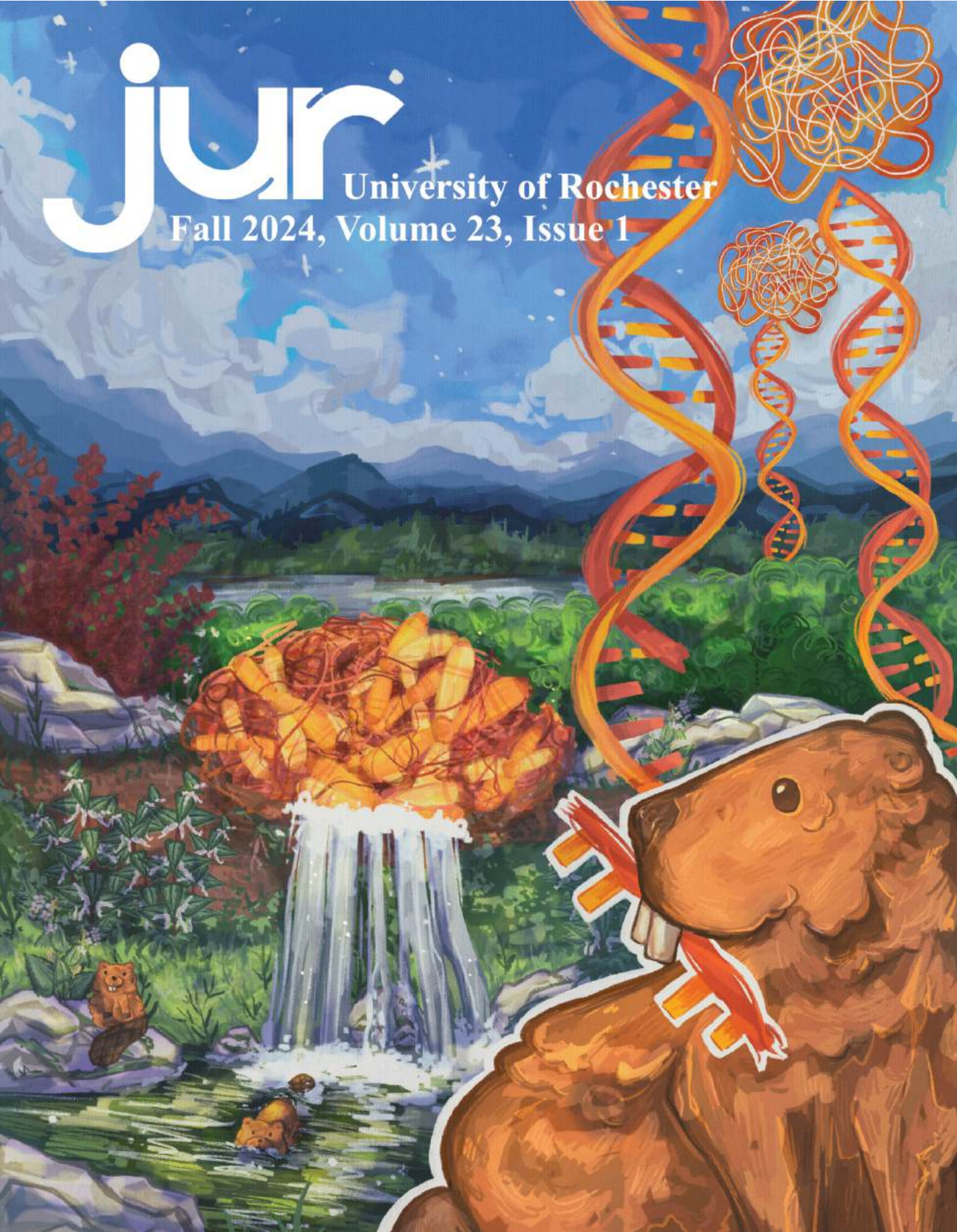
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University of Rochester
Fall 2024, Volume 23, Issue 1

# Journal of Undergraduate Research


*Fall 2024, Volume Twenty-three*
*Issue One*


UNIVERSITY of ROCHESTER

*The Journal of Undergraduate Research (JUR) is dedicated to providing the student body with intellectual perspectives from various academic disciplines. JUR serves as a forum for the presentation of original research, thereby encouraging the pursuit of significant scholarly endeavors.*

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# Letter from the Editors

Conducting research – much like a beaver building a dam– is a cumulative process that deepens our understanding of the world and nurtures the curiosity that drives scientific inquiry. Just as beavers chip away at wooden logs, undergraduate students make small, yet impactful contributions that can ultimately lead to exciting discoveries. To our readers, faculty, and amazing staff, it is with great excitement that we present the Fall 2024 issue of the Journal of Undergraduate Research. Herein, you will find the impressive research accomplishments of our undergraduate community that have culminated in four senior theses and six articles spanning multiple disciplines.

From the natural sciences and engineering, Carlina Velicer ‘26 discusses the role of the North American beaver in invasive plant growth, highlighting the issues caused by tree canopy damage. Also studying beavers, among other rodent species, Natasha Sieczkiewicz ‘26 investigates the role of SIRT6 in regulating mobile elements and demonstrates how these elements may contribute to aging. Within the scope of viral genetics, Daniel Kuo ‘24 presents a comprehensive analysis of how CRISPR gene editing systems can be used to edit viral genomes, particularly focusing on the trial and error process of adapting such systems. In E.coli, Kevin Zheng ‘24 dissects the factors that induce DNA condensation, revealing multiple DNA conformations that can induce condensation. Finally, pertaining to medicine, Katherine O’Leary ‘25 (Take-5 Scholar) reveals the role of estrogen signaling in neutrophil-tumor crosstalk in Lymphangioleiomyomatosis and Haolin Wang ‘25 demonstrates the applications of statistical models to reveal the risk factors of heart disease.

From the humanities and social sciences, Erin Hess ‘25 discusses the role of Saint Thecla in early Christianity, with focus on her conflicting portrayals throughout history. Albenys Díaz Hernández ‘25 probes into the effects of bilingualism in childhood English proficiency, while Seth Cutler ‘24 tackles the relationship between exposure to accented speech and its impact comprehension. Sophia Nguyen ‘27 examines the correlation between sleep apnea and bradycardia.

We also are delighted to share an interview with Professor Emeritus Franklin Stahl, an alumnus of the Biology program. He speaks on his experience as a founding figure in the field of Molecular Biology, his time at Rochester, and much more.

We would be remiss if we did not express our gratitude to our outstanding editorial board: our managing editors for ensuring our articles were of the highest quality and our layout chair, Phoebe Shin. We would like to especially thank the content and layout editors for their hard work and contributions to the journal. Of course, we are ever grateful for the continuous support from the Office of Undergraduate Research, Emily Rendek and Ann Robinson. Finally, we thank Cat Crawford and the Wilson Commons Students Activities Board for their guidance.

Without further delay, to our readers, we hope you enjoy and draw inspiration from the research featured in this issue.

Sincerely,

*Catherine Lan and Wimeth Dissanayake*

# Journal of Undergraduate Research

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# Alumni Interview

## Dr. Franklin Stahl, PhD

### A Perspective on Research: Franklin Stahl

The 1950's was the beginning of a revolution in the field of genetics. The discovery of the DNA as the carrier of genetic information by Alfred Hershey and Martha Chase in 1952, and the uncovery of the DNA double helix structure by James Watson and Francis Crick in 1953, had transformed the field from the study of Mendelian crosses and heredity into the study of DNA and the mechanisms of life at the molecular level. Collaborating with Professor Matthew Meselson to discover semiconservative replication, Professor Franklin Stahl has cemented himself as one of the pioneering figures in this new field of Molecular Genetics. Their experiments, using heavy and light isotopes of nitrogen to label newly formed DNA molecules, established the current understandings of DNA replication. Yet, as Professor Stahl often pointed out, his seminal role in genetics was not only due to his intellect, but also lucky encounters. An alumnus of the University of Rochester, Professor Stahl has graciously accepted an interview with the Journal of Undergraduate Research, to reminisce about his time here as a graduate student and to pass on advice to undergraduate researchers.

When pursuing his bachelor's degree, Professor Stahl initially intended to enter into a medical program. However, his grades and interests led him to consider a doctorate degree instead. At the time, he had not had the opportunity to see what a laboratory was like, but knew that if he was going to pursue a Ph.D. it would be in genetics.

"I didn't like the genetics courses, they were taught poorly, and worst of all they were boring. But I knew that I liked genetics, Mendelian Genetics, because I thought it was interesting … It was the predictability and orderliness of Mendelian Genetics that drew me to it, unlike my other subjects, it was predictable."

In search for graduate programs, Professor Stahl was to some extent drawn to the University of Rochester by its Genetics program and to some extent drawn to it by chance:

"I had applied to some graduate programs, and the one that worked out, the only one I seemed to get right, was to the University of Rochester … the school had a Genetics program, and that's what I was interested in, so I applied."

The acceptance was a relief to Stahl, as he would much rather work in the laboratory than be sent off to Korea.

Though initially assigned to Professor Charles' lab, who was studying mice genetics, Stahl found himself much more interested in bacteriophage, or phage, genetics after a course he took over the summer at Cold Spring Harbor (CSH).

"The course was designed for faculty, for retiring atomic physicists, who wanted to learn molecular biology, so that they could turn from studying death to studying life. This meant I had very bright classmates that helped make the course exciting."

Stahl, who became completely enamored by phage genetics, was particularly fascinated by the simplicity of the systems. His perspective was that if the same basic processes occurred in both phage and higher organisms, then studying phage, a system with few bells and whistles, could uncover the basic mechanisms of life. As his interest developed, Professor Charles had been suffering from complications due to Hodgkin's lymphoma and withdrawn from the laboratory. This had prompted Stahl to transfer to a group investigating phage genetics.

As Stahl was applying for transfer, the Department of Biology was recruiting new professors, and Stahl had suggested Professor Gus Doermann, one of the CSH phage course instructors. Doermann was already well-known for his work with T4 phage at Oak Ridge National Laboratory. His recruitment to the University of Rochester halted Stahl's plans of transferring and Stahl immediately asked to join the Doermann Lab, to which Professor Doermann recommended for Stahl to train at Oak Ridge before Doermann moved to Rochester.

"Gus (Doermann) wanted me to come over to the Oak Ridge lab so that I could get trained on all the techniques, and make mistakes, in the old lab. He was leaving that lab, so he didn't care how much of a mess I made, but he didn't want me making those mistakes in his shiny new lab at Rochester."

Professor Stahl completed his doctoral work in the Doermann Lab, honing skills in working with T4 phage. However, he encountered a problem with his qualifying committee:

"I was all set to graduate, but then my qualifying board wanted me to be held back to take physiology, which I knew I was not so good at. Now, I didn't want to take physiology, because I knew I didn't like the dumb professor who taught physiology, so I told the Chair [of Biology] that there was no way that I was taking this course. After some time, we came to the agreement that I could be sent off to the Woods Hole Marine Biology Laboratory (MBL) to take the physiology course there."

By luck, the lab section of the course was taught by Professor James "Jim" Watson, and it was also at MBL where Stahl and Meselson first became acquainted.

"I was sent to Woods Hole, which gave me the good luck of getting to know Jim Watson better, and meet Matt Meselson, so that I could later collaborate with them at Caltech."

After completing the physiology course and earning his doctorate degree, Stahl headed off to Caltech, the "Meca of Molecular Biology, where Max Delbruck had gotten biologists, geneticists, and biochemists all under the same roof," and the institution where Stahl made his seminal discoveries with Meselson. The idea of the final experiment was simple and beautiful: take bacteria grown in heavy 15N containing media and move it onto light 14N media before lysing cells and spinning down the DNA in an ultracentrifuge. The logic was that for replication to occur, new nitrogenous bases must be produced. Therefore, the different isotopes from the growth media would be incorporated into the bacterial DNA, and the relative amount of each isotope incorporated into the DNA could be measured using ultracentrifugation. If replication was conservative (i.e., both strands of the original parent molecule stay bound together), then a heavy 15N band was expected to persist through multiple replication cycles. But if replication was semiconservative (i.e., a parent strand becomes bound to a daughter strand), then a mixed band between 14N and 15N would be expected to persist through multiple replication cycles. While the Meselson-Stahl experiment succeeded through the use of bacteria, they had initially planned to use phage.

"So, we had just gotten a new ultracentrifugation machine, and we wanted to use phage in a Cesium Chloride (CsCl) gradient, because we knew that CsCl was just heavy enough (to separate out 14N and 15N), and we had hoped to transfer the phage from bacteria grown in heavy nitrogen (15N) to light (14N) media. As a test, Meselson tried to use the ultracentrifuge to separate the phage particles, but two things happened that he was not expecting. First, the CsCl moved, which by-mistake created the gradient that was so crucial to separating out the light and heavy strands of DNA. Second, the phage broke apart, and it made this band in the middle." Soon after, the pieces fell into place for the two, and after switching to E. coli as a model, they successfully validated the semiconservative model of replication.

Yet soon Stahl would find himself leaving Caltech. Finding the weather in Pasadena unbearable, Stahl would head to the University of Missouri where he wanted to start a lab of his own:

"The smog was insufferable in Pasadena, and my wife and I were just having kids. I knew that the air could not be good for them, and it was not the way I wanted them to grow up. Back in those days people would burn their trash in backyard incinerators, so you could imagine how bad it was."

Relocating to Columbia, Missouri, Stahl thought he could find respite from the California smog and settle down to raise his family. In fact, his sister-in-law had married Professor Lewis Stadler, a renowned geneticist at the University of Missouri, and both had settled in the Midwestern state. Unfortunately, a litany of issues emerged there:

"We had just gotten to Columbia (Missouri), and me and my wife needed clothes, so we looked for the nearest clothing shop and in the yellow pages we kept seeing – white only – and my wife and I didn't like that. I came to realize that the town was fundamentally racist, and so that convinced my wife that we could not raise our children there. The other thing which I didn't like about Missouri was the way they wanted me to teach, because I had been asked to teach the Introductory genetics course for undergraduates. When I tried to teach the students genetics I started with Mendel, but it was too deep for these students, because what people don't get is that Mendel was a genius. So, I rewrote the whole course, so that these students could start from something simple, like Bacteria. I went through how they replicate, how that meant that bacteria must have this genetic information, and I got the students acquainted with these ideas. The university did not like that, they were fundamentally reactionary towards genetics, and they wanted me to teach genetics in a way in which I was not comfortable teaching it, because the students were not able to really understand the material that way. They wanted me to teach heritability and Mendel, and I couldn't stand for that."

These complications prompted Stahl to look for another institution to establish his lab. Fortunately, he had many offers.

"Well, word must have gotten out that I wasn't very happy at Columbia (Missouri), because I was getting invitations to visit from a lot of fine schools. Cornell wanted me to come, and Cornell is a lovely place, Wisconsin wanted me to come, and Berkley wanted me to come, but nobody came to visit me and tell me why I should go there, except for Aaron Novick, from the University of Oregon. Novick had accepted the responsibility of creating an Institute of Molecular Biology in Oregon, and while he was still considering whether he should (take on that responsibility) he asked Jim Watson for advice. First, Jim said that two positions is not enough for an institute, and that he should ask for double that at least, and second, you should hire Frank Stahl as your first appointment. So, Aaron asked the higher ups in Eugene (University of Oregon) and came to Missouri to ask me to be the first guy to fill the role, and he told me why I should. He said that an Institute of Molecular Biology, as he envisioned it, would be Physicists, Chemists, and Biologists, especially Geneticists, all who are interested in understanding life at the molecular level, housed next to each other so that they could not help bumping into each other. They would share many facilities, centrifuges and other expensive things, so like it or not, conversations would arise. Collaborative chemical, biological, physical investigations that might not otherwise occur, and that sounded exactly like what I needed. I felt comfortable with Genetics, but my Chemistry was woefully impeded. This was why I couldn't take on graduate students at Columbia, because there weren't any chemists that gave a darn about Biology; I simply couldn't find any chemists or physicists who could be collaborators, so I had to forgo graduate students there. Aaron came to me with exactly what I wanted, which convinced me to go to Eugene (Oregon) to look at the situation. Once you set foot in Eugene, you never want to leave, it is a beautiful place, and it was just

the type of place I wanted to work. Furthermore, I could live in the forest, within bicycle distance of my laboratory, which was very attractive. I couldn't find anything wrong with the invitation, so I became his first appointment."

At the University of Oregon, Novick and Stahl established a revolutionary institute for advancing Molecular Biology and Genetics. Additionally, it was at Oregon where Stahl began turning his attention to a new phenomenon: recombination, the process by which homologous chromosomes exchange genetic information between one another through crossing over. Professor Stahl was particularly interested in uncovering the mechanisms behind this process:

"When you look at the chromosomes of a bean plant, when undergoing mitosis, they look like big, furry things; they don't look like straight highways, instead looking like a bundle of yarn that has been played with by a cat for some time. How could two homologs find each other with precision in that mess, and then break and rejoin properly so that nothing is lost, nor extra is produced? Even more, how do they swap these parts, it would be like swapping parts between two messed up balls of yarn by just mushing them together, that's what it looked like in the microscope? It must be that there was some underlying simplicity that was not apparent in the complexity of higher organisms, and I thought that underlying simplicity should be accessible in bacteriophages, because they are so much smaller, so they can't have big balls of yarn. They must be simple enough to get at the answer of how two chromosomes line up, very precisely and neatly, and make an exchange, so that's why I chose bacteriophage to study recombination in."

Stahl soon started also trying to move into higher order organisms, such as yeast, to further probe into the complexities introduced by these systems.

"There is some added stuff that complicates the issue, but the basic thinking of how the underlying skeleton, which are the DNA molecules and how they react to each other, are the same. In organisms like yeast, now you have to deal with higher order questions, which aren't manifested in the phage, with things such as crossover interference: where a crossover event at one gene can have an effect on the likelihood of a recombination event at a separate gene. Why some genes have interference, while others do not, are interesting questions which are not manifested in bacteriophage, so you have to go to yeast to answer those. Even working with yeast, I was not looking in a microscope, oh no, I was looking at mutants. The same as phage, I was pecking away at what was required for crossing over, and then relate what I saw to the double helix".

While conducting recombination research, Professor Stahl finally felt comfortable establishing a lab, a process that was greatly supported by Professor Aaron Novik, a "director who knew that his primary job was not his research, but instead supporting the young people that he had hired, which is the most important ingredient in a job as a youngster". Very soon, Stahl had opened his laboratory and recruited his first student:

"The first day I was in my laboratory, a lady came into my lab, approached me, and introduced herself and asked if she could work in my laboratory. The student was a fugitive from Poland, who had been through the starvation in 1945, and came to America. She was such a cracker jack student that they threw her out of high school, because she didn't need high school, and sent her right to the University of Utah, and she got her bachelor's degree there and got married. Her husband had a job in Oregon, in Chemistry, and she came with him. Before I had come to Oregon, she had been working with Geneticist, because genetics was what she liked, but he was a notorious bastard. He was very famous, but he was not a good guy, and she was the kind of person who made it clear when she thought something was wrong. So she had to leave his lab, and come to my lab. She didn't want a Ph.D., she just wanted to work, and that's extremely attractive. She doesn't need a project that is guaranteed to give her a thesis, she could try to go for very fundamental understanding in one big leap. Even if it didn't work, she will have learned a lot, and she can try another one. Then one day, after a year and a half, she came to me and said "my husband is leaving, and we are going to Iowa, in about 5 months, and I want a Ph.D." now that's a problem. So, I had to find her a Ph.D. question that she could take with her, so I gave her one. It was a question that Max Delbruck said that needed to be answered, or he would never believe that T4 phage had a circular linkage map, a linear chromosome, with circularly mutant sequence and terminal redundancy. The goal was to show all of this in one cross. What she needed was a fourth genetic marker, which she found, and with that she went off to Iowa. In 2 years, she had her thesis, she came back to Oregon and defended it, and got an Oregon Ph.D. She eventually came back to volunteer at the lab, she loved the work, and she was good at it."

At Oregon, Stahl had initially taught several advanced discussion groups, but later transitioned to teaching the introductory biology course.

"My job was to design an introductory biology course that was future looking. You can't teach this kind of Biology course to freshmen, because they don't know enough chemistry to know what you are talking about, so chemistry must be a prerequisite. The course starts with microbes, the genetics of eukaryotes, and then we get into evolution. First, the studies of evolution in microbes, and then moving rather cleanly into the analysis of evolution and speciation of the classical sort, as it is understood in higher organisms. After evolution, the course would go into physiology, and by then, they will have enough genetics and enough chemical thinking, that they can handle physiology not at a descriptional level, but instead at an analytical level. For the good students, that worked beautifully, but some students got blown out, because it wasn't what they had in mind when they signed up for a biology course. They wanted to study flowers or the cross-sections of root hairs, or something like that, so they disappeared. Soon the course gained the reputation of being a good hard course, where you had to work hard, but it was worth it. Once the

medical school had heard about it, they told students to take that course, or not apply to medical school here."

Stahl tried to promote the same level of analytical understanding in the undergraduates who came to work in his lab.

"When they came to work in my laboratory, they would work on a bacteriophage system, or yeast system later on. They would be initiated into the underlying logic of the experiment, and they were expected to understand them and pursue their research with that in mind. Often, they rose to this challenge and went on to go to good graduate schools. I never had more than 4 undergraduates in my lab, and we had group meetings with them. We tried to integrate them into the ongoing research, and they would pick up a piece of it, which they identified as their own. An interesting story is when I was close to retiring, I only had undergraduates in the lab, no graduate students, because the department was afraid that I would die and leave the graduate students halfway to a Ph.D. with nobody fit to guide them (similar to the position Stahl nearly found himself at Rochester). I had 4 fine undergraduates, working on different aspects of a question that was identified by that first Ph.D. student, who became a volunteer at our laboratory after returning from Iowa. She took on the yeast part of the laboratory, she was far brighter than I, and with yeast she had a far better intuition. She had taken on these undergraduates, but they were pretty independent. Once she kicked them in the right direction, they were collecting the data and trying to get enough data to have meaningful statistical analysis. In the middle of that, I decided that I was too tired to go on, and that I was closing the laboratory in June, leaving them just with 5 months warning. They looked a little panic stricken, and the undergraduates got together, decided which of the experiments which were philosophically related (bore on the same question) had to be trashed, and which ones they would complete with adequate data and analysis before the lab closed on June 1st. And that they did! That's exactly what they did, and they sort-of cut us out of it. They did it all themselves, and the truncated work was nevertheless enough to float 3 papers, which we wrote after we closed the lab. They had done such a fine job organizing and analyzing the data, so we related the data to each other in a coherent way, and we wrote group papers. When these kids came looking for letters for graduate school, it was great fun to write the letters. It works to take on undergraduates, as long as you give them a direction which is analytical and challenging enough, like a Sudoku, its DNA afterall, it can't be that complicated. I really liked how they cooperated, when they knew they didn't have time, they switched responsibilities. The woman who was good at tetrad analysis, became the tetrad dissector for all the projects, the guy who was good at other stuff took that stuff. It was just wonderful, I never felt better in my life, to see what undergraduates could do, once you give them a good direction and get the hell out of their way."

## Some Quick Questions:

**JUR:** What is your favorite Molecular Biology/Genetics Textbook?

**Stahl:** I haven't been keeping up with the new textbooks that are coming out, but of course I still like the first book that I wrote: The Mechanics of Inheritance, let me tell you a story about that. The book came out in 1953, and it taught Genetics starting with DNA, essentially bacteria and transformation, and then built up to Mendel. That was my Genetics book, and it was reviewed by Science, now that's quite a nice treat, to be reviewed by Science Magazine. So, I was flattered that they chose it, and they chose it as their Chief, first review, but the reviewer was a sharp guy. He had no sympathy, and he didn't like my style of writing, because it wasn't dignified. Now, I was writing for the students, I wasn't writing for a reviewer who thought himself so damn important. I thought it would kill the book, it didn't, but I was at Cold Spring a few days after the review came out. I was there with my family, down at the beach with my two little boys, and it was the end of the season, there was no one else on the beach. After a while, I saw a little figure walking down the beach, obviously walking toward me, and she was cutting a B-line. I recognized her as Barbara McClintock, with whom I was friendly, as I had met her a couple of times. As she was sitting, she said "Franklin, I know it must be hard, it must be hurting you, but you mustn't worry about it. In a few years people will have forgotten what a really bad book it was." After I caught my breath, I asked Barbara what she didn't like about it, "Franklin," she said, "you've taken all the mystery out of it." Well, the couple of times I went to Cold Spring, I tried to one up her: "Barbara, why did you write your papers in such a way so no one could understand them?" and she would respond "Alright, but I sure didn't write them for you to understand." Another time: "Barbara, why did you such and such?" and she asked "Franklin, will you never grow up?" Anyway, I still like that little book, it has to be added onto since we know so much more, but I think it takes the right approach to Genetics. Now, the trouble with up-to-date books is that they tend to get bigger and fatter. Soon the student drowns in them, too many pages of stuff; my book was small and got to the essentials.

*The Mechanisms of Inheritance* by Franklin Stahl, can be found at Carlson Library on River Campus.

**JUR:** What is your favorite animal as a pet?

**Stahl:** I love the llamas! We've had 4 llamas in our pasture, but now we are down to one llama because I am getting old, and the llamas get old and die. The one llama we do have is beautiful. She got untrained when one of my caregivers started feeding her when she was out of the barn. So, the caregiver is in the process of retraining her, to get her used to feeding in the barn. That way, when the veterinarian comes for the biannual checkup, we will be able to get her [the llama] in the barn for her shots and stuff. She's made great progress, on the first trip she wouldn't do it, but by the fourth or fifth trip she was comfortable and went right into the barn. They learn fast [just like undergraduates]. They are great pets, they are friendly,

they are sociable, and they don't spit at you unless you spit at them first.

**JUR:** What is your favorite model organism?

**Stahl:** Phage Lambda, it's beautiful. If lambda goes into a cell and there are lots of other lambdas, they immediately recognize that life outside is not good, and if they go outside the cell, it will be slim pickings, because the bacteria will all be dead by now. So, what do we do? Alright, we make a truce, and one of us (who is lucky) will integrate into the bacterial chromosome, and be carried in the bacterial genome, until we (lambda phage) change our mind. The lambda phage and bacteria enter a deal, as long as the bacteria keeps multiplying in a happy way, any other lambda phage enters the cell, the integrated phage lambda will paralyze it. It's basically a protection racket. If only one lambda finds itself in a bacterium, then it knows that there must be a lot of bacteria around, so it eats the bacteria, and gets on with the job of procreating and making 200 progeny. It does this until the bacterium is so scarce that it finds itself in the other situation, and they then hunker down. This first option, where lambda integrates into the genome, is the lysogenic phase, and the second option, where the virus gets to procreating, is the lytic cycle. Lambda phage is a temperate phage, which switches between the two. Now, there is also the option where the bacterium carrying the lysogenic-dormant lambda is in the sunlight for too long, and can no longer replicate efficiently. Now the deal is off, and lambda kills that bacterium, like a rat jumping off a sinking ship. Now, something that small has figured this out, through evolution of course, but it seems so smart that you have to study this phage.

**JUR:** If you were to restart your research career today, what would you study?

**Stahl:** I would first probably look at lambda, because lambda probably has a few tricks which it hasn't shown us yet. On the other hand, the competition is very steep in phage lambda, because a lot of people feel this way about it. We did accidently find something else about phage lambda, and the fact that we could just stumble onto this confirms that there must be more to lambda. It started when we looked at these very sick lambda mutants, which had a knockout of Rec (the bacterial recombination machinery) and gamma (an important repair factor). During replication one prong of the fork formed can break off, due to the complexity of moving the fork in the same topological direction, in addition to the size of the bacterial chromosome. The recombination machinery puts the fork back together by having gamma grab a very lethal Red recombinase. By doing this, gamma turns Red recombinase, which normally induces double-strand breaks, into a repair enzyme that fixes the fork using recombination. In the mutants, there was no gamma factor to "tame" Red recombinase, and no bacterial machinery to help gamma out, so the colonies did not grow so well. An undergraduate took the little titers they could get from the colonies and kept recycling them. Eventually, they started to grow, what they had done was select for a mutant in lambda which had allowed for the phage to be rescued from loss of gamma. There were 4 mutants that were found, all of which produced a specific sequence, and it was found that not only did that sequence tame Red recombinase, but also was found in the E. coli genome. Without looking in lambda, we would have never expected, or looked, for such a thing.

# Engineering Invasion: Do Canopy Gaps Created By The North American Beaver (*Castor canadensis*) Facilitate Terrestrial Plant Invasions?

**Carlina Velicer '26**
Advised by Dr. Mark Lesser, Center of Earth and Environmental Science, SUNY Plattsburgh, *Department of Ecology*

## Abstract

The North American beaver (*Castor canadensis*) is a keystone ecosystem engineer whose dam-building and foraging activities create or sustain riparian habitats. Beavers influence the competitive dynamics of the forest understory through tree felling, which creates canopy gaps with increased light availability. Although invasive vegetation is common in riparian ecosystems across the United States, it is unknown whether the canopy gaps created by beavers promote terrestrial plant invasions. Our objectives were to assess: (1) the extent to which beaver felling influenced light availability, (2) whether canopy gaps created by beaver felling facilitated the spread of non-native herbaceous plants and woody shrubs, and (3) which invasive species took advantage of these gaps. This study took place at the Huyck Preserve and Biological Research Station in east-central New York State. We surveyed five-meter radial plots around a total of 38 beaver-felled and unfelled control trees, measuring tree diameter, canopy openness, soil pH, and the percent abundance of invasive plant species present. Plots with beaver-felled trees had significantly higher light levels than unfelled control plots. The total percent abundance of invasive herbaceous plants and shrubs was significantly higher in beaver-felled plots than in controls. However, only three of the ten invasive species surveyed were drivers of this difference (*Celastrus orbiculatus, Lonicera spp.*, and *Berberis thunbergii*). These results reinforce the importance of light availability resulting from canopy disturbance, and facilitated by beaver activity, in enabling terrestrial plant invasions.

## Introduction

North American beavers (*Castor canadensis*) mold the riparian ecosystems they engineer and inhabit. Through selective foraging and the use of woody vegetation for dam and lodge creation, they can significantly impact the composition and structure of near-shore canopy and understory (Donkor & Fryxell, 1999; Rosell et al., 2005). Reduced tree density creates gaps that allow for greater light penetration through the forest canopy while decreasing competition for soil and nutrients (Barnes & Dibble, 1988; Johnston & Naiman, 1990).

Invasions of non-native plants have been demonstrated to increase in frequency and severity in the wake of canopy disturbance (Belote et al., 2008; Lee & Thompson, 2012). In Pennsylvania and New Jersey, Eschtruth and Battles (2009) found that canopy disturbances resulting from hemlock woolly adelgid (*Adelges tsugae*) infestation and propagule pressure were associated with intensified invasions of garlic mustard (*Alliaria petiolata*), Japanese barberry (*Berberis thunbergii*), and Japanese stiltgrass (*Microstegium vimineum*). Gaps created by logging in Hawaiian forests resulted in greater availability of light and nitrogen that promoted the spread of invasive plants (Loh et al., 2008). In California's redwood forest, increased light availability was also a vital resource in plant species invasions, as unshaded plots had a greater proportion of invasive species than nearby shaded plots (Blair et al., 2010).

Although beavers are considered generalist herbivores, their preference for more palatable woody plant species, such as willows and poplars, has earned them recognition as choosy, opportunistic foragers (Haarberg & Rosell, 2006; Vorel et al, 2015). A well-documented effect of selective and choosy herbivory is the proliferation of non-preferred vegetation, as herbivores with a stronger preference for native species over non-native species can encourage invasions (Augustine & McNaughton, 1998; Keane & Crawley, 2002). Previous studies have demonstrated a relationship between beaver foraging preferences and invasions of non-native trees and shrubs. In Hungary, the Eurasian beaver (*Castor fiber*) may expedite the shift in canopy composition toward non-native hardwood shrubs through its preference for softwood species (Juhasz et al., 2022). Beavers in Ohio may aid amur honeysuckle (*Lonicera maackii*) invasion by selectively felling its competition (Deardorff & Gorchov, 2020). Invasive tamarisk trees (*Tamarix ramosissima*) thrive where beavers are abundant in the Grand Canyon National Park (Mortenson et al., 2008).

While invasive vegetation is common in the riparian ecosystems of the northeastern United States, whether the canopy gaps created by beavers promote invasions of non-native herbaceous plants and shrubs has yet to be thoroughly investigated. In this study, we examined the influence of beavers on facilitating invasive colonization and spread at the Huyck Preserve and Biological Research Station in New York. Lincoln Pond, situated within the Preserve, has a long history of beaver activity, making it an ideal location for this study (Muller-Schwarze et. al, 1983; Tevis 1949; Tevis, 1950).

Our objectives were to determine: (1) the extent to which beaver felling influenced light availability, (2) whether canopy gaps created by beaver felling facilitated the spread of non-

native herbaceous plants and woody shrubs, and (3) which invasive species took advantage of these gaps. To investigate these questions, we surveyed plots centered around beaver-felled trees and unfelled controls to characterize canopy openness, soil pH, and percent abundance of invasive species. Since beaver-felled gaps increase light availability—potentially creating conditions conducive to the spread of non-native species—we hypothesized that beaver herbivory promotes the spread of invasive herbaceous plants and shrubs at the Preserve.

## Methods

*Study Site*

This study took place at the Huyck Preserve and Biological Research Station in east-central New York State (Fig. 1a). Beavers are well-established on Lincoln Pond, with evidence of their presence first being recorded by Eugene P. Odum in 1939 (Tevis, 1950). By 1947, two beaver lodges were observed nested along the banks of the 4 hectare-pond (Odum, 1939; Tevis, 1950). Since then, a third has been established. Over this timeframe, beavers have had a marked effect on the landscape. In a preliminary survey of beaver activity surrounding the pond, 835 trees with evidence of beaver interaction (felling or gnawing) were identified (Fig. A1, Campbell & Velicer, 2024).

The north and east sides of Lincoln Pond are dominated by old-growth eastern hemlock (*Tsuga canadensis*). Marshes and alder (*Alnus incana*) thickets cling to the north, west, and, to a lesser extent, east shorelines. The southwest shore is characterized by a red pine plantation dating back to 1928 (Tevis, 1950) that is now overgrown with mixed deciduous hardwood, namely sugar maple (*Acer saccharum*), American hornbeam (*Carpinus caroliniana*), American hophornbeam (*Ostrya virginiana*), and white ash (*Fraxinus americana*).

Invasive species are actively managed on the Preserve, through the use of a variety of techniques from manual cutting to spot-torching. Fieldwork for this study was conducted in the summer of 2024 before the annual management of invasive herbaceous plants and shrubs began around the pond area.

*Field Sampling*

Five-meter radial plots were surveyed around a total of 38 selected trees—19 felled and 19 unfelled control trees (Fig. 1b). Felled tree plots were selected based on the initial assessment of beaver activity around Lincoln Pond (Fig. A1, Campbell & Velicer, 2024). Only woody stems greater than 15 cm were considered, as felled trees of that size created a canopy gap large enough to alter the understory light environment. Plots were selected to avoid overlaps with a hiking trail surrounding the pond to minimize the effects of increased light availability from the path. Due to the high levels of beaver activity immediately surrounding the pond, randomly selected unfelled controls were, on average, farther from the pond than felled trees, but still within the riparian zone.

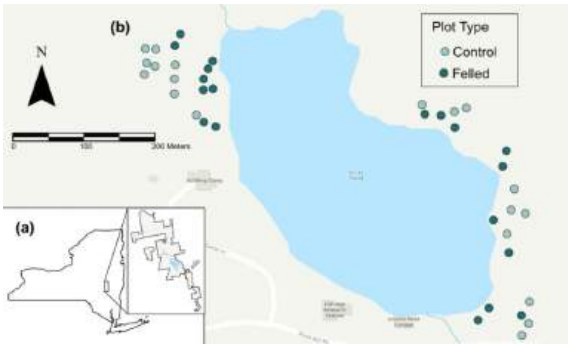


**Figure 1.** (a) Location of the Huyck Preserve and Biological Research Station in east-central New York State. (b) Location of surveyed plots (n=38) around Lincoln Pond, summer 2024 (plot symbols not to scale).

Within each plot, we estimated the percent abundance of all invasive terrestrial herbaceous plants and shrubs. Invasive trees were not present at the study location, and therefore not included in the study design. While observed in some plots, aquatic invasive species were not considered in this analysis. The abundance and growth form of some non-native species, particularly the Asiatic bittersweet (*Celastrus orbiculatus*), rendered counts impractical. Tree diameters were measured 20 cm from the ground, which is below beaver felling height (Belovsky, 1984; Janiszewski, 2017). We used Avenza Maps version 5.3.3 (243.1) to record tree location and a spherical densiometer to measure canopy openness. Soil samples were taken at the base of each tree, and their pH was measured with a HANNA H198107 pH meter in the lab. This controlled for differences in soil pH between plot types that may have accounted for observed differences in invasive species abundance.

*Statistical Analysis*

Analysis of Variance (ANOVA) was performed in R version 4.3.2 (R Core Team, 2023) to test whether the mean percent abundance of invasive plants varied according to soil pH or canopy openness. Student's t-tests were conducted to determine whether the total percent abundance of invasive species, soil pH, and canopy openness were significantly different between felled and control plots. A simple linear regression model was used to assess how well canopy openness predicted the total percent abundance of invasive species plants across all plots.

## Results

Plots centered around a total of eight different tree species were surveyed (Table 1). Two felled trees could not be identi-

fied due to significant rotting. The vast majority of felled plots were centered around deciduous trees, with eastern hemlock encompassing only ~11% of felled trees surveyed. In contrast, ~68% of control trees were eastern hemlock (Table 1). This difference can be attributed to diet preferences, as beavers generally favor more easily digestible deciduous trees over conifers (Fryxell & Doucet, 1993; Gallant et al., 2004). The study site's high levels of beaver activity, undoubtedly a consequence of prolonged beaver residency at Lincoln Pond, rendered very few large deciduous trees left standing within the riparian zone that could serve as controls.

There were no significant interactions between plot type, soil pH, and canopy cover. However, canopy cover was highly correlated with plot type (Table 2, Fig. A2). Plots with beaver-felled trees exhibited gaps in the forest canopy, resulting in significantly higher light levels than unfelled control plots (~74% versus ~91%, respectively; Fig. 2). The total percent abundance of invasive species decreased as canopy cover increased (Fig. 3), with canopy cover explaining ~41% of the variability in the total percent abundance of invasive species ($p < 0.001$; Table 3).

| Plot Type | Species | Common Name | Species Code | Relative Abundance (%) | Average Diameter (cm) |
|---|---|---|---|---|---|
| Felled | *Acer saccharum* | Sugar maple | ACSA | 5.26 | 29.60 |
| | *Betula alleghaniensis* | Yellow birch | BEAL | 10.53 | 23.85 |
| | *Carpinus caroliniana* | American hornbeam | CACA | 5.26 | 17.00 |
| | *Ostrya virginiana* | American hophornbeam | OSVI | 31.58 | 25.79 |
| | *Tilia americana* | Basswood | TIAM | 21.05 | 42.93 |
| | *Tsuga canadensis* | Eastern hemlock | TSCA | 10.53 | 26.00 |
| | *Ulmus rubra* | Slippery elm | ULRU | 5.26 | 23.50 |
| | Unknown | N/A | N/A | 10.53 | 37.00 |
| Control | *Acer saccharum* | Sugar maple | ACSA | 5.26 | 20.85 |
| | *Ostrya virginiana* | American hophornbeam | OSVI | 5.26 | 20.30 |
| | *Quercus rubra* | Red oak | QURU | 21.05 | 46.28 |
| | *Tsuga canadensis* | Eastern hemlock | TSCA | 68.42 | 28.23 |

**Table 1.** Average relative abundance and size of tree species found in the felled (n=19) and control plots (n=19).

| Variable | Sum of Squares | df | Mean Square | F value | Pr (>F) |
|---|---|---|---|---|---|
| Plot type | 5338 | 1 | 5338 | 32.352 | < 0.001*** |
| Soil pH | 361 | 1 | 361 | 2.118 | 0.150 |
| Canopy cover | 1387 | 1 | 1387 | 8.403 | < 0.01** |
| Plot type x Soil pH | 177 | 1 | 177 | 1.072 | 0.309 |
| Plot type x Canopy cover | 57 | 1 | 57 | 0.345 | 0.561 |
| Soil pH x Canopy cover | 459 | 1 | 459 | 2.779 | 0.106 |
| Plot type x Soil pH x Canopy cover | 6 | 1 | 6 | 0.037 | 0.848 |
| Residuals | 4950 | 30 | 165 | | |

** $p < 0.01$, *** $p < 0.001$

**Table 2.** Percent abundance of invasive plants as a function of soil pH and canopy cover, tested using an ANOVA.

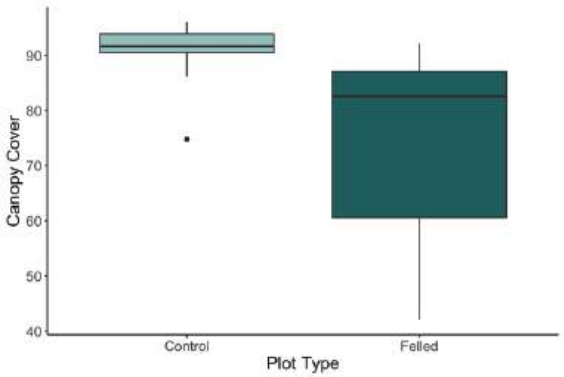


**Figure 2.** A boxplot of canopy cover (%) between control and felled plots ($p < 0.001$). Thick black line indicates the median value. Boxes represent the 1st to 3rd quartile range. Whiskers extend to the most extreme values within 1.5 times the interquartile range. Outliers are represented by black dots.

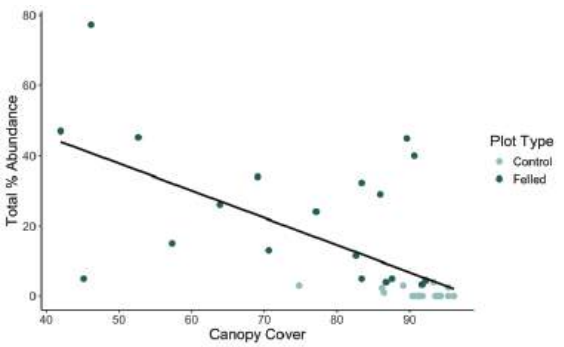


**Figure 3.** A scatterplot of canopy closure as a predictor of total percent abundance of invasive species plants across all plots. The line represents the fitted values from the linear regression.

| Variable | Estimate | Standard Error | p-value |
|---|---|---|---|
| Intercept | 76.5951 | 12.8657 | < 0.001*** |
| Canopy cover | -0.7759 | 12.8657 | < 0.001*** |

*** $p < 0.001$

**Table 3.** Estimated regression parameters, standard errors, and p-values for the simple linear regression model (R Square = 0.4148).

The total percent abundance of invasive herbaceous plants and shrubs was significantly higher and more variable in beaver-felled plots than in controls ($p < 0.001$; Fig. 4, Table 4). However, of the ten invasive species identified, only Asiatic bittersweet, honeysuckles (*Lonicera spp.)*, and Japanese barberry were significantly more prevalent in felled plots than in control plots (Figure 5; Table 5). Thus, only a small portion of the invasive species surveyed are drivers of the difference in invasive species prevalence by plot type.

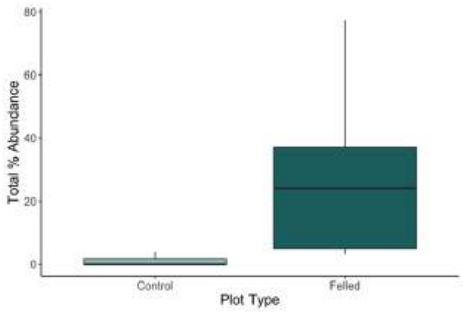


**Figure 4.** A boxplot of total percent abundance of invasive herbaceous plants and shrubs between control and felled plots ($p < 0.001$). The thick black line indicates the median value. Boxes represent the 1st to 3rd quartile range. Whiskers extend to the most extreme values within 1.5 times the interquartile range. Outliers are represented by black dots.

| T-test | t-value | p-value |
|---|---|---|
| Total % abundance | -5.097 | < 0.001*** |
| Canopy cover | 4.267 | < 0.001*** |
| Soil pH | -2.253 | 0.031* |

* p < 0.05, *** p < 0.001

**Table 4.** T-test for differences in total percent abundance of invasive species, canopy openness, and soil pH between felled and control plots.

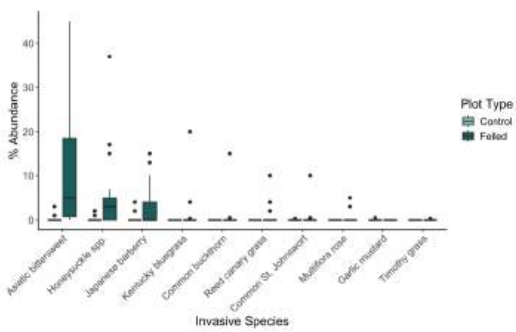


**Figure 5.** A boxplot of percent abundance of observed invasive herbaceous plant and shrub species between control and felled plots ($p < 0.001$). A thick black line indicates the median value. Boxes represent the 1st to 3rd quartile range. Whiskers extend to the most extreme values within 1.5 times the interquartile range. Outliers are represented by black dots.

| Species | Common Name | t-value | p-value |
|---|---|---|---|
| *Celastrus orbiculatus* | Asiatic bittersweet | -3.538 | 0.002** |
| *Lonicera spp.*[a] | Honeysuckle spp. | -2.643 | 0.016* |
| *Berberis thunbergii* | Japanese barberry | -2.236 | 0.037* |
| *Poa pratensis* | Kentucky bluegrass | -1.205 | 0.244 |
| *Rhamnus cathartica* | Common buckthorn | -1.035 | 0.315 |
| *Phalaris arundinacea* | Reed canary grass | -1.509 | 0.149 |
| *Hypericum perforatum* | Common St. Johnswort | -1.084 | 0.293 |
| *Rosa multiflora* | Multiflora rose | -1.407 | 0.177 |
| *Alliaria petiolata* | Garlic mustard | 1.000 | 0.331 |
| *Phleum pratense* | Timothy grass | -1.000 | 0.331 |

[a]There are potentially up to three species of invasive honeysuckle (*Lonicera morrowii*, *Lonicera tatarica*, and *Lonicera xylosteum*) located at the study site which were not able to be practically distinguished in the field.
* p < 0.05, ** p < 0.01

**Table 5.** Student's t-tests comparing the mean percent abundance of each invasive herbaceous plant and shrub species in felled versus control plots.

## Discussion

Beaver-created canopy gaps exhibited significantly increased understory light availability, a finding consistent with previous observations in the midwestern United States (Barnes & Dibble, 1988; Johnston & Naiman, 1990). As predicted, higher light levels, resulting from beaver felling, were associated with a greater prevalence of invasive herbaceous plants and woody shrubs. This observed correlation between canopy openness and invasive species abundance reinforces light as a crucial resource for invasive species proliferation (Blair et al., 2010). Other studies have also highlighted the association between increased light availability due to canopy disturbance and the spread of invasive plants (Belote et al., 2008; Eschtruth & Battles, 2009; Lee & Thompson, 2012).

The dominance of Asiatic bittersweet, honeysuckles, and Japanese barberry in driving the observed differences in total invasive species abundance between plot types suggests that not all invasive plants are equally skilled in exploiting the gaps created by beavers. The success of the most prevalent invader in felled plots, Asiatic bittersweet, is likely bolstered by its rapid seed production, high germination rates, and rapid growth in high-light environments (Ellsworth, 2003). The hardy invader may endure years in heavily shaded understory before rapidly colonizing canopy gaps created by disturbance—likely giving it an advantage over other invaders such as common St. Johnswort (*Hypericum perforatum*), whose seedlings are small and slow-growing, or even common buckthorn (*Rhamnus cathartica*), whose growth rate is considered medium to fast (Campbell, 1985; Dirr, 1998; Patterson, 1974).

The honeysuckles found on the Preserve are also fast-growing and tolerant of a wide range of light conditions. Their seeds are readily dispersed by white-tailed deer populations, and, like the seeds of Asiatic bittersweet and Japanese barberry, by frugivorous birds. Morrow's honeysuckle (*Lonicera morrowii*), one of the Preserve's invasive honeysuckle species, can germinate beneath 2 inches of leaf litter (Hidayati, 2000). This ability may provide it the edge needed to invade dense

hemlock forest, where thick leaf litter typically inhibits colonization by most herbaceous plants and shrubs. While not a particularly fast grower, Japanese barberry has been observed exploiting canopy gaps in hemlock forest, and its seedling survival and growth rate is also positively correlated with light level (D'Appollonio, 2006; Eschtruth & Battles, 2009; Silander, 1999).

In contrast to the more successful invaders, garlic mustard is most competitive in well-shaded understory (Dhillion, 1999), and was only observed in a single control plot in this study. Reed canary grass (*Phalaris arundinacea*) and timothy grass (*Phleum pratense*) were restricted to plots on a thin strip of wetland sandwiched between the pond's edge and the beginning of hemlock forest. Thus, the non-significant increase in the percent abundance of these grasses in felled plots may be influenced by the slight difference in distance from the pond between felled and control plots.

The lack of significant interactions between soil pH and invasive species abundance further underscores the dominant role of light availability in facilitating non-native plant invasions. Soil pH was measured to account for any possible confounding effects of soil chemistry on invasive colonization across plot types. Although, it is possible that the increased presence of invasive plants may also be a contributing factor to the higher soil pH levels observed in felled plots. Asiatic bittersweet and Morrow's honeysuckle have been observed to significantly increase soil pH (Hicks, 2004).

While beavers are typically recognized as wetland engineers with positive impacts on biodiversity and are often introduced to habitats as part of ecological restoration efforts (Law et al., 2019; Smith & Mather, 2013; Stringer & Gaywood, 2016), this research sheds light on an understudied aspect of their ecological impact: their potential to aid terrestrial plant invasions. Common invaders in the northeastern United States, such as Asiatic bittersweet and Japanese barberry, are known to monopolize resources and reduce biodiversity by out-competing native vegetation (Delisle & Parshall, 2018; Dibble & Rees, 2005; Ellsworth, 2003; McNab and Meeker, 1987).

The findings of this study may be context-dependent based on forest type and the legacy of invasive plants in the area. More studies, particularly across larger sites without annual invasive plant management, are needed to gain a more comprehensive understanding of the possible long-term impacts of beaver felling on plant invasions and implications for invasive plant management at a broader scale. Investigating the relationship between near-shore tree felling and aquatic invasive species abundance also warrants further consideration. Beavers' influence on invasive plant spread may be important to consider in wetland restoration and management.

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## Appendix

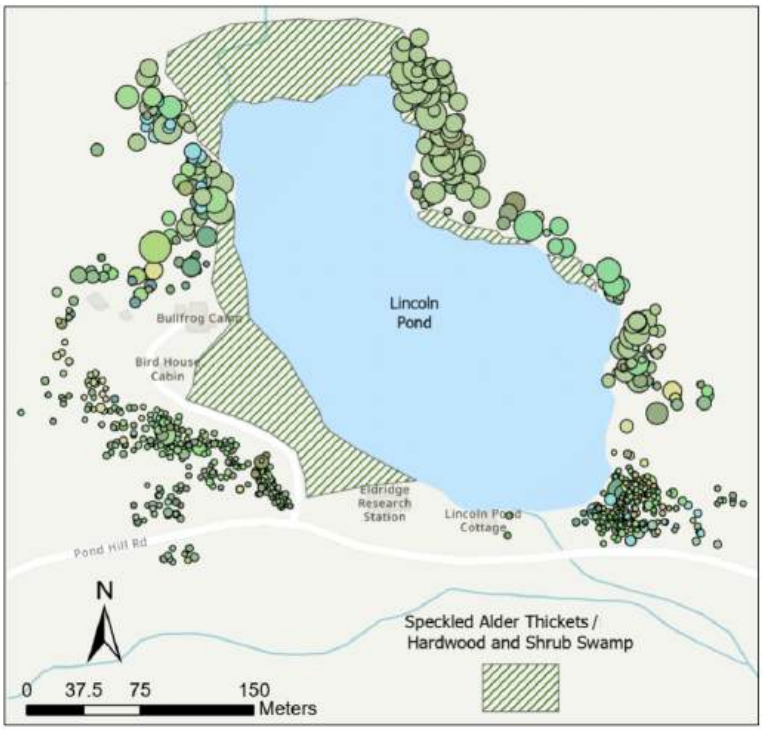


**Figure A1.** Map of trees fully felled (n=627) and gnawed (n=208) by beavers surrounding Lincoln Pond, summer 2024. Dot color corresponds to tree species (n=22) and dot size corresponds to tree size (Campbell & Velicer, 2024).

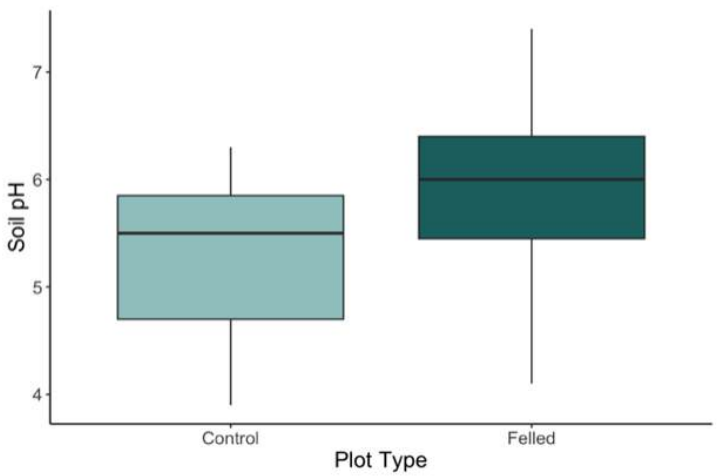


**Figure A2.** Boxplot of soil pH between control and felled plots ($p < 0.05$). Thick black line indicates the median value. Boxes represent the 1st to 3rd quartile range. Whiskers extend to the most extreme values within 1.5 times the interquartile range. Outliers represented by black dots.

# Investigating the Relationship of LINE-1 Expression and SIRT6 Rescue Across Rodent Species

**Natasha Sieczkiewicz '26**
Advised by John Martinez, Dr. Vera Gorbunova, and Dr. Andrei Seluanov,
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## Abstract

SIRT6, a sirtuin protein, regulates various cellular processes including aging, DNA repair, metabolism, and inflammation (Tian et al., 2019). SIRT6 expression is intricately linked to the expression of transposable element LINE-1 (L1), which constitutes a substantial portion of the genomic DNA in mice and humans. L1 activity in somatic tissues has been implicated in various age-related diseases, including neurodegeneration and cancer (Simon et al., 2019). SIRT6 regulates L1 expression by engaging the KAP1 protein, which facilitates packaging of L1 in heterochromatin, thereby preventing its expression. Mice deficient in SIRT6 exhibit elevated levels of L1 expression, leading to shortened lifespan and chronic inflammation. Comparative analysis of SIRT6 and L1 expression across rodent species with varying lifespans revealed a correlation between SIRT6 rescue efficiency and maximum lifespan. Our investigation reveals that in longer-lived rodent species, SIRT6 may have evolved to efficiently repair double-strand breaks, underscoring its significance in longevity research. Longer-lived species exhibit lower L1 expression, indicating the effectiveness of SIRT6-mediated repression Immunofluorescence staining further corroborated these findings, highlighting differences in SIRT6 rescue ability and L1 expression among rodent species. Our findings highlight the importance of understanding species-specific variations in SIRT6-mediated L1 repression. For non-rodent species, further investigation is needed to expand our findings. Additionally, further conducting additional immunofluorescence analyses will be needed to validate these conclusions.

## 1. Introduction

This experiment builds off of previous research investigating DNA double-strand break repair in rodent species by the protein SIRT6 (Tian et al., 2019). This study determined that in longer lived rodent species, SIRT6 has been optimized to repair double strand breaks, indicating its potential importance in the study of longevity. SIRT6 is a type of protein known as a sirtuin, which plays a role in regulating cellular processes such as aging, DNA repair, metabolism, and inflammation (Guo et al., 2022). SIRT6 acts as a deacetylase, mono-ADP-ribosyltransferase, and long fatty deacylase, engaging in diverse cellular signaling pathways ranging from the initial stages of DNA damage repair to disease advancement (Guo et al., 2022). These properties of SIRT6 cause DNA wrapped around histones to tighten, leading to more heterochromatin regions of the genome. SIRT6 also has anti-inflammatory properties, as it can suppress cytokines, which are small signaling molecules that help to regulate immune responses (Liu et al., 2020). SIRT6 helps to mitigate inflammation and maintain immune homeostasis, thereby contributing to healthy aging (Zhang et al., 2020). To understand the effects of SIRT6 on aging in rodent species, the aging of knockout SIRT6 mice can be monitored.

SIRT6 expression is directly linked to the expression of transposable element LINE-1 (L1) (Simon et al., 2019). In mammalian genes, the presence of this element is extensive, comprising approximately 20% of the genomic DNA in mice and humans (Lander et al., 2001). Because of the ability of transposable elements to cut and paste within a host's genome, L1 activity causes breakages in DNA, which results in serious consequences for the host. While previous research on L1s has focused on their activity in the germ line, recent evidence has suggested that L1 activity in somatic tissues contributes to a number of age-related diseases, such as neurodegeneration and cancer (Simon et al., 2019). Overexpression of L1 is linked to inflammation, so eukaryotic cells have adapted ways to prevent it. Cells commonly use the SIRT6 protein to regulate expression of L1: it functions by employing the KAP1 protein, which contributes to the packaging of LINE1 in heterochromatin and thus preventing expression (Van Meter et al., 2014). Previous studies have found that mice deficient in the SIRT6 protein have higher levels of L1 expression, leading to shortened lifespan and chronic inflammation (Simon et al., 2019).

SIRT6 and L1 expression studies also highlight the differences in expression between longer lived and shorter lived rodent species. Rodents have drastically different life spans that are not specifically correlated to size, for example, Rats can live up to 4 years, whereas Naked Mole Rats can live for up to 30 (AnAge: The Animal Ageing and Longevity Database, n.d.). The considerable difference between these two maximum lifespans makes rodent species good candidates for cross-species experiments. The similarity in rodent genomes also allows for successful plasmid incorporation from different species. This experiment tested the success of the rescue of SIRT6 plasmid by using multiple species of rodents' SIRT6. These plasmids were created using the same endogenous CMV promoter, because it induces very strong expression SIRT6 rescue functions by incorporating a SIRT6 plasmid into knockout mouse embryonic fibroblasts (MEFs) with the use of lipofectamine reagent. The goal of this experiment was to measure the change in SIRT6 production in these MEFs after transfection by different rodent species plasmids with varying

lifespans. By transfecting these knockout MEFs with a plasmid vector, they can successfully uptake the new species SIRT6 plasmid into the host cell genome, where it can be expressed and therefore "rescue" SIRT6 protein production.

By using multiple species for this experiment, conserved biological mechanisms of the L1 repression can be better understood for its effect on lifespan. Using a cross-species study also highlights the differences in an animal's ability to rescue the SIRT6 protein when introduced as a plasmid. Differences in maximum lifespan are supported by a species' ability to rescue SIRT6, linking aging to the expression of this protein. Comparing data of SIRT6 knockout DNA from multiple species supports prior research on the direct relation between a species' ability to produce or rescue SIRT6 and their maximum lifespan. Furthermore, investigation of the relationship between SIRT6 and maximum lifespan of multiple species can allow the understanding of aging to be better translated to humans.

## 2. Methods

*Cell Passaging*

This investigation utilized cell culture techniques to cultivate and maintain cells of multiple species. The mouse embryonic fibroblasts, MEFs, were plated on two separate 10 cm plates, one wild type (WT) and one SIRT6 knockout, using Dulbecco's Modified Eagle Medium (DMEM) These MEF lines were used through the length of this experiment. First, the DMEM was aspirated out, and the cells were washed with PBS. Then, cells were trypsinized, and placed in an incubator for 3-5 minutes. When the majority of cells were no longer confluent, the trypsin was inactivated with (DMEM) and the cells were collected and spun down for 5 minutes to create a pellet. The supernatant was aspirated off and the pellet was resuspended in 1 mL of DMEM. The pellet was then broken and a cell count was recorded.

*Transfections*

Over the course of this experiment, SIRT6 MEFs were passaged and plated onto 6-well plates and multiple IF slides. They were then transfected with the following species' SIRT6 plasmid, one per well: Hampster, Deer Mouse, Mouse Porcupine, Beaver, Naked Mole Rat, Red Squirrel, Paca, Capybara, Gerbil, Guinea Pig, Rat, and Human. The Mouse MEFs were transfected with Lipofectamine and Opti-Mem. First, Tube A was prepared using a 3:100 ratio of Lipofectamine and Opti-Mem. A Tube B was prepared for each of the plasmids with the following: 250 uL of Opti-Mem, 5 uG plasmid DNA, and 5µL of P3000 reagent. Tube A was distributed evenly to each Tube B and left to incubate for 10 minutes at room temperature, after which 250uL of each tube B was added to each well.

*RNA Isolation*

The cells collected in transfection then underwent further RNA isolation and purification techniques. MEF cells were collected from plates using cell passaging methods, and centrifuged to form a pellet. The supernatant was poured off, and the pellet was left to air dry. The RNA of each plasmid was isolated and purified with the Addgene kit.This process included washing the pellet with multiple buffers and spinning them down to first isolate RNA. After this series of washes, the RNA was eluted using a spin column, it was collected and the concentration determined, and the samples were stored at -20℃.

*cDNA synthesis for RT-qPCR*

The isolated RNAwas then used for Superscript III cDNA synthesis. For each species, 5µg of the isolated RNA was mixed with 1µL of dNTPS and 1µL of OligodT primer in a labeled PCR tube. This was briefly spun down and incubated at room temperature for 5 minutes. Separately, 2µL of each DTT and 10x Buffer were mixed with 4µL MgCl2. Then, 1µL of the enzymes RNaseOUT and Superscript III were added. The second mixture was added to each of the tubes with RNA and mixed. The tubes were incubated in the PCR block at 50℃ for 50 minutes, then 85℃ for five minutes, and finally 12℃ for five minutes. An aliquot of RNaseH was diluted with ddH2O in a 1:1 ratio; 2µl of this was added to each sample, and PCR tubes were incubated for 30 minutes at 37℃. The samples were diluted as needed, using ddH2O to achieve qPCR dilutions. The samples were stored at -20℃ until they were analyzed using RT-qPCR.

*Immunofluorescence Staining*

The IF slides for each species were then fixed and stained. Samples were aspirated and then washed twice with cold PBS. The slides were fixed with 3.7% PFA (4℃) and placed in an incubator (37℃, 5% CO2) for 10 minutes. The slides were then aspirated and washed 3 times with PBS. Then, 0.5% Triton X-100 was added and each slide was left on the shaker at room temperature for 10 minutes. Slides were washed 4 times with PBS for 10 minutes on a shaker. 2 mL of 100% ice-cold methanol was added to each slide and placed in a -20℃ freezer overnight. The slides were washed with PBS, and a blocker (10% FBS and 5% BSA in PBS) was added. Subsequently, the slides were placed on the shaker for 1 hour. The primary antibody was added and left to shake at 4℃ overnight. Next, the cells were washed with 0.1% Triton X-100 4 times at room temperature on a shaker for 10 min each.

The secondary antibody (1:1000 in the blocker) was added, and left to shake for one hour at room temperature,covered. The cells were washed with PBS for 10 minutes on a shaker at room temperature 4 times. The PBS was first removed, followed by the chambers from the slides. After drying, 30 µl of DAPI mounting medium was added to each chamber and a cover slip was placed on top for storage at -80℃.

## 3. Results and Discussion

This experiment further validated data gathered from previous experiments; SIRT6 expression decreases LINE1 expression in mouse MEFs. These experiments also further validated that

SIRT6, the longevity regulating protein, is a powerful repressor of L1 activity (Van Meter et al., 2014). The qPCR results, though not significant, suggest that longer lived species, on average, have lower relative L1 expression (Figure 1.1).

While there are some outliers, such as the Rat, a short lived species, the general pattern of Figure 1.1 supports the conclusion that the longer a species' maximum lifespan is, the lower the L1 expression. This experiment validated this throughout multiple rodent species, as well as confirmed the relationship between protein SIRT6 and L1 expression. SIRT6 attaches to the 5′-UTR of L1 loci, where it performs mono-ADP ribosylation on the nuclear corepressor protein, KAP1 (Van Meter et al., 2014). This modification aids in KAP1's interaction with the heterochromatin factor, thereby assisting in the formation of repressed heterochromatin around L1 elements (Van Meter et al., 2014). Decreased L1 expression is due to the increase of repressed heterochromatin in the regions surrounding these L1 elements, which occurs when SIRT6 is expressed at a higher level. Short lived species have significantly higher L1 expression ($p = 0.0368$) than longer lived species (Figure 1.2), which is likely due to the ability of MEF's to rescue SIRT6 from other species. This further supports the conclusion that longer lived species are more effective at repressing L1 transposons through production of SIRT6.

This is further supported by the immunofluorescence staining of rescue SIRT6 MEFs, from prior experimentation (Figure 2.1) and this experiment (Figure 2.2). In both figures, the shorter lived rodent species had higher hybrid presence, indicating L1 expression in these MEFs (John Martinzed, unpublished). Additionally, both shorter lived species SIRT6, the Hamster and Mouse, were not rescued as successfully by the MEFs. These stains were compared to a negative control, where no SIRT6 plasmid was incorporated.

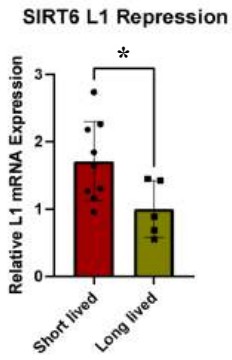


**Figure 1.2** Condensed results from qPCR run. Each black dot represents a different species' S6 plasmid, and trends grouped together in larger boxes. Relative L1 mRNA expression vs longevity of species measured. Standard error bars included. Lower levels of expression indicate higher levels of SIRT6 L1 repression. P value is 0.0368, indicating significance and rejecting null hypothesis.

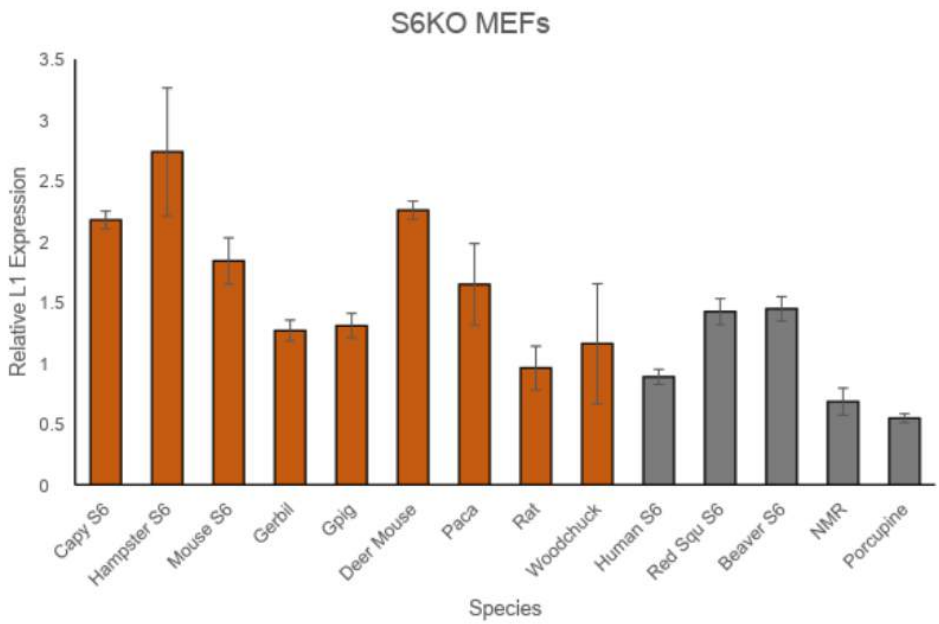


**Figure 1.1** Results of qPCR on multiple species S6 plasmid incorporated into SIRT6 knockout MEF's. Longer lived species are gray, and shorter lived species are rust color. NMR refers to the naked mole rat. The graph shows relative L1 expression once successful transfection of new species SIRT6 plasmid. Standard error bars included. Highest expression is in Hamster at around 2.75. Lowest expression is in Porcupine at around 0.6.


**Figure 1.3** Images, common names, *latin names*, and maximum lifespans of plasmids of each species used. Four longer lived species: Beaver, Porcupine, Red Squirrel, and Naked Mole Rat. 8 shorter lived species above: Mouse, Hamster, Gerbil, Rat, Guinea Pig, Paca, Capybara, Woodchuck. Images shown for relative size and appearance of each species. Longest maximum lifespan is the Naked Mole Rat, and shortest maximum lifespan is in the Hamster (AnAge: The Animal Ageing and Longevity Database, n.d.)..

Both the Hamster and Mouse show similarities to the negative control; there is a significantly higher presence of hybrid proteins compared to longer lived species (Figure 2.1), indicating that SIRT6 was not successfully rescued, and L1 expression occurred at relatively the same rate as the negative control. In both longer lived species, the rescue of SIRT6 was higher (Figure 2.1 & 2.2), and the hybrid protein levels were lower. Additionally, both Beaver and Porcupine SIRT6 were more present in the images, suggesting a successful rescue. These results indicate that something about short lived species' SIRT6 proteins are less effective at L1 repression than longer lived species' SIRT6 proteins.

It is important to understand the differences in the ability of SIRT6 to repress L1 expression across species and how this could be correlated to lifespan. DNA damage is a cause of aging, and organisms that are able to better repair this damage are likely to live longer. The differences can be attributed to variations in the structure, expression levels, and enzymaticactivity of SIRT6 across different species. Additionally, genetic variations or mutations in the SIRT6 gene among different species may result in alterations in its ability to bind to L1 loci or modify associated proteins, ultimately influencing the efficacy of L1 repression (Simon et al., 2019). Furthermore, differences in the regulatory mechanisms or interacting partners of SIRT6 in different species could also contribute to variations in L1 repression (Simon et al., 2019). Based on previous research and supported by this experiment, it can be confirmed that longer-lived species have more effective and

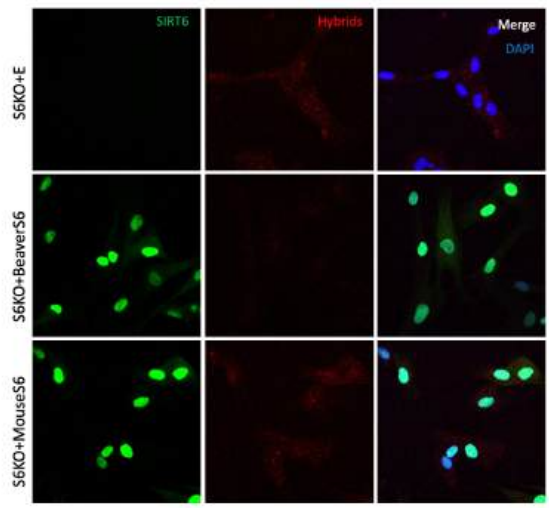


**Figure 2.1** Previous experimental Immunofluorescence of mouse and beaver SIRT6 in S6 knockout mice. Beaver SIRT6 is more hyperphosphorylated than mouse and is better at repressing L1s IF for SIRT6, DNA/RNA hybrids, and DAPI in human fibroblasts knocked out for SIRT6. Top row is stably-transfected with either empty vector, or mouse SIRT6, shown by prevalence of staining. Mouse knockout shows less present SIRT6 (green) and prevalence of hybrid. (John Martinzed, unpublished)

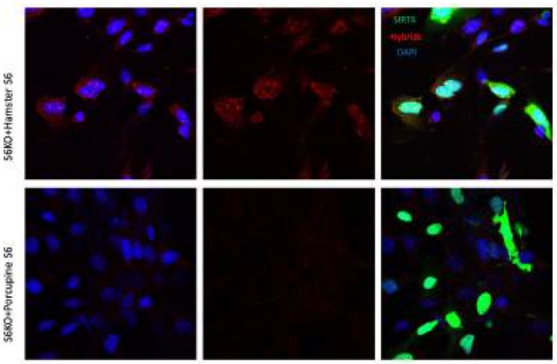


**Figure 2.2** New experimental data with a new long and short lived species. Immunofluorescence of Porcupine S6 vs Hamster S6. Staining shows protein DAPI (blue) and SIRT6 transfection (green), and hybrid RNA present after S6 transfection. S6KO Hamster MEFs depict higher presence of hybrids compared to the S6KO Porcupine. Porcupine also shows higher prevalence of SIRT6 protein (green) than the Hamster. Immunofluorescence of other species was not obtained nor published.

abundant SIRT6 proteins present in their cells. This is likely an evolutionary adaptation; mammals with longer maximum life spans have better adapted SIRT6 expression to decrease L1 expression to reduce chronic inflammation and DNA damage, and therefore are able to live longer. Future work will consist of repeating transfection experiments with more non-rodent species SIRT6 plasmids. Additionally, more IF slide samples of other short and long lived rodent species should be stained and imaged to confirm these conclusions.

## 4. Acknowledgements

The author of this paper would like to thank Professor Vera Gorbunova and Professor Andrei Seluanov for their support as PI's in the lab and this experiment, Seyed Ali Biashad for his continuous support and help throughout the semester, John Martinez for his excellent mentorship and guidance throughout this experiment, and all other lab members for all their help.

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# Exploring Poxvirus Genome Packaging using CRISPR-Cas Systems

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## Abstract

The vaccinia virus genome is an approximately 195-kb linear double-stranded DNA genome that is AT-rich (~67%) and covalently closed at both ends. How this genome is replicated and packaged is still being determined. Many viruses rely on genomic elements called packaging signals to selectively encapsidate viral DNA/RNA over cellular DNA/RNA. To identify the location of packaging signals, the vaccinia virus genome can be targeted by CRISPR-Cas endonucleases that cleave DNA preceded by a short PAM. The CRISPR-Cas system can fragment the vaccinia virus genome and, with the help of fluorescent protein reporters, determine packaged fragments, if there are any. The process of developing a FnCas12a system and SpCas9 system that targets the vaccinia virus genome is described in this paper.

## Introduction

The compaction and packaging of genetic material are intricate and important processes shared among all three domains of life and viruses (1). The specific mechanisms by which viruses package their genome vary from species to species. For most DNA and RNA viruses with small genomes (<20 kb), an energy-independent system is used, where the capsid is assembled around the genome (1). In contrast, viruses with larger genomes tend to use energy-dependent systems, where ATP-driven motors pump the genome into a preformed capsid (1). Furthermore, many viruses use packaging signals to selectively package viral RNA or DNA as opposed to host RNA or DNA. These signals are present in several notable large double-stranded DNA viruses. For example, adenovirus genomes contain an AT-rich packaging domain that interacts with viral and cellular proteins to mediate genome packaging (2). Additionally, herpesvirus genomes contain two sequence motifs named *pac*1 and *pac*2 that are involved in concatemer cleavage and genome packaging (3). While poxviruses are also large double-stranded DNA viruses, similar packaging motifs have yet to be identified.

The *Poxviridae* family consists of large DNA viruses that replicate entirely in the cytoplasm of vertebrate or invertebrate cells (4). Vertebrate poxviruses are grouped into six genera, with the *Orthopoxvirus* genus being the most extensively studied (4, 5). Several notable viruses belong to this genus, including mpox, vaccinia virus (VACV), and variola virus, which is the causative agent of smallpox (6). VACV serves as the prototypic member of the *Poxviridae* family, and the extensive study of this virus has contributed to several key developments, such as the mpox and smallpox vaccines (6).

Transcription of the VACV genome occurs in a temporally-regulated fashion, divided into early, intermediate, and late stages (7). Early genes, which are expressed shortly after infection and before uncoating, encode proteins involved in host immune evasion, viral DNA synthesis, and intermediate gene expression (4). DNA replication is required for intermediate and late gene expression; intermediate genes encode enzymes and factors required for late gene expression (4,7).

A common characteristic of poxvirus genomes is the presence of AT-rich inverted terminal repeats (ITRs) which are identical, but oppositely oriented sequences found at the two ends of the genome (4). For VACV specifically, the entire genome is a 195-kb linear duplex that is AT-rich (~67%) (7). The genes contain 5' and 3' untranslated regions, lack introns, and can be encoded on either strand of the genome (7). ITRs of about 10 kb are present at the distal ends of the genome to link the two strands into a covalently closed molecule (7). After early gene transcription, uncoating inside the cell takes place which releases the genome. Thus replication undergoes in discrete cytoplasmic structures called replication factories (7). Nascent genomes are then delivered into immature virions (IVs) before the membrane closes, forming IVs with nucleoids that further mature to form infectious mature virions (MVs) (7).

The replication and packaging of the VACV genome remains poorly understood. Since no *in vitro* replication system has been established, models of poxvirus replication remain speculative. Most discussions focus on a self-priming model where a nick is introduced in the ITRs (7), providing a 3'-OH that can serve as the site of initiation for DNA synthesis. The self-priming model labels infected cells with [3H]thymidine and observes that the radiolabel was first incorporated within the ITR (8). Furthermore, prior research has demonstrated that the ITR is involved in replication and certain VACV proteins (I1, I6, K4) interact with the ITR (9). However, it remains unclear whether these proteins are involved in DNA replication or marking the genome for encapsidation (9).

A proposed model for genome packaging, shown in Figure 1, posits that the I6 viral protein marks the viral genome for encapsidation by binding the genomic termini, which is AT-rich, contains extra helical bases, and forms hairpin structures (7). Afterward, I6 brings the viral genome to the A32 viral protein, a viral ATPase positioned at the empty viral membrane to pump the genome into the virion (7). While packaging signals

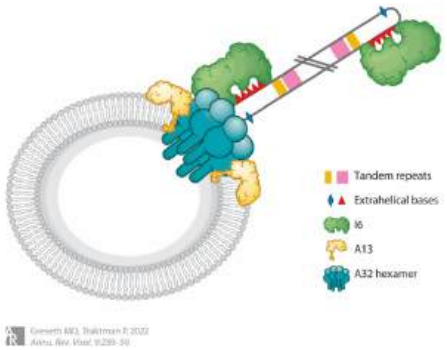



**Figure 1. Model for genome encapsidation.** It was proposed that the I6 viral protein marks the viral genome for encapsidation by binding to specific regions of the ITR. The A32 viral protein is a viral ATPase positioned on the empty viral membrane through its interaction with the A13 viral protein. I6 brings the viral genome to A32 on the empty viral membrane, which then pumps the genome into the virion. Figure reproduced from Greseth and Traktman 2022 (4).

have been identified in other double-stranded DNA viruses like herpes simplex virus type 1 and adenovirus, similar sequences have not been identified in poxviruses (8).To explore the location of packaging signals, clustered regularly interspaced short palindromic repeats and CRISPR-associated protein (CRISPR-Cas) systems, in conjugation with fluorescent protein reporters, can be utilized to fragment the VACV genome and determine packaged fragments.

Many archaea and bacteria utilize CRISPR-Cas systems composed of Cas effector proteins and CRISPR RNAs (crRNAs) to defend against attacks by foreign mobile genetic elements (MGEs) like plasmids or phages (10, 11). CRISPR-Cas systems work by their proteins forming an adaptation complex that recognizes the protospacer sequence on invading DNA/RNA via the protospacer adjacent motif (PAM) (10, 11). This sequence is incorporated into the CRISPR array, an area of the prokaryotic genome composed of short and conserved repetitive sequences, called repeats, between unique spacer sequences. The CRISPR array is transcribed into a long precursor crRNA (pre-crRNA) and processed into a shorter crRNA molecule that guides the Cas endonuclease to the target sequence for cleavage (10, 11). CRISPR-Cas systems are divided into two groups that are subdivided into six types (I to VI) based on their effector nucleases: Class 1 systems use several Cas proteins while class 2 systems use a single-component Cas protein (10). Class 2 systems (types II, V, and VI) comprise approximately 10% of all identified CRISPR-Cas loci and are found almost exclusively in bacteria (11).

Two notable examples of Class 2 systems are Cas12a and Cas9. Cas12a is a type V Cas protein that forms a ribonucleoprotein complex with a single crRNA molecule (11). Unlike type II systems which utilize bacterial RNase III with trans-activating crRNA (tracrRNA) to process the pre-crRNA, Cas12a has endoribonuclease activity and can process its pre-crRNA into mature crRNA without r tracrRNA (11). This gives it an advantage over CRISPR-Cas systems that require tracrRNA, like Cas9, as the system's design can be simplified due to needing one less component. Additionally, for the expression of CRISPR-Cas systems, for genome editing in plants and animals, the RNA polymerase type III U6 promoter is utilized to express precise, uncapped, non-polyadenylated crRNA molecules. The ability of the Cas12a endonuclease to process its pre-crRNA into crRNA means that crRNA expression can come from a wide range of promoters, including viral ones. Furthermore, it has been demonstrated that Cas12a can generate a staggered DNA double-stranded break with a 4 or 5 nucleotide 5’ overhang on target DNA preceded by a short T-rich PAM (10). This gives CRISPR-Cas12a an advantage over other CRISPR-Cas systems for targeting sequences in the orthopoxvirus genome that have an AT-rich nature by providing an increased number of targets. The specific PAM sequence can vary between species. For example, the PAM for LbCas12a (*Lachnospiraceae bacterium*) and AsCas12a (*Acidaminococcus*) has the sequence 5’-TTTN-3'; for FnCas12a (*Francisella novicida*), the PAM sequence 5’-TTN-3’ is located upstream of the 5' end of the non-target strand (11). FnCas12a has been demonstrated to cut the 18th base after the PAM on the non-targeted (+) strand and the 23rd base on the targeted (-) strand (10). This break can be repaired through nonhomologous end joining (NHEJ), typically leading to short insertion/deletion (indels) near the cut site that inactivates protein-coding genes by inducing frameshifts (10,12). If template DNA homologous to the break site is present, this double-stranded break can also be repaired through homology-directed repair (HDR) (11). HDR allows exogenous sequences with homology to the cut site to be introduced into the genome, allowing for the incorporation of specific sequences at a target site (12).

Cas9 is a type II Cas protein that forms a ribonucleoprotein complex with two noncoding RNAs, the crRNA and tracrRNA (12). These two RNA molecules can be fused into a single guide RNA (sgRNA) for genome editing (12). The Cas9/sgRNA complex then binds double-stranded DNA sequences that match the first 17-20 bases of the sgRNA only if the sequence is followed by a PAM, which has the sequence 5’-NGG-3’ for SpCas9 (*Streptococcus pyogenes*) (12). Once bound, the Cas9 endonuclease cleaves both DNA strands 3 bases upstream of the PAM, resulting in a blunt end DNA double-stranded break (12). Like CRISPR-Cas12, this break can be repaired through NHEJ, typically leading to indels near the cut site that inactivates protein-coding genes by inducing frameshifts (12). Since VACV early gene expression occurs before uncoating, early gene ORFs may be sequestered from the CRISPR-Cas system by the capsid and as a result, cannot be accessed before early gene expression. While the VACV genome–located in the cytoplasm–can be efficiently cut with CRISPR-Cas9, prior research did not observe NHEJ repair suggesting that the viral genome can be cleaved into smaller fragments (13). We hypothesize that bona fide packaging signals exist within the VACV genome. To map these signals, we initially designed a CRISPR-Cas12a system, but ultimately

developed a CRISPR-Cas9 system to cut the VACV genome into smaller fragments. By adding different fluorescent protein reporters to each fragment, it will then be determined which, if any, of these smaller fragments are packaged. Reported here is the progress made toward developing a FnCas12a system and SpCas9 system that cleaves the Emerald green fluorescent protein (EGFP) gene inserted in the VACV genome.

## Results

**No observed differences in EGFP expression between treatment with the CRISPR-Cas12a system and the negative control.**

Initially, we sought to observe the cutting of the EGFP gene in the VACV genome by a virally-expressed CRISPR-Cas12a system. Two crRNA, or guide RNA (gRNA), oligonucleotides targeting the 3' and 5' regions of the EGFP gene were designed based on Cpf1 parameters described in Zetsche et al. 2015 (10). These oligonucleotides were cloned into the pMiniT 2.0 backbone under the control of the I1 viral promoter and termed p3gRNA (3' gRNA) and p5gRNA (5' gRNA) (Table 1). The pCas12a is the plasmid that expresses the FnCas12a endonuclease, with expression of both gRNAs and the FnCas12a driven by the I1 viral promoter, an intermediate VACV promoter. The 5' crRNA binds starting at nucleotide 45 and the 3' crRNA binds starting at nucleotide 607 on the EGFP open reading frame (ORF), with cleavage generating a 5 nucleotide 5' overhang with some complementarity (Figure 2).

**Table 1: gRNA oligonucleotide sequences and corresponding plasmids**

| Oligonucleotide Name | Sequence (5'-3') | Plasmid Name |
|---|---|---|
| EGFP 3' gRNA Rev | CTGAGCACCCAGTCCGCCCTGAGATCTACAA<br>CAGTAGAAATTTAAGTTCACC | p3gRNA |
| EGFP 5' gRNA Rev | CCTGGTCGAGCTGGACGGCGACGATCTACAA<br>CAGTAGAAATTTAAGTTCACC | p5gRNA |

**Table 1. gRNA oligonucleotide sequences and corresponding plasmids**

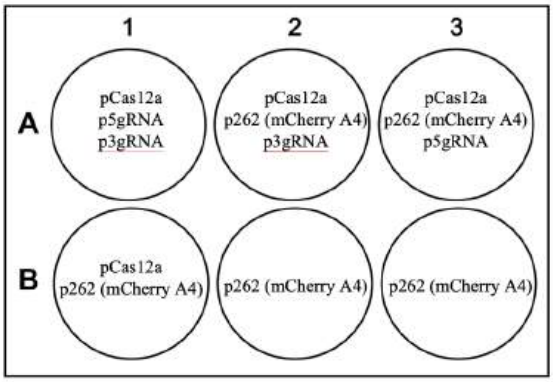



**Figure 3. Infection and transfection experimental setup.** All wells were infected at a multiplicity of infection (MOI) of 3 with vA4mKateF13GFP for two hours. Infections occurred over the course of two hours. Cells were transfected with the full CRISPR-Cas12a system (pCas12a, p3gRNA, and p5gRNA), parts of the system, or none of the Cas12a plasmids.

HeLa cells were infected with a recombinant VACV that expresses red fluorescent protein (RFP) tagged A4 proteins and EGFP-tagged F13 proteins (vA4mKateF13GFP). It was expected that when the EGFP ORF was fragmented, little to no EGFP would be expressed compared to the control cells. Regardless, infected cells should express RFP. The cells were then transfected with plasmids as shown in Figure 3. Well A1 contained the following plasmids: pCas12a for FnCas12a expression, p3gRNA for the 3' gRNA expression, and p5gRNA for the 5' gRNA expression. Expression of these components was driven by the I1 intermediate viral promoter. All components necessary for the FnCas12a protein to cleave the EGFP gene were present in wells A1, A2, and A3 but showed no difference in EGFP expression compared to the control wells (wells B1, B2, B3), which lacked plasmids encoding elements of the CRISPR-Cas12a system.

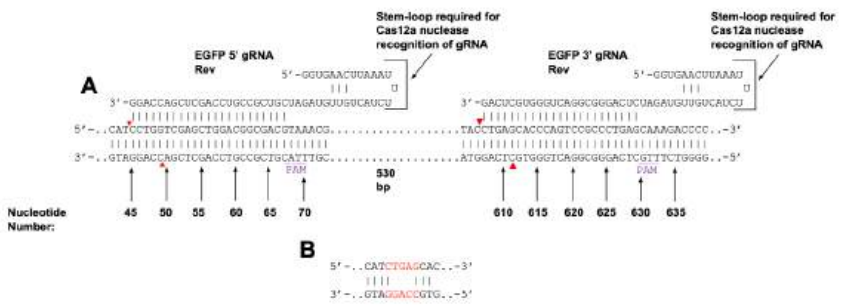



**Figure 2. gRNA annealing to the EGFP open reading frame and 5' overhang alignment. (A)** The 5' gRNA binds starting at nucleotide position 45 on the EGFP open-reading frame, while the 3' gRNA binds starting at nucleotide position 607. Cleavage sites are indicated by red arrowheads. The gRNA forms a stem-loop required for the Cas12a nuclease to recognize the gRNA. **(B)** DNA cleavage by CRISPR-Cas12a forms 5-nucleotide 5' DNA overhangs (depicted in red) with some complementarity.

No difference in the number of cells fluorescing RFP and EGFP was observed between well A1 and the negative control wells, which suggested that the FnCas12a system was not cutting the EGFP in a measurable way. We theorized that this could be related to factors such as the high multiplicity of infection (MOI), the CRISPR-Cas protein not expressing, the crRNAs not targeting the EGFP ORF, or the crRNAs not being expressed. A high MOI can induce antiviral responses and cytotoxicity, potentially causing undesirable effects that may interfere with the CRISPR-Cas system. Additionally, the genome's replication rate may outpace the CRISPR-Cas12a system's ability to locate and cleave it. This means cutting the template genome before replication will result in fewer replicated genomes being made, compared to fragmenting it after several rounds of replication have already taken place. Moreover, FnCas12a-mediated cleavage requires the expression of both the FnCas12a nuclease and the crRNAs. Without proper expression of the FnCas12a endonuclease, it is not expected that DNA cleavage will be observed. Additionally, without proper expression of the gRNAs, DNA cleavage will be absent because the FnCas12a endonuclease requires the gRNAs to target a specific DNA sequence. If either of these components is not properly expressed, the FnCas12a system will not be able to cut the EGFP gene.

**No observable difference in EGFP expression and the number of progeny virions produced at different MOIs.**

The high MOI of the previous experiment may induce cellular responses that interfere with the CRISPR-Cas12a system. To optimize the MOI and determine how it impacts the CRISPR-Cas system, HeLa cells were infected at different MOIs (3, 0.3, or 0.03) and transfected with either the full FnCas12a system (pCas12a, p3gRNA, p5gRNA) or only the FnCas12a effector protein (pCas12a). Again, there was no clear difference in EGFP expression between the wells transfected with the complete FnCas12a system and the wells that lacked the crRNAs. Thus, comparing the fluorescent proteins alone may not be an adequate way of assessing if the CRISPR-Cas12a system is working because the fluorescent proteins may not be sensitive enough. Double-stranded breaks in the viral genome should not be repaired because NHEJ was not previously observed after the VACV genome was cleaved with CRISPR-Cas9 (13). If the viral genome is not repaired and remains fragmented before encapsidation, fewer infectious progeny virions may be produced since the genes required for an efficient replication cycle will be absent if only a fragment of the genome is packaged. As a result, the progeny virions were quantified by titration to determine the efficiency of the viral replication process (Figure 4).

It was expected that infected cells transfected with the full Cas12a system should produce fewer progeny virions compared to the negative control group. However, no significant difference between the number of progeny virions was observed between the experimental and control groups for all MOIs, suggesting that the CRISPR-Cas12a system did not cut the viral genome (Figure 4).

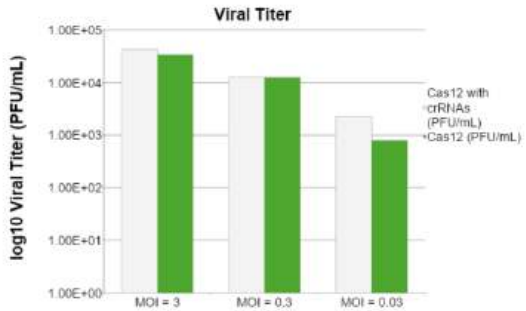



**Figure 4. Viral titer results.** All wells were infected for two hours with vA4mKateF13GFP at the MOIs indicated (3, 0.3, or 0.03). Infections occurred over the course of two hours. The wells were transfected with the plasmids indicated. Each well contained a total of 1 μg of DNA. After 24 hours, the cells were harvested, and the amount of progeny virions produced was determined by titration and plaque assay.

**FnCas12a is expressed in infected cells.**

The CRISPR-Cas12a system requires two components: the FnCas12a endonuclease and the gRNAs. If the CRISPR-Cas protein was not expressed, there would be no EGFP gene cleavage and the observed similarity in the PFU concentration between the experimental and control groups. To confirm the FnCas12a protein expression, a western blot was performed using an antibody that recognized the hemagglutinin (HA) epitope on FnCas12a after lysing the cells in the previous experiment, shown in Figure 4. The western blot revealed a band at approximately 152 kDa, consistent with the predicted size of the FnCas12a protein (Figure 5).

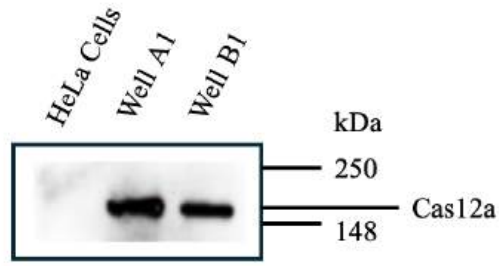



**Figure 5. Western Blot.** HeLa cells, well A1 from Figure 4, and well B1 from Figure 4 were lysed with RIPA buffer. The band observed between 250 and 148 kDa is consistent with the published kDA of Cas12a, 152 kDA.

**No observed difference in EGFP expression when gRNAs were shortened with a restriction enzyme and infected/ transfected in the presence of AraC.**

If the FnCas12a endonuclease was expressed but not cleaving, it could indicate that the crRNAs may not be expressed. The CRISPR array is transcribed as a continuous transcript (pre-

crRNA), which is then processed into mature crRNAs, 42 to 44 nucleotides in length, by the Cas12a nuclease without the need for tracrRNA (10). The Cas12a nuclease has intrinsic endoribonuclease activity that enables it to cleave the pre-crRNA directly upstream of a repeat-derived stem-loop structure, as shown in Figure 6 (14).

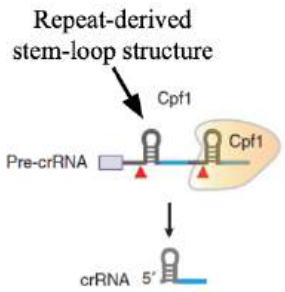



**Figure 6. Cas12a processing of pre-crRNA.** Illustration of pre-crRNA processing for Cas12a (formerly known as Cpf1). The Cas12a endonuclease cleaves the pre-crRNA directly upstream of a repeat-derived stem-loop structure. Cleavage sites indicated with red triangles. Figure reproduced from Zetsche et al. 2016 (15).

The plasmids that express the 5' and 3' crRNAs (p5gRNA and p3gRNA respectively) using the I1 viral promoter do not possess RNA-transcription termination sequences after the crRNA sequences. This can lead to run-on transcription and the formation of a crRNA molecule, which lacks the necessary secondary structure and a 3' end that may be too long for FnCas12a to effectively process. Without adequately processed crRNA molecules, the FnCas12a protein may not efficiently target and cleave the DNA target. To eliminate run-on transcription and shorten the 3' end, p3gRNA and p5gRNA were cut with the restriction enzyme NotI, which targets nucleotide 583 on the parental pMiniT 2.0 vector, located immediately downstream from the gRNA insertion site. Transfection with these shortened sequences was compared to transfection with the uncut plasmids and transfection with p331 (irrelevant plasmid). Furthermore, each condition was infected and transfected in the presence or absence of cytosine arabinoside (araC). AraC reversibly inhibits vaccinia virus DNA replication and subsequent morphogenesis, potentially allowing more time for the CRISPR-Cas12a system to cut the VACV genome before replication.

Poxviruses have multiple infectious forms, one being intracellular mature virions (IMVs), which are found inside cells and released via lysis (4). To isolate the IMVs and assess fluorescent protein expression, cells in wells A3 and B3 were lysed (Figure 7). Wells A3 and B3 were transfected with the FnCas12a plasmid and crRNA plasmids after NotI digest. Well A3 was infected in the presence of araC (40 µg/mL) while Well B3 was infected without araC. A plaque assay was performed using BSC-40 cells, a line of African green monkey cells derived from BSC-1 cells that are routinely used for VACV plaque assays, which were then infected with the progeny virions and imaged after 24 hours. These virions encode for EGFP and RFP-tagged proteins. If no EGFP gene cleavage occurs, all plaques will fluoresce green and red. If EGFP gene

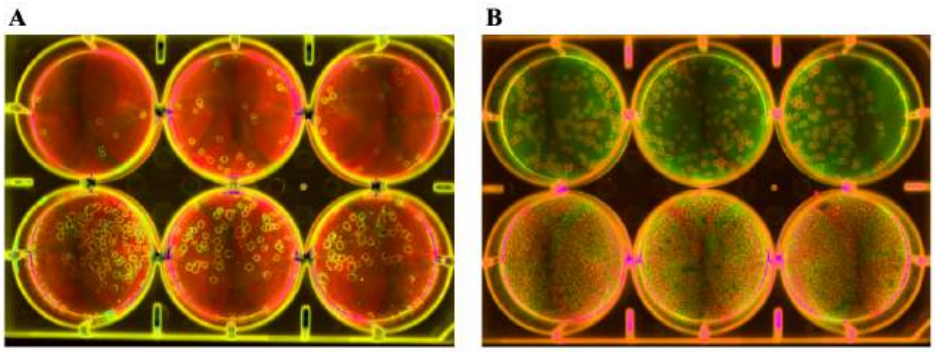



**Figure 7. Infection of BSC-40 cells.** All wells were infected at an MOI of 3 with vA4mKateF13GFP for two hours. Wells A1, A2, and A3 were infected in the presence of araC (40 µg/mL) for two hours. Wells A1 and B1 were transfected with the FnCas12a plasmid and no crRNA plasmids. Wells A2 and B2 were transfected with plasmids expressing FnCas12a and the crRNAs. Wells A3 and B3 were transfected with the FnCas12a plasmid and crRNA plasmids after NotI digestion. Transfections for wells A1, A2, and A3 occurred in the presence of araC (40 µg/mL) for two hours. Each well contained a total of 1 µg of DNA. (A) The top row was infected with IMVs from well A3 from the infection/transfection experiment described above after the virus had been diluted by a factor of 10-4. The bottom row was infected with IMVs from well A3 from the infection/transfection experiment described above after the virus had been diluted by a factor of 10-3. Well A3 cells were infected with vA4mKateF13GFP at an MOI of 3 in the presence of araC (40 µg/mL). Well A3 cells were also transfected in the presence of araC with plasmids expressing FnCas12a and crRNA plasmids after NotI digests. (B) The top row was infected with IMVs from well B3 from the infection/transfection experiment described above after the virus had been diluted by a factor of 10-4. The bottom row was infected with IMVs from well B3 from the infection/transfection experiment described above after the virus had been diluted by a factor of 10-3. Well B3 cells were infected with the vA4mKateF13GFP virus at an MOI of 3 in the presence of araC (40 µg/mL). Well B3 cells were also transfected in the presence of araC with plasmids expressing FnCas12a and crRNA plasmids after NotI digest. Plaques fluoresced red, green, or both red and green when cells were infected with a virus that encoded RFP, EGFP, or both RFP and EGFP respectively. Overlap of green and red signal is shown as yellow.

cleavage and knockout occur, plaques that fluoresce only red but not green will be present. Given the presence of plaques that only fluoresce both red and green, there was no clear indication that the EGFP gene was being cut (Figure 7).

**FnCas12a-mediated cleavage of the EGFP gene was not observed with transient EGFP expression.**

It is possible that the FnCas12a system cannot target the EGFP gene present in recombinant VACV genomes, as the genomes are sequestered in the replication factory. To account for this, HeLa cells were transfected with the CRISPR-Cas12a system and pT7GFP, a plasmid that encodes EGFP under the control of the T7 promoter. pT7GFP expression was driven by the VACV T7 expression system (16).

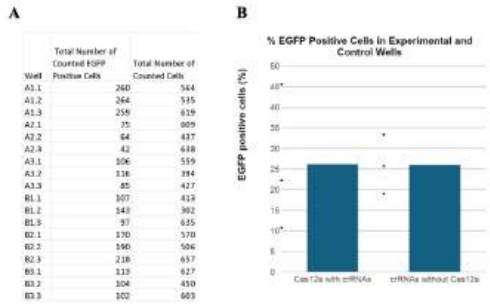



**Figure 8. Imaging results. (A)** Three different fields of view of each well were imaged. EGFP positive cells and total cells in each field of view were counted. Row A wells contained Cas12a with both crRNAs. Row B wells contained crRNAs without the Cas12a. **(B)** The percentage of EGFP positive cells was determined for each well. EGFP positive percentages in row A wells (Cas12a with crRNAs) were averaged. EGFP positive percentages in row B wells (crRNAs without Cas12a) were averaged. Graph depicts the percent EGFP positive cells in each well (data points) and the average percent EGFP positive cells for each condition (bars).

After 24 hours, three different fields of view of each well were imaged. The total number of cells and the total number of EGFP-expressing cells were counted and compared (Figure 8). The percentage of EGFP-expressing cells in the experimental group (FnCas12a with both crRNAs) and the negative control group (crRNAs without FnCas12a) were 26.19% and 26.01%, respectively. No significant difference between these values suggests that there was no significant cleavage of pT7GFP by FnCas12a. Examining the percentage of cells that expressed EGFP for each specific well, A2 had a lower percentage of EGFP-expressing cells compared to the negative control wells. This result would be consistent with the Cas12a endonuclease cutting the EGFP gene, thus reducing EGFP expression.

**PCR amplification of a segment of the EGFP ORF targeted by CRISPR-Cas12a revealed a single band, suggesting that FnCas12a did not cut the gene.**

It is possible that the difference between wells A2 and B2 was obtained through random chance and not a functional CRISPR-Cas12a system. To further analyze our experiment, specific EGFP primers were designed to bind outside the CRISPR-Cas12a cut sites and amplify a region of 714 base pairs between nucleotide 7 and nucleotide 721 of the EGFP coding region via polymerase chain reaction (PCR) (Figure 9). For cells that were transfected with pT7GFP without the Cas12a system, the expected PCR amplicon size was 714 base pairs. For cells transfected with the CRISPR-Cas12a system, Cas12a-mediated double-stranded breaks are repaired with NHEJ which may introduce indels. As a result, the expected PCR amplicon size may be either larger or smaller than 714 base pairs. Since 2 separate sgRNAs were used, it may be possible to simultaneously generate two staggered double-stranded DNA breaks on the plasmid at each cut site. The sgRNAs target sequences are approximately 560 bases apart, meaning that if NHEJ occurs between the two staggered ends of the plasmid, PCR amplification of pT7GFP should produce a distinct band of approximately 560 bases smaller than the uncut EGFP gene band.


**Figure 9. EGFP open reading frame (ORF) map with alignments of PCR primers and crRNAs.** EGFP Forward Primer for PCR binds starting at nucleotide 7 on the GFP ORF. EGFP Reverse Primer for PCR binds starting at nucleotide 721 on the EGFP ORF. The EGFP 5' crRNA binds nucleotide starting at 45 on the EGFP ORF. The EGFP 3' crRNA binds starting at nucleotide 607 on the EGFP OR.

PCR amplification of the EGFP coding region in wells A1 and A2 revealed only a single band approximately 700 bases in length (Figure 10). A band with a similar size was observed for PCR amplification of negative controls pT7GFP and well B2. This result suggests that the CRISPR-Cas12a system did not cut the EGFP coding region on pT7GFP in wells A1 and A2.

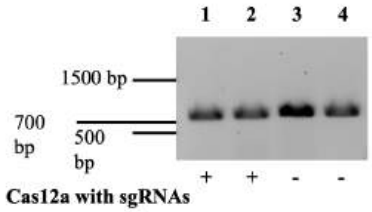



**Figure 10. Agarose gel electrophoresis of DNA fragments from pT7GFP.**

1: Cells transfected with pT7GFP, pCas12a, p3gRNA, p5gRNA,

2: Cells transfected with pT7GFP, pCas12a, p3gRNA, p5gRNA,

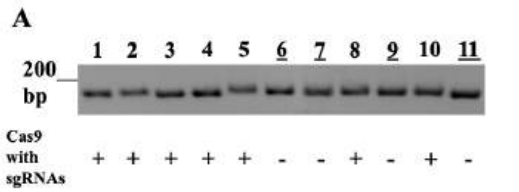
3: Cells transfected with pT7GFP, pCas12a, p3gRNA, pcDNA3,

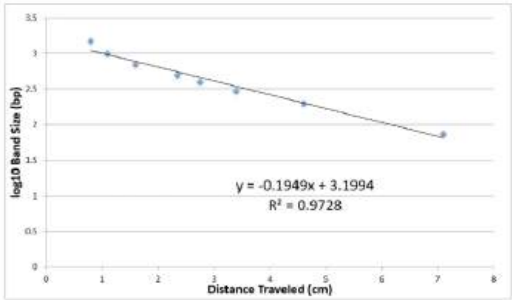
4: pT7GFP. 4 hours after transfection, cells were harvested, the DNA was isolated, and analyzed by PCR. PCR products were analyzed via 1.8% *MetaPhor* agarose gel in 1x TBE electrophoresis.

**PCR amplification of the EGFP ORF on pT7GFP after transfection with a Cas9 and sgRNA-encoding plasmid revealed differences in DNA band sizes.**

Since there was no clear indication that our CRISPR-Cas12a system was working, we switched to a CRISPR-Cas9 system. HeLa cells were transfected with pT7GFP and one or more pLentiCRISPR-EGFP-sgRNA plasmids, which express human codon-optimized Cas9 protein with an EGFP-targeting synthetic sgRNA (*Addgene*).pLentiCRISPR-EGFP-sgRNA 1, 2, 4, and 5 targets the EGFP ORF between nucleotides: 28 and 29; 69 and 70; 304 and 305; 358 and 359 respectively. After 24 hours, the cells were collected and the EGFP ORF was amplified via PCR (Figure 11). PCR amplification was performed using EGFP Forward Primer 255, which binds the EGFP ORF starting at nucleotide 255, and EGFP Reverse Primer 420, which binds the EGFP ORF starting at nucleotide 420. For cells transfected with pT7GFP without the Cas9 system, the expected PCR amplicon size was 165 base pairs. For cells transfected with pT7GFP and the pLentiCRISPR-EGFP-sgRNA, CRISPR-Cas9-induced blunt end DNA double-stranded breaks were expected to be repaired via NHEJ, which typically leads to indels. As a result, HeLa cells that expressed both the Cas9 protein and EGFP-targeting sgRNA were predicted to produce a DNA band different in size compared to PCR amplification of pT7GFP without Cas9 or the sgRNA.

PCR amplification of the EGFP-coding region on pT7GFP revealed one band of varying lengths in each lane (Figure 11). Cells transfected with the full CRISPR-Cas9 system had a larger mean and range of estimated PCR band sizes compared to cells that were not transfected with the system. This is consistent with indel formation during NHEJ, suggesting that pT7GFP was successfully targeted and cleaved by the Cas9 endonuclease and gRNAs.




C

| Lane | Distance Traveled (cm) | Size (bp) |
|---|---|---|
| 1 | 5.1 | 160.5 |
| 2 | 5 | 167.8 |
| 3 | 5.2 | 153.4 |
| 4 | 5.2 | 153.4 |
| 5 | 5 | 167.8 |
| 6 | 5.15 | 156.9 |
| 7 | 5.2 | 153.4 |
| 8 | 5.1 | 160.5 |
| 9 | 5.15 | 156.9 |
| 10 | 5.1 | 160.5 |
| 11 | 5.25 | 150 |

D

| | Mean Size (bp) | Range (bp) |
|---|---|---|
| Transfection of pT7GFP without Cas9 or sgRNA (Lanes 6, 7, 9, and 11) | 154.3 | 6.9 |
| Transfection of pT7GFP with Cas9 and sgRNA (Lanes 1, 2, 3, 4, 5, 8, and 10) | 160.56 | 14.4 |

**Figure 11. Agarose gel electrophoresis of DNA fragments from pT7GFP. (A)** PCR products from amplification of the EGFP ORF were analyzed via 3% MetaPhor agarose gel in 1x TBE electrophoresis. Transfection of pT7GFP without Cas9 or sgRNA includes gel lanes 6, 7, 9, and 11. Transfection of pT7GFP with Cas9 and sgRNA includes gel lanes 1, 2, 3, 4, 5, 8, and 10. Underlined lanes indicate transfection without Cas9 or sgRNA. Lanes with no underlining indicate transfection with Cas9 and sgRNA. 1: Cells transfected with pT7GFP, pLentiCRISPR-EGFP-sgRNA 1, and pcDNA3, 2: Cells transfected with pT7GFP, pLentiCRISPR-EGFP-sgRNA 2, and pcDNA3, 3: Cells transfected with pT7GFP, pLentiCRISPR-EGFP-sgRNA 4, and pcDNA3, 4: Cells transfected with pT7GFP, pLentiCRISPR-EGFP-sgRNA 5, and pcDNA3, 5: Cells transfected with pT7GFP, pLentiCRISPR-EGFP-sgRNA 1, pLentiCRISPR-EGFP-sgRNA 2, pLentiCRISPR-EGFP-sgRNA 4, pLentiCRISPR-EGFP-sgRNA 5, 6: Cells transfected with pT7GFP, pLenti-Cas9, and pcDNA3, 7: Cells transfected with pT7GFP and pcDNA3, 8: Cells transfected with pT7GFP, pLentiCRISPR-EGFP-sgRNA 2, and pcDNA3, 9: Cells transfected with pT7GFP and pcDNA3, 10: Cells transfected with pT7GFP, pLentiCRISPR-EGFP-sgRNA 2, and pcDNA3, 11: pT7GFP only. (B) Line of best-fit formula from linear regression analysis of GeneRuler 1 kb DNA Ladder migration. (C) Calculated size of PCR fragments. (D) Mean size and range of DNA fragments for each condition.

**PCR amplification of the F13-GFP ORF on a recombinant vaccinia virus after transfection with a Cas9/sgRNA-encoding plasmid revealed differences in DNA band intensity.**

It was hypothesized that the CRISPR-Cas12a system showed no clear fragmentation of the VACV genome because the genome was made inaccessible to the CRISPR-Cas12a system by the capsid or replication factory. To determine if the CRISPR-Cas9 system could access the EGFP ORF on the VACV genome, HeLa cells were transfected with pLenti-CRISPR-EGFP-sgRNA 2 for 24 hours to allow for the expression of the CRISPR-Cas system off of cellular promoters. Sp-Cas9 endonuclease expression was driven by the elongation factor 1α short (EFS) promoter and gRNAs expression was driven by the RNA polymerase type III U6 promoter. The cells were then infected with vA4mKateF13GFP at an MOI of 3 for 24 hours. pLentiCRISPR-EGFP-sgRNA 2 was specifically chosen because PCR amplification of the EGFP ORF in pT7GFP after pLentiCRISPR-EGFP-sgRNA 2 transfection produced the largest bands compared to the other pLenti-CRISPR-EGFP-sgRNA plasmids (Figure 11). The band's size increase is likely due to nucleotide additions during NHEJ after Cas9-mediated cleavage. Since the efficiency of NHEJ for the VACV genome after Cas9 endonuclease cleavage has been suggested to be an inefficient process, most of the VACV genomes cut by the Cas9 endonuclease should remain fragmented at the EGFP ORF (13). If the EGFP ORF remains fragmented, full-length EGFP mRNA will not be transcribed, reducing EGFP expression. Additionally, PCR amplification using primers that bind upstream and downstream of the cut site will not amplify the segment if it is fragmented. Lastly, it remains uncertain whether the viral genome, when cut approximately in half, will be encapsidated or not. If it is not packaged, fewer progeny virions will be made. If the fragment is packaged, the progeny virions will not effectively infect and replicate inside cells since half of the genome is missing. As a result, it was expected that cells transfected with the Cas9 and gRNA expressing plasmid would have decreased EGFP mean fluorescence intensity (MFI), amplicons following PCR amplification of the EGFP ORF, and decreased infectious progeny virus production after infection with vA4mKateF13GFP.

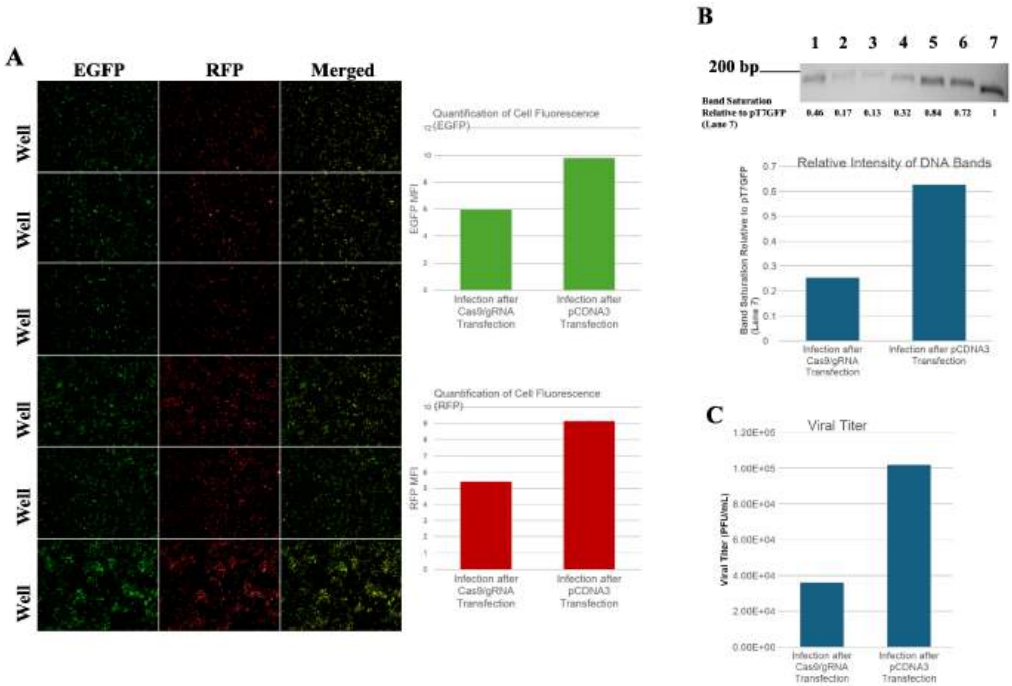



**Figure 12. Fluorescent imaging, PCR, and viral titer of cells.** (A) Wells Al, A2, and A3 were transfected with pLentiCRISPR-EGFP-sgRNA 2. Wells B1, B2, and B3 were transfected with pCDNA3. After 24 hours of transfection, wells were infected With vA4mKateF13GFP. Separated and merged fluorescent emissions (EGFP in green, RFP in red, and overlap in yellow). MFI of the EGFP and RFP images was quantified by *Fiji*. (B) PCR amplification of the EGFP ORF in each well of HeLa cells. PCR products were analyzed via 3% *MetaPhor* agarose gel in 1x TBE electrophoresis. Lanes 1, 2, and 3 correspond to wells A1, A2, and A3. Lanes 4, 5, and 6 correspond to wells B1, B2, and B3. Lane 7 corresponds to pT7GFP, a plasmid that expresses EGFP. Band saturation was quantified via *Fiji*. (C) PFU per milliliter in each well was determined via a viral titer.

Transfected and infected cells were imaged 24 hours post-infection. Reduced fluorescence for both EGFP and RFP was observed for cells transfected with pLentiCRISPR-EGFP-sgRNA (Wells A1, A2, and A3) compared to cells transfected with pCDNA3 (Wells B1, B2, and B3) (Figure 12A). Further analysis and quantification of the fluorescence intensity revealed that the mean fluorescence intensity was reduced for cells transfected with pLentiCRISPR-EGFP-sgRNA compared to cells transfected with pCDNA3 (Figure 12A). Cells were harvested as before and analyzed by PCR. PCR amplification of the EGFP ORF resulted in fewer amplicons for cells transfected with pLentiCRISPR-EGFP-sgRNA compared to cells transfected with pCDNA3 (Figure 12B). Lastly, cells transfected with pLentiCRISPR-EGFP-sgRNA produced fewer PFU per mL compared to cells transfected with pCDNA3 (Figure 12C).

## Discussion

To explore poxvirus DNA genome replication and packaging, a CRISPR-Cas12a system and CRISPR-Cas9 system were utilized to fragment the viral genome at specific sites. To determine the necessary conditions under which these systems would function, crRNAs targeting the EGFP gene were used, and the impact on EGFP expression was analyzed.

When cells were infected with a recombinant VACV expressing EGFP and then transfected in plasmids expressing the CRISPR-Cas12a system from a viral promoter, there were no observed changes in EGFP expression between the experimental and control groups. The reason for this outcome could be the high MOI used for the infection (Figure 3), as the increased viral genomes in cells during infections at high MOIs can trigger double-stranded DNA sensors that stimulate cytokine expression, which increase cell stress and cell death compared to infections at lower MOIs. Additionally, a high MOI may induce antiviral cellular responses, complicating experimental results. Moreover, the genome's replication rate may outpace the CRISPR-Cas12a system's ability to locate and cleave it. For example, if the template genome is fragmented before replication, fewer replicated genomes will be produced. In contrast, if the viral genome is cut after many replication cycles, the number of replicated genomes will be higher. No difference in EGFP expression was observed between the experimental and the control well when different MOIs were used. A viral titer was performed to determine the replication process efficiency and overall success of the initial infection procedure. The CRISPR crRNAs target EGFP sequences on the viral genome for recombinant VACVexpressing EGFP-tagged proteins, which should lead to unpackaged viral genome cleavage and the subsequent reduction of infectious progeny virions. However, the concentration of plaque-forming units in the experimental conditions (CRISPR-Cas12a with crRNAs) was either the same or greater than the control conditions (CRISPR-Cas12a without crRNAs), indicating that the system was not sufficient to reduce viral replication.

Initially, we hypothesized that the failure of FnCas12a to cleave the viral genome was because the Cas endonuclease was not expressed or unable to correctly process the crRNAs. Western blot analysis revealed a band consistent with the size of FnCas12a (Figure 5), and EGFP expression remained similar between the experimental and control groups after the crRNA plasmids underwent restriction digestion. Transitioning from a recombinant vaccinia virus expressing EGFP to transgenic EGFP expression plasmids, we saw similar results, with little variation in the percentage of EGFP-expressing cells between the experimental and control conditions. In both experimental and control samples, PCR amplification of 714 base pairs of the EGFP coding region revealed only a single band of approximately 700 base pairs in length. If Cas12a had cut pT7GFP, it would have resulted in staggered cuts that could be repaired via NHEJ, introducing variable indels. Furthermore, two bands–one band at around 714 base pairs and another band 560 base pairs shorter–were expected if two Cas12a-mediated staggered cuts at different sites were generated simultaneously on the pT7GFP plasmid. This would result in two different staggered ends on the plasmid that could be repaired and annealed together, resulting in the removal of around 560 base pairs of the EGFP ORF. This smaller band was not observed, suggesting that multiple simultaneous Cas12a-mediated cleavages of DNA were not observed in our system (Figure 10). Notably, the PCR amplicon produced from cells transfected with the full CRISPR-Cas12a system appears slightly larger than the PCR amplicons of pT7GFP (Figure 10). While this suggests that additions to the EGFP ORF may have occurred–likely as a result of NHEJ repair after Cas12a-mediated cleavage–the gel used did not have a high enough resolution for us to make any conclusions. Moving forward, nucleotide additions could be verified by sequencing clones from the agarose gel bands.

Our CRISPR-Cas12a system's ability to cut plasmid DNA but not viral DNA could be explained by the timing of the CRISPR-Cas12a system expression during viral infection. The CRISPR-Cas12a plasmids utilize intermediate VACV gene promoters, and intermediate genes are expressed after viral DNA replication. Upon entry into a cell, the viral genome is surrounded by a proteinaceous shell that later undergoes a poorly understood uncoating process, when the core dissolves and the genome is released (7). Genome replication occurs in discrete cytoplasmic structures called replication factories (7). By the time the Cas12a endonuclease and crRNAs are expressed, the VACV genome is already sequestered in the viral factory and replicating. As a result, the VACV genome may not be accessible to the CRISPR-Cas12a system for cleavage. Efficient VACV genome cleavage was strongly suggested by the results in Figure 12 when cells were transfected with the CRISPR-Cas9 system and then infected with VACV 24 hours later. Transfecting before infecting the cells allows the CRISPR-Cas9 system to be present in the cell before any viral DNA is present. After virion entry and uncoating, the genome localizes to replication factories (7). During this process, it is exposed to the cytoplasm and may be targeted by the CRISPR-Cas9 system.

Another explanation for the observed inefficiency of the CRISPR-Cas12a system could be the transfection protocol. Using 5 µl of Lipofectamine with 0.25 µg of pT7GFP, an EGFP-expressing plasmid, resulted in approximately 20% of the cells fluorescing green. In comparison, using 5 µl of Lipofectamine 2000 with 0.25 µg of the same plasmid led to approximately 70% of the cells fluorescing green. Moving forward, revisiting prior infection and transfection experimental setups and replacing Lipofectamine with Lipofectamine 2000 to improve transfection efficiency may lead to different results. The low transfection efficiency seen with Lipofectamine and pT7GFP indicates that many of the cells in earlier experiments did not receive all the components necessary for the CRISPR-Cas12a system to function properly. After transfection, CRISPR-Cas12a functionality could be determined through counting cells and PCR amplification of the EGFP coding region. Once the optimal conditions for FnCas12a activity are determined, crRNAs for specific areas of the VACV genome can be developed to fragment the genome and explore the mechanisms of poxvirus genome replication and packaging.

Plasmids expressing human codon-optimized Cas9 protein were transfected with EGFP-targeting synthetic sgRNA. Cells are then infected with a verified VACV expressing EGFP-tagged proteins to decrease fluorescence, viral EGFP template for PCR, and progeny virion production compared to the negative control. This strongly suggests the VACV genome was targeted by the Cas9 endonuclease. To further optimize Cas9 endonuclease activity, cells could be transfected with a plasmid expressing a Cas9 that does not possess nuclear localization signals (NLS). NLS is an amino acid motif that mediates protein transport into the nucleus (17), and is often appended to Cas9 endonucleases so the Cas9 protein can be transported into the nucleus, where DNA is present. These NLS may hinder VACV genome cleavage because the VACV genome replicates in the cytoplasm, not the nucleus, and the NLS signal reduces the number of Cas9 endonucleases in the cytoplasm. It would be beneficial for future studies to design a new plasmid for expressing a Cas9 endonuclease without NLS. Moving forward, to minimize variations in background fluorescence, which result from imaging the cells when covered in DMEM, images will be taken with cells covered with PBS or a media without neutral red (which autofluoresces). As well, quantitative PCR can be used to measure the quantity of present DNA more precisely.

It is hypothesized that within the large dsDNA genome of VACV, there may be packaging signals present. It is unclear if these signals are present and where they may be located. To explore the location of packaging signals and VACV genome packaging, a recombinant VACV expressing mKate-tagged A4 proteins, EGFP-tagged thymidine kinase (TK) proteins, and blue fluorescent protein (BFP) tagged F13 proteins will be utilized. Since the TK-GFP ORF lies near the VACV genome center, transfecting cells with pLentiCRISPR-EGFP-sgRNA 2 before infecting them with this virus should fragment the viral genome near the middle. If NHEJ does not occur, this should produce two distinct fragments, with one containing the A4-mKate ORF and the other containing the F13-GFP ORF. If packaging signals are present on one half but not the other, cells infected with progeny virions should fluoresce predominantly in one color, either red or blue. If packaging signals are present in both halves, an equal number of cells infected with progeny virions should fluoresce red and blue. Moreover, by placing the EGFP ORF in different parts of the genome, different VACV genome fragments can be generated after the EGFP ORF has undergone Cas9-mediated cleavage and tested for packaging.

Understanding VACV genome encapsidation is important for several reasons. First, despite smallpox eradication, there are concerns that it may re-emerge from forgotten stocks, de novo synthesis, or melting permafrost resulting from global warming (6). This is especially concerning given the low rates of smallpox vaccination since the 1980s and the low number of smallpox-specific drugs (6). By understanding poxvirus genome packaging, replication, and the proteins involved in those processes, novel drugs that target essential proteins can be developed. Furthermore, many biotechnology products have been developed based on enzymes identified from the vaccinia virus, like the vaccinia capping system for in vitro synthesized RNA and TOPO cloning, which is based on vaccinia DNA topoisomerase (6). Identifying the enzymes involved in replication or encapsidation may lead to novel poxvirus-based biotechnology products. Lastly, poxviruses are being developed as vaccine vectors, oncolytic therapeutics, and gene delivery vehicles (6). By better understanding viral processes like genome replication and encapsidation, more efficient systems can be designed.

In conclusion, the CRISPR-Cas12a system we used did not efficiently cut the EGFP-coding region in our tests. Related to this outcome could be the low transfection rates seen with Lipofectamine, so the decision was made to replace Lipofectamine with Lipofectamine 2000. Furthermore, the Cas12a sgRNAs designed may not be effectively processed and recognized by the Cas12a endonuclease. Lastly, the CRISPR-Cas12a system was expressed using the I1 promoter, an intermediate VACV promoter. Intermediate genes are expressed after viral genome replication, so by the time the CRISPR-Cas12a system is expressed, the viral genome may be sequestered and unavailable for the CRISPR-Cas12a system to cleave. Since there was no clear indication that pT7GFP was being cut by the CRISPR-Cas12a system, we switched to a CRISPR-Cas9 system. With this system, we observed decreased fluorescence, decreased viral EGFP DNA template for PCR, and decreased progeny virion production compared to the negative control, suggesting that the Cas9 endonuclease was able to fragment the VACV genome. By placing the EGFP ORF in specific regions of the VACV genome and using the CRISPR-Cas9 system to target the EGFP ORF, the VACV genome can be fragmented. Fluorescent protein reporters can help to determine which fragments, if any, are packaged. Understanding how these fragments are packaged can provide

insight into the location of packaging signals in the VACV genome.

Please find supplementary materials online at jur.rochester.edu.

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Daniel Kuo graduated from the University of Rochester with a Bachelor of Science in Microbiology. From 2021 to 2024, Dan conducted poxvirus research under the mentorship of Dr. Brian Ward. As an aspiring medical student and physician, Dan hopes to continue his exploration of virology, with a particular focus on oncolytic viruses and viral vectors for gene therapy.

# The Effect of Modifications to DNA Structure on the Architecture of Bacterial DNA Condensed with a Nucleoid-Associated Protein

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## Abstract

Bacteria are adaptive organisms that have developed various protective mechanisms to respond to a range of environmental stressors like nutrient deprivation. One distinct protective mechanism in *Escherichia coli* that will be discussed further involves the overproduction of a nucleoid-associated protein called DNA-binding protein (Dps) from starved cells. The high concentration of Dps protects the bacteria's genetic material by binding to the DNA and forming a Dps-DNA condensate. The morphology of these Dps-DNA condensates in an in-vitro system will change under different environmental conditions. To better understand how altering Dps-DNA interactions independent of environmental stressors affects the resulting morphology of the Dps-DNA condensate in an in-vitro system, fluorescence microscopy imaging was utilized to examine the characteristics of the condensate in response to different DNA features. Upon varying the length of linear DNA fragments incubated with Dps protein, the lengths of the DNA did not change the morphology/structure of the condensate. However, incubation with linear 5k DNA fragments resulted in a greater amount for larger sized condensate formation. When Dps was incubated with DNA fragments that were either supercoiled or relaxed, minimal differences were observed in the size and structure of condensates. These findings demonstrate that Dps can bind to and condense multiple DNA conformations, helping better explain the function of Dps in organisms other than E. coli.

## Introduction

Dps serves multiple functions in bacterial cells, but one of its primary functions is binding to and condensing DNA. This function protects the genetic material of the bacteria from environmental stressors, allowing the bacteria to survive in a wide range of harsh conditions. Under both stress and non-stress conditions, Dps monomers are commonly assembled into a spherical dodecamer that is 90 Ångstroms in diameter, with a hollow core cavity that is 45 Ångstroms in diameter (1). Each Dps monomer consists of a bundle of four alpha-helices interacting with each other (2). The surface of the dodecamer is negatively charged, making it unsuitable for interactions with the negatively charged phosphate backbone of DNA molecules (3). Studies suggest that the N-terminal tails of the Dps dodecamer may mediate the interaction with DNA (10). These N-terminal tails are largely disordered and extend outwards from the 12-mer quaternary structure of the Dps dodecamer. In addition, they contain three positively charged lysine residues at the 5$^{th}$, 8$^{th}$, and 10$^{th}$ positions required for DNA binding (4).

The interaction between the lysine residues on the N-terminal tails of the Dps with the DNA occurs when one Dps oligomer interacts with multiple DNA molecules simultaneously. Numerous interactions are possible because the Dps oligomer has multiple N-terminal tails projecting out of it. The size of the tails is small compared to the overall size of the Dps dodecamer, resulting in minimal opportunities for interactions between the residues on the tails and DNA if there is only one strand of DNA. As a result, DNA plectonemes have been shown to have the most stable condensate formation between Dps and DNA (S2 Figure 1A-B). When one DNA strand is twisted around itself, it allows for the distance between neighboring regions of the plectoneme to be roughly the same length as the dodecamer diameter, allowing the Dps dodecamer to interact with both strands (5).

Since electrostatic interactions drive Dps-DNA interactions, it is most likely that the different morphologies observed in Dps-DNA condensate under various environmental conditions are due to changes in these electrostatic interactions (6). Using an in-vitro system and different buffer conditions, we have discovered that the Dps-DNA condensates can take on three different morphologies: globular, liquid-like, and spongiform (S1 Figure 1A-C). When using in-vivo systems where the cells have been deprived of nutrients for an extended period, others have observed additional morphologies of the condensates: nanocrystalline, liquid crystalline, and folded nucleosome-like type (7). These different condensate structures vary in their DNA packing architecture and potential DNA accessibility.

Despite obtaining a better understanding of the formation of Dps-DNA condensates and how the morphologies of these condensates change under various environmental conditions, it is still not understood how the morphologies of the condensate change when the DNA itself is altered. Upon exposure to *Escherichia coli* genomic DNA, which is circular and of a specific length by nature, Dps will condense DNA and form a Dps-DNA condensate. However, not all organisms share the same features of DNA, and little is known about how Dps will interact with these different features of DNA that deviate from that of *Escherichia coli*. Features of DNA that could vary include fragments of various lengths and supercoiling states, as some are circular while others are linear by nature. This study examines the question of how Dps interacts with different

features of DNA, which is essential to better understand the function of Dps in organisms besides E. coli and how those organisms respond to environmental stressors.

## Materials and Methods

Using lambda (λ) phage DNA, two fixed forward primers and multiple reverse primers were designed to create DNA fragments of different lengths (Table 1). The reverse primers were designed to create five DNA fragments of different lengths. These lengths were determined based on the distance between the forward primers and respective reverse primers. One forward primer was used to develop the 500, 1000, and 2000 bp, and the other was used to create DNA fragments of 5000 and 10,000 bp in length. λ DNA was used as a control, roughly 50,000 bp in length.

We created a master mix consisting of the forward primer, DNA polymerase, the template DNA being λ DNA, and other components necessary to synthesize new DNA strands before PCR amplification. After distributing the master mix into five PCR tubes for each of the five different DNA lengths being tested at equal amounts, the reverse primers were added to each of their respective PCR tubes (8). After each DNA fragment was amplified, each group of DNA fragment lengths was purified using a PCR cleanup kit to remove the impurities and components that were not the DNA fragments. A sample from each group of purified DNA fragments was analyzed via gel electrophoresis to ensure they were synthesized at their intended lengths. The resulting DNA bands were compared to a DNA ladder, where each DNA product corresponded correctly to its size (Figure 1A-B).

## Creation of Circular and Linear DNA Fragments

Three circular plasmids similar to the DNA lengths tested in previous experiments on different-length DNA fragments were identified. Three bacterial strains, each with the plasmid of interest were selected. The three strains separately containing CD8 plasmid (4894 bp), pET17b plasmid (2070 bp), and pShew:mtrC (10,500 bp). LB medium was inoculated with the three bacterial strains along with their respective antibiotics: for the strain containing the CD8 and pShew:mtrC plasmid, the antibiotic used was Kanamycin, while for the strain containing the pET17b plasmid, Chloramphenicol was used. The strains were then shaken at 37 °C overnight to allow for the growth of a saturated culture. After the incubation period, the plasmids were isolated from the bacteria using a plasmid miniprep kit.

A portion of the extracted plasmids of each of the three lengths was then utilized to undergo restriction enzyme digest to create relaxed DNA, (which will be henceforth referred to as linear DNA). Restriction enzymes were chosen based on their ability to only cut the plasmid at one position, thereby creatinga linear piece of DNA of that length. If a restriction en

| Primers | Sequence |
|---|---|
| Forward Primer #1 | GCTGGGTGTTTATGCCTAC |
| 500 Reverse Primer | CGCCAAATTTAAGATACTGCTCC |
| 1000 Reverse Primer | GTCAATCAGCCAGCTTTCC |
| 2000 Reverse Primer | GCATCCACACTTTCACTCG |
| Forward Primer #2 | GCTTGGTGTACCTCATCTAC |
| 5000 Reverse Primer | GTTATCAAGCACTGCACTGG |
| 10000 Reverse Primer | GCTGGCAACTAATTCAGTCC |

**Table 1. Sequences for the forward and reverse primers used to create the different lengths of DNA fragments.**

(A) Forward Primer #1 was used with the 500, 1000, and 2000 reverse primer, while forward primer #2 was used with the 5000 and 10000 reverse primer. (B) All primers satisfy the following requirements: at least 18 bp long, G-C content ranging from 50% to 55%, and having a melting temperature of around 50 °C.

zyme cut the plasmids at multiple positions, it would result in numerous linear fragments of much smaller lengths. The CD8 and pShew:mtrC plasmid was cut using the restriction enzyme *Eco*R1. In contrast, the pET17b plasmid was cut using the restriction enzyme *Xbal*. In the control group, the CD8 plasmid was incubated with a restriction enzyme for which it has no cut site, so it could not cut the plasmid. After obtaining the relaxed DNA from a portion of each extracted plasmid of different lengths through restriction digest, the resulting linear DNA was purified using a PCR cleanup kit to remove all the impurities. To ensure the plasmids were cut at the expected length, each circular and linearized plasmid was loaded onto a gel and analyzed via gel electrophoresis. The resulting bands were then compared to a DNA ladder, where each DNA product used corresponded correctly to their respective sizes and location on the gel, with circular plasmids migrating further down the gel than their respective linear counterparts (Figure 1C).

## Image Preparation and Imaging using Fluorescence Microscopy

Nine samples were made to assemble the Dps-DNA complexes for imaging. Each sample contained the same buffer containing 5% polyethylene glycol 8k (PEG), 4 mM MgCl2, 50 mM HEPES-KOH, and 100 mM KCl. Then, we added 1x sytox green and 10 ng/uL of the aforementioned DNA fragments (final concentrations). Finally, 1 mM Dps was added to begin forming condensates. We made three control groups: a sample with all components but DNA, another with all components but Dps, and the final sample containing neither Dps nor DNA. We ensured that the amount of DNA was consistent between samples. The use of sytox green allows for the visualization of DNA. Some of the Dps we used is a variant chemcally labeled with Alexa fluor 647, enabling the Dps to be visualized. Most, however, were wild-type Dps (9). All samples were incubated overnight at 37 °C and imaged after one day using fluorescent microscopy.

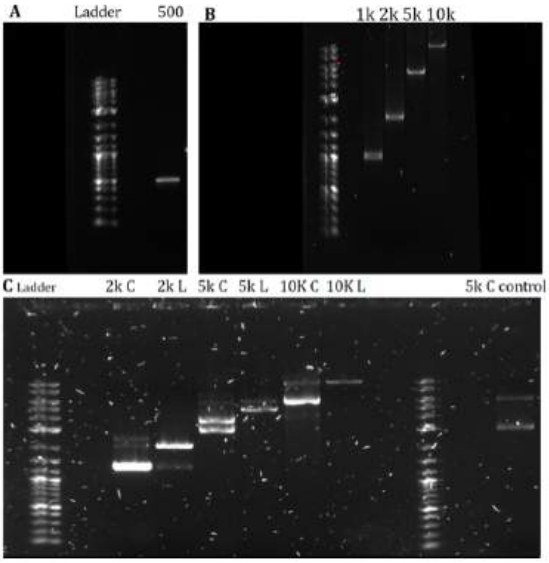


**Figure 1. Measurement of the size of purified DNA products of different lengths and supercoiling state relative to the 1 Kb Plus Ladder.**

(A-B) Gel electrophoresis of the purified DNA products of different lengths after PCR amplification and cleanup. (C) Gel electrophoresis of the purified DNA products of different supercoiling states after selected plasmids of specific lengths were extracted from bacteria, and some undergo a restriction digest. The C represents the circular form of the DNA, while the L represents the linear form of the DNA.

Following the same procedure described above, nine samples were made to assemble the Dps-DNA complexes for imaging. Each sample had the same buffer at the same concentration described above. The sole differences were the lengths of DNA fragments, structure of DNA (circular or linear), and the final DNA concentration. Two samples shared the same size and concentration of DNA fragments but differed in the DNA topology as one consisted of supercoiled DNA, while the other sample consisted of relaxed DNA. This setup was applied to three DNA fragment lengths (2000, 5000, and 10000-bp).

The three control samples included a mock-digested 5000-bp plasmid incubated with EcoR1(a restriction enzyme for which it has no cut sites), a sample with no DNA, and a sample with neither DNA nor Dps. The samples were incubated overnight at 37 °C. The following day, the samples were imaged using fluorescence microscopy. For each sample, images of the condensates were taken at five random positions. Bright-field and green-fluorescent images were used for each position. This procedure was done for three trials.

## Data Analysis Using Software

The green images of the condensates, representing the DNA, were analyzed using a custom Fiji program that detects fluorescence distribution throughout the surface of the Dps-DNA condensates. Fiji sums the intensity distributed throughout a condensate, referred to as integrated density (IntDen), as well as the area and perimeter of the Dps-DNA condensate. Data regarding area and perimeter was used to calculate the circularity of each condensate formation. The formula for circularity that was used was:

$$\frac{12.56 \times (Area\ of\ the\ Condensate)}{(Perimeter\ of\ the\ Condensate)^2}$$

The 12.56 represents 4 times pi, which should equal 1 if the shape is a perfect circle when multiplied by this value. The distance from 1 of this value is commonly used to describe circularity.

## Results

### Binding affinity and cooperativity differences between Dps and circular and linear DNA remain statistically insignificant.

Dps, a protein commonly found in bacteria, typically binds to circular DNA. However, the affinity of Dps for linear DNA has yet to be tested. To test this affinity, we performed a gel shift assay. The bands will appear at similar concentrations if Dps binds to linear or circular DNA with the same affinity.

No binding was observed between Dps and DNA when 0.1 μM Dps was present. The well containing 0.3 μM Dps displayed binding, indicating that the concentration required for Dps to bind to circular DNA is within the 0.1 to 0.3 μM range. The mock digest and linearized plasmid indicate comparable binding affinity (Figure 2A-B).

We calculated the dissociation constant (KD) and Hill coefficient by creating a binding curve. The mock digest and circular DNA fragment shared similar values, with the KD being roughly 0.25 (Figure 2C). The linear DNA fragments had a slightly lower KD value of around 0.23; the slightly lower KD value between Dps and linear DNA fragments indicates that Dps has a slightly higher binding affinity for linear DNA fragments than circular DNA fragments (Figure 2D). However, the difference is statistically insignificant, and does not conform to expectation, as we would expect Dps to bind to circular DNA at a similar or higher affinity. All experimental groups displayed a Hill coefficient greater than 1, indicating that Dps exhibits positive cooperativity whether interacting with circular or linear DNA fragments (Figure 2D).

### The morphologies of the Dps-DNA condensate remain consistent when changing the length of the DNA fragments.

To test the impact of DNA length on the size and morphology of Dps, DNA condensates were created in vitro and imaged with fluorescence microscopy. By analyzing these images, condensate morphology and size could be approximated using

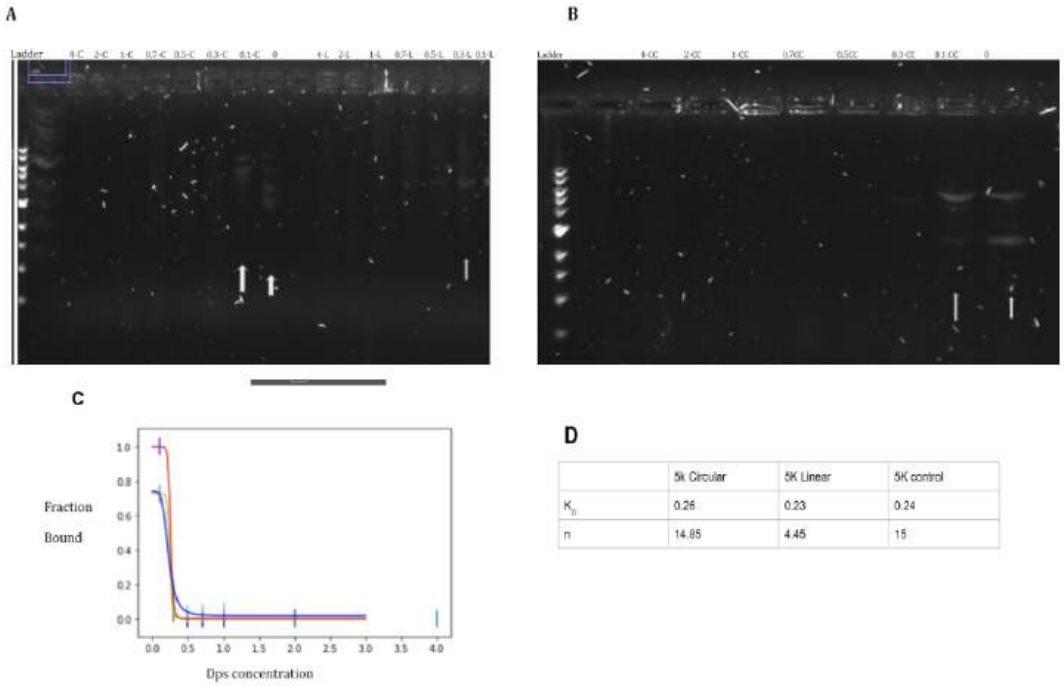


**Figure 2. Dissociation and Binding Cooperativity between Dps and DNA fragments of different supercoiling states. Data provided by Azra Walker**

(A-B) Gel shift displaying the concentration of Dps needed to bind to DNA. The category of the wells can be read as the (concentration of Dps - whether it is circular (C), linear (L), or control group (CC)). (C) The resulting binding curve was obtained using data from the gel shift (D). The dissociation constant and Hill coefficient values were used for the interaction between Dps with the control group, circular DNA fragment, and linear DNA fragment of 5000 in length.

the summed intensity of DNA fluorescence across them. Condensates share the same spongiform morphology regardless of DNA fragment size (Figure 3A-F). This suggests that changing the DNA length does not alter the electrostatic interaction between the Dps and DNA (11), as it was previously discussed that this was one of the main driving forces in forming the condensate morphology (6).

## There is a marked increase in condensate size when formed using intermediate DNA lengths.

While little appears to change morphologically in Dps-DNA condensates when using longer or shorter DNA fragments, there are differences in the level of total fluorescence (representing DNA) inside these condensates. This indicates that altering the DNA's size affected condensate formation but was unrelated to its morphology. The average summed intensity of DNA fluorescence in each condensate was used to represent the average condensate size. Through this analysis, Dps-DNA condensates were observed to be, on average, larger when Dps was incubated with 5000-bp DNA fragments compared to other DNA lengths (Figure 4A-F). This suggests that changing the size of the DNA may not alter the electrostatic interactions between DNA and Dps (7) but could still change the level of accessibility between the DNA fragments and Dps. This, in turn, changes how frequently Dps will find and bind to DNA (5).

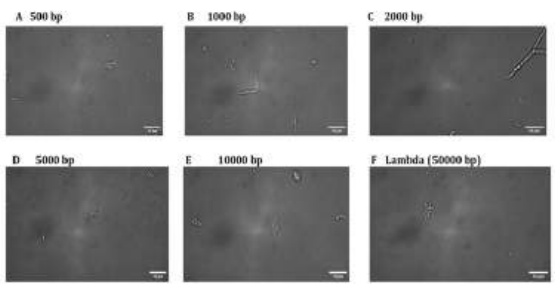


**Figure 3. Dps-DNA condensates adopt spongiform morphologies regardless of differences in DNA fragment lengths**

(A-F) The Dps-DNA condensates formed when Dps is incubated with DNA fragments of different lengths.

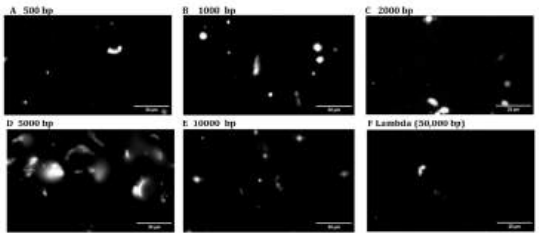


**Figure 4. Fluorescent images of the DNA in condensates formed using different lengths of DNA**

(A-F) Distribution of fluorescence of the Dps-DNA condensate formed from incubating Dps with different lengths of DNA fragments. The level of fluorescence indicates the DNA present in each of the condensates.

## Optimal DNA fragment lengths for Dps-DNA condensate of the largest size and area.

In all three trials, after taking the average integrated density of all the condensates visible in each image, there was a prominent peak in the average integrated density of the Dps-DNA condensate formed from 5000-bp DNA fragments (Figure 5B). This peak suggests that when incubated with 5000-bp DNA fragments, it alters the DNA in a manner that changes the number of binding opportunities Dps can have when in contact with DNA fragments of this length. In addition, all three trials showed the integrated density value of the condensate formed when Dps is incubated with DNA fragments that are shorter and longer than 5000-bp being drastically smaller than the values of that when the condensate is formed with 5000-bp DNA fragments. These lower values indicate a significantly smaller condensate (Figure 5B).

When comparing the average area of the Dps-DNA condensate across all the different DNA fragment lengths used, it can be visibly seen that the average area value for condensates formed when Dps is incubated with 5000- bp DNA fragment was larger than that of other groups (Figure 5A). This indicates that these condensates have larger surfaces, supporting our hypothesis that they are the largest. However, it is essential to note that the error bar does overlap, signifying that there is no significant difference in the area among condensates formed when Dps is incubated with 2000, 5000, and 10000-bp DNA fragments. When comparing the average circularity, there is no significant difference in the circularity of the condensates formed when incubating Dps with different DNA fragment lengths (Figure 5C).

## No significant change in size or structure of Dps-DNA condensate when under different supercoiled states

Samples of DNA molecules of the same size but different topologies were prepared to determine whether DNA supercoiling would affect the morphology and characteristics of resulting Dps-DNA condensates. The fluorescent images of these samples were then analyzed in the same manner as previous images.

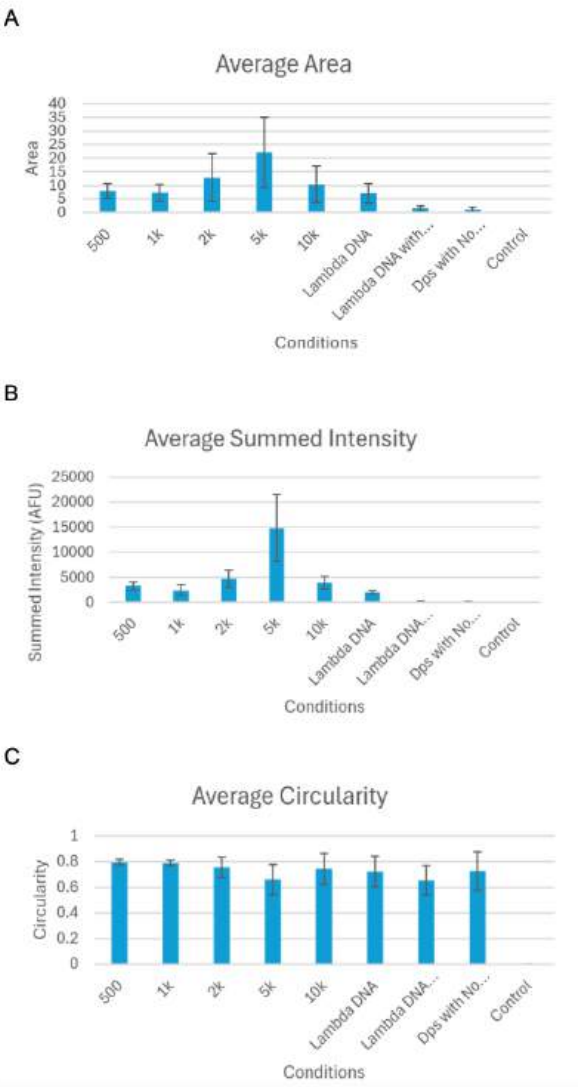


**Figure 5. A graph displaying the average area, summed intensity, and circularity of Dps-DNA condensates present in samples using specific DNA lengths**

(A) Average area of the Dps-DNA condensates formed from each of the different DNA fragment lengths used (B) Average IntDen of the Dps-DNA condensates formed from using different lengths of DNA fragment (C) Average Circularity of the Dps-DNA condensates formed from using different DNA fragment lengths

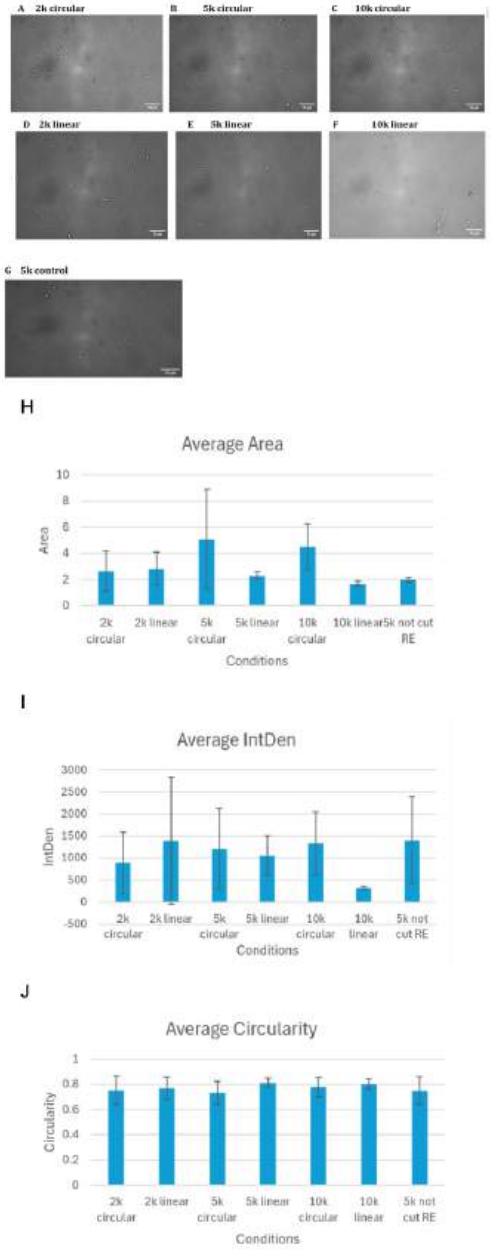


**Figure 6. Condensates formation when Dps is incubated with DNA of different lengths and whether DNA is supercoiled or not**

(A-G) Condensate formation when Dps is incubated with DNA molecules that share the same length but different DNA topology (H) Average area for the Dps-DNA condensates formed under different DNA length and supercoiling states (I) Average IntDen of the Dps-DNA condensates formed under different DNA length and supercoiling states (J) Average Circularity of the Dps-DNA condensates formed under different DNA length and supercoiling states.

Similar to how varying lengths of DNA did not affect the overall morphology of Dps-DNA condensates, the state of DNA molecules (supercoiled or relaxed) had no observable effect on the structure of the condensates, as all condensates took on a spongiform morphology (Figure 6A-6G). When comparing the total average summed integrated density value, there was no significant difference in the size of condensates formed with supercoiled DNA compared to condensates formed with relaxed DNA. Condensates formed when Dps were incubated with 2000-bp circular DNA plasmids were roughly the same size when formed with 2000-bp linear DNA plasmids. The same is true of 5000 and10000-bp plasmids, regardless of whether it was supercoiled or relaxed. (Figure 6I).

There was no significant difference in the total area and circularity among the condensates formed from the circular and linear DNA fragments of varying fragment lengths (Figure 6H and J). This further manifests the lack of notable difference between condensates formed with different supercoiling states. Condensates formed from both the mock-digested 5000-bp plasmid and 5000-bp circular DNA fragments appeared to be relatively similar (Figure 6G). Overall, no significant differences were noted across all experiments comparing condensate formation using same-size DNA molecules, but they differed in supercoiling states (Figure 6A-G).

## Discussion

The findings show that changing the DNA length at our concentration affects the size and number of condensates, without changing the overall structure. This suggests that there is likely some difference in the ability to form complex structures between smaller and larger DNA fragments, which affects the capacity of Dps to bind to these DNA. Samples with larger DNA fragments may be more accessible for Dps to attach to and condense, creating smaller structures. Samples with smaller DNA fragments could condense more effectively up to a point. With more free DNA ends, less DNA needs to wrap around the Dps for condensation to occur (11). The lesser need for DNA bending would mean that it is faster and more efficient for small DNA fragments to wrap themselves around the Dps completely.

DNA fragments smaller than 5000 bp in length have more free DNA ends, helping them condense more easily by the Dps, and DNA fragments larger than 5000 bp in length are more accessible for Dps to attach and condense. DNA fragments that are 5000-bp in length are at a point between where these two effects occur. Our data suggests that DNA fragments of 5000-bp in length are either the most difficult to condense, condense less effectively when done, or both. DNA fragments of 5000-bp may condense less effectively and are harder to condense due to their limited length. Small DNA fragments can result in more effective condensing. Small DNA is short enough that its free DNA end can wrap around the Dps dodecamer without bending. This makes it easier for an array of small DNA fragments to line up, which in turn promotes inter-

actions between the Dps dodecamer and multiple DNA fragments simultaneously. It is thus more likely for all the N-terminus tails in the Dps dodecamer to interact with the DNA. More interaction leads to a higher level of condensing.

Medium-length DNA fragments, like the 5000-bp fragment have ends that aren't long enough to bend completely, unlike longer-length DNA fragments where the free DNA ends are long enough to bend completely to reach the Dps that are already interacting with multiple DNA strands. As a result, the inability to bend completely back will cause the DNA fragment to bend instead, making it harder for the DNA region to be accessible to some sites of the Dps dodecamer. This reduces the number of interactions between Dps and DNA, resulting in less wrapping and less condensing.

Small differences were observed in the overall size of the Dps-DNA condensate between the condensates formed when Dps was incubated with circular plasmids, and the condensates formed when Dps was incubated with linear plasmids. However, the data does not display a significant difference between the sizes of the condensates, indicating that Dps can condense both linear and circular plasmids in roughly the same manner and form. Similar to how the Dps interacts with DNA fragments of different lengths, the overall morphology of the Dps-DNA condensate remains consistently spongiform.

Whether the DNA is in a supercoiled state did not impact the resulting condensate's overall size or structure greatly. This is most likely because supercoiled DNA contains multiple DNA regions interwound that were initially distal to each other (12). Similar to how medium-sized linear DNA fragments may bend in a manner that makes it harder to be accessible to some sites of the Dps dodecamer, distal DNA regions that are interwound multiple times, potentially limit the accessibility of DNA to Dps. As a result, Dps can only interact with DNA in certain regions. With most of the DNA already intertwined with each other, very few DNA will wrap around the Dps, resulting in bigger or similar size condensates as those formed with medium-sized DNA fragments.

Recent research has provided a more in-depth understanding of the structure of Dps itself, how it interacts with DNA to protect it, the different structures the Dps-DNA condensate can take on, and how changing environmental conditions could alter these structures. Although studies have focused on how changes to the environment have affected the interaction between Dps and DNA, there has been minimal research on whether altering one of the key components of the condensate DNA, would change its overall morphology. However, our results have indicated that altering the DNA lengths and supercoiling states does not affect the overall morphology of Dps-DNA condensates. Still, it alters the interaction between the DNA and Dps in a manner that changes some condensate characteristics. These results could have certain implications for non-bacterial organisms. Not all organisms share the same structure and length of DNA as E. coli, which posses circular and significantly longer DNA than other bacteria. Observing how Dps binds to and interacts with these different DNA modifications y helps provide a better understanding of the possible function of Dps in other organisms and environmental conditions. A complete understanding of the role of Dps in other organisms would require extensive testing of additional DNA modifications, such as methylated DNA, and their effect on Dps-DNA interactions.

Please find supplementary materials online at jur.rochester.edu.

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## About the Author

Kevin Zheng recently graduated in May 2024, earning a BS in Biochemistry with a minor in psychology. He started conducting research at the Meyer Lab in the fall of 2022 and contributed to the project involving the effect of different DNA features on the formation of DNA-Dps condensate up until graduation. His undergraduate research interest focuses on better understanding the function of Dps in various organisms. Currently, Kevin is pursuing an EdM at the Harvard Graduate School of Education, where his research examines the impact that mentorship has on the likelihood of undergraduate students pursuing teaching as a postgraduate career.

# Estrogen Receptor α (ERα) Signaling and Tumor Cell-Derived Factors Mediate Pro-Tumor Neutrophil Activation in the Setting of Lymphangioleiomyomatosis (LAM)

**Katherine O'Leary '25 (T5), Erin Gibbons, Briaunna M. N. Minor, Thomas Henson**

Advised by Dr. Stephen R Hammes, *Department of Medicine*

## Abstract

Lymphangioleiomyomatosis (LAM), is a rare disease characterized by the abnormal proliferation of smooth muscle-like cells containing tuberous sclerosis complex (TSC) gene mutations. This proliferation leads to tumor formation and cyst development in the lung parenchyma, resulting in declining respiratory function. Notably, LAM primarily affects biological females and appears to be estrogen-sensitive. Recent work from our laboratory suggests that estradiol not only stimulates TSC-null smooth muscle cells but also promotes neutrophil production via estrogen receptor-alpha (ERα) signaling in the bone marrow, which in turn stimulates TSC-null tumor progression. While prior studies have showed that estradiol influences tumor-induced production of leukocytes, the direct effects of ERα signaling in neutrophils are not well understood. In concert with tumor-derived factors, we hypothesized that estradiol may augment pro-tumorigenic and immunosuppressive functions of neutrophils. To address this, we employed real-time quantitative PCR to assess for ERα-mediated transcription-level expression changes in several genes associated with alternative neutrophil activation in other tumor microenvironments. Our results show that neutrophils cultured in tumor-conditioned media (TCM) require ERα for full expression of neutrophil elastase (Elane) and nitric oxide synthase 2 (Nos2) mRNA, while programmed death-ligand 1 (Cd274) mRNA expression is mediated byTMC) alone. These findings indicate that, in addition to stimulating neutrophil production in bone marrow, estradiol signaling through ERα receptors on neutrophils and tumor-derived factors significantly influences the quality of tumor-exposed neutrophil activation, promoting a pro-tumorigenic immunosuppressive phenotype.

## Introduction

Lymphangioleiomyomatosis (LAM) is a rare multisystem disease that predominantly manifests in the lungs. [1]. It is characterized by the abnormal growth of smooth muscle tumors, which leads to cyst formation in the lungs and the progressive loss of respiratory function [1].

Common symptoms include shortness of breath, coughing, and chest pain, with impacted individuals also being susceptible to recurrent lung collapse. Diagnosis is typically confirmed through a biopsy of lung tissue or lymph nodes, as pulmonary function tests may appear normal in earlier stages of the disease [2, 3]. Computed tomography (CT) scans are also helpful when diagnosing LAM, as they visualize cystic changes in the lungs [2]. Extrapulmonary manifestations of LAM include the presence of benign smooth muscle tumors in the kidney or uterus, lymphadenopathy, and large cystic lymphatic masses (lymphangioleiomyomas) [2]. There are a few notable features of LAM that inform ongoing investigations into its mechanisms. First, genetically, LAM is caused by mutations in the tuberous sclerosis complex (TSC) genes, TSC1 and TSC2, with most cases linked to inactivating mutations in TSC2 [4]. These mutations may occur sporadically or as inherited germline mutations in tuberous sclerosis. Regardless of origin, defective TSC signaling leads to hyperactivation of the mammalian target of rapamycin complex 1 (mTORC1) promoting excessive cell growth and proliferation. The main treatment for this disease includes rapamycin-like drugs which target the hyperactivity of mTORC1 to attenuate disease progression and provide symptom relief [2]. In severe cases of respiratory decline secondary to disease progression, lung transplantation may be required. Some patients experience adverse effects from rapalogs or recurrence of LAM following lung transplantation, highlighting the need for further investigations into the disease mechanism to identify potential therapeutic targets [2].

Second, LAM is considered to be a metastatic disease, as LAM cells have been found in the blood and lymph vasculature of patients. Recurrence in transplanted lungs is also common [1]. Third, LAM exhibits a marked female sexual dimorphism in disease presentation, as it predominantly occurs in biological females and rarely affects males (less than 10 cases of significant disease reported). When it does present in males, it generally has no to mild symptoms [3]. Finally, LAM is an estrogen-sensitive disease, causing it to worsen during reproductive years, with pregnancy, and with oral contraception use, though improving after menopause [1].

The estrogen responsiveness of LAM has been the subject of studies aiming to elucidate the potential benefits of hormone-targeting therapies to combat this disease. For instance, prior preclinical studies have shown that estrogen enhances lung transplantation, highlighting the need for further investigations into the disease mechanism to identify potential therapeutic targets [2].

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The estrogen responsiveness of LAM has been the subject of studies aiming to elucidate the potential benefits of hormone-targeting therapies to combat this disease. For instance, prior preclinical studies have shown that estrogen enhances lung colonization of TSC2-null tumor cells, derived from Eker rat leiomyomas (ELT3), in a SCID.NOD metastasis model [3, 5]. Using the (Progesterone Receptor) PRCre/+ TSC2fl/fl uterine-specific TSC2-null mouse model, which develops LAM-like tumors in the myometrium, our previous work has demonstrated that estrogen removal causes tumor regression [6]. These in vivo data, along with others, highlights that LAM-like tumors are not just estrogen-responsive, but estrogen-dependent [5-8]. Interestingly, while in vivo response to estradiol is significant, recent studies show mild responsiveness of TSC2-null tumor cells to estradiol stimulation in vitro. Using a novel in vitro model of TSC2-null cells derived from the uterine tumors of the TSC2fl/fl PRCre/+ mouse (LTM3 cells), we previously found that these LAM-like tumor cells were transcriptionally reactive to estradiol stimulation, but did not augment pro-tumorigenic functions such as migration, invasion, or proliferation in response to estradiol treatment [3]. With these results in mind, recent studies have shifted to investigating the estrogen sensitivity of other cellular components of the tumor microenvironment to elucidate the mechanisms of estrogen-mediated LAM progression.

LAM tumors have a diverse microenvironment consisting of fibroblasts, adaptive immune cells (B and T lymphocytes), and innate immune cells (monocytes, macrophages, dendritic cells, mast cells, and neutrophils) [1, 9]. Similar to other malignancies, crosstalk within the LAM microenvironment enables these non-tumor cells to support tumor growth through immunosuppressive and direct pro-tumorigenic mechanisms [1, 3].Interestingly, recent single-cell RNA characterization of LAM-diseased lungs revealed that CD45-expressing immune cells expressed a high level of ESR1 (the gene encoding ERα) much like the LAM tumor cells [10]. These data substantiated previously reported modulations in immune cell-related mRNAs in the preclinical TSC2fl/fl PRCre/+ murine model [3, 6]. Specifically, estradiol stimulation in uterine tissues led to increased expression of neutrophil elastase (NE), a serine protease reported to be associated with tumor progression for other malignancies [6, 11, 12]. Further immunophenotyping of myometrial tumors in TSC2fl/flPRCre/+ mice revealed significant increases in CD11b+Ly6CloLy6G+ (neutrophil) cell infiltration into uteri burdened with LAM-like tumors compared to tumor-naïve uteri [11]. These neutrophils were also identified as potent regulators of TSC2-null tumor growth in the myometrium, as evidenced by marked tumor growth reduction following the depletion of myeloid cells, inhibition of their recruitment, and inhibition of neutrophil-derived neutrophil elastase (NE) [11]. It is well-established that neutrophils play a crucial role in fighting infections [13, 14]. However, research suggests that environmental stimuli can modulate neutrophil functions, allowing them to utilize a diverse repertoire of functions that, in some instances, can support disease progression [15]. In cancer, neutrophils have been shown to promote tumor growth by releasing a variety of different factors that stimulate tumor cells directly, such as Mmp9 and ELANE, or by supporting evasion of cytotoxic immune cells via immunosuppressive means through Nos2, Cd274, and S100a9 [12, 15-18]. Given the evidence implicating both estrogen and neutrophils as drivers of LAM-like tumor progression, understanding how estrogens influence neutrophil accumulation and function in the LAM tumor microenvironment is critical. Our lab has previously demonstrated that estradiol increases neutrophil levels, facilitating greater colonization of LAM-like tumors in the lungs of SCID.NOD mice [3]. Neutrophil plasticity, which allows for the versatility of neutrophil functions and phenotypes,likely supports neutrophil and tumor cell clusters [19]. Since neutrophils play a role in estrogen-mediated lung colonization, the influence of estrogen on neutrophil plasticity is an important feature of neutrophils that must be explored when looking at the LAM-microenvironment. Based on the all the data mentioned, we hypothesize that estradiol signaling through estrogen receptor-alpha on neutrophils is promoting a pro-tumorigenic immunosuppressive phenotype, which in turn supports the pro-

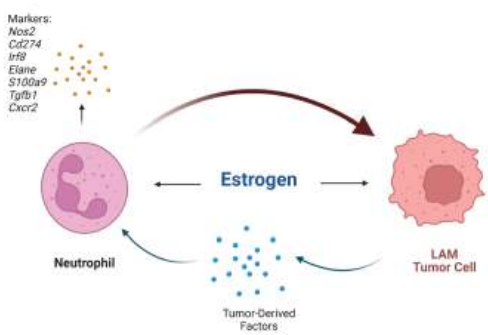


**Figure 1. The model of crosstalk between Neutrophils and LAM cells is modulated by estrogen.**

We hypothesize that estrogen supports the crosstalk between LAM tumor cells and neutrophils in the tumor microenvironment, which in turn supports the progression of LAM. LAM tumor cells secrete tumor-derived factors that alter the neutrophil phenotype, making the neutrophils express pro-tumorigenic and immunosuppressive markers that support the tumor cells.

gression of LAM. Supporting this, our results indicate that ERα positively influences the expression of neutrophil elastase (Elane) and nitric oxide synthase 2 (Nos2), markers of neutrophil activation, to support tumor progression.

## Materials and Methods

### Mouse Studies

Mouse studies were performed following AALAC guidelines and approved by the University of Rochester Committee on Animal Resources (UCAR).

### Tumor Cell Culture and Reagents

The LAM-like TSC2-null Myometrial (LTM3) cells are derived from TSC2-null myometrial tumors of TSC2fl/fl PRCre/+ mice [4]. LTM3 cells were maintained in DF8 media with 10% FBS and were incubated in incubators at 37ºC in air humidified with 5% CO2 and passaged every 4-5 days. Tumor-conditioned media (TCM) was collected after culturing the LTM3 cells to confluency in regular DF8 with 10% FBS.

### Neutrophil Isolation

Bone marrow was harvested from both the tibia and femur of three 18-week-old C57BL/6J (#000664, Jackson Laboratory) or B6N(Cg)-ESR1tm4.2Ksk/J ERα-null (#026176, Jackson Laboratory) mice and pooled for neutrophil isolation. The bone marrow was then placed into ACK lysis buffer to remove blood cells, neutralized, passed through a 40mm filter, and resuspended in FACs buffer. Single-cell suspensions of filtered bone marrow were stained with anti-Ly6G microbeads (#130120337, Miltenyi), and neutrophils were isolated according to the protocol for MS column MACS positive-selection separation (#130042201, Miltenyi). Once separated, neutrophils were counted and resuspended in the appropriate media at 1x106 neutrophils per mL.

### Neutrophil Stimulation Cultures

1x106 neutrophils per mL were seeded into 6-well plates with either DF8 media supplemented with 10% FBS or TCM. Stock Fulvestrant (#ICI182780, MedChemExpress), an estrogen receptor antagonist, was diluted to a working concentration of 1 mM using DMSO and stored in 10 mL aliquots at -80°C. Neutrophils were stimulated with 100 nM Fulvestrant or vehicle (DMSO). Cultures were incubated for 24 hours at 37°C in air humidified with 5% CO2.

### Western Blot

In preparation for Western Blot, samples were homogenized in RIPA buffer (#89900, Thermo Fisher Scientific, Waltham, MA) supplemented with 1×Halt™ protease and phosphatase-inhibitor cocktail (7843036, Thermo Fisher Scientific) and mixed with 4x-sample buffer. Samples were mixed with 4x sample buffer, boiled for five minutes, and were then loaded into gradient polyacrylamide gels to separate protein (4561084, Bio-Rad, Hercules, CA). Samples were then transferred to PVDF membranes (#1620177, Bio-Rad), and blots were blocked using 5% milk in TBST. The primary antibody was 1:1000 Erα (8644S, Cell Signaling). Secondary antibodies included 1:4000 Goat anti-rabbit (#1706515, Bio-Rad). Clarity Western ECL Substrate (1705062, Bio-Rad) was used to detect the blot.

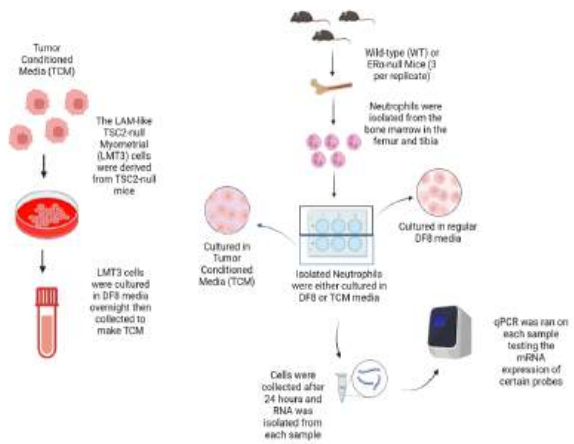


**Figure 2. Transforming Growth Factor Beta 1 (Tgfβ1), S100 Calcium Binding Protein A9 (S100a9), and Interferon Regulatory Factor 8 (Irf8) mRNA expression does not change in both WT and KO mouse models cultured in DF8 or TCM.**

Transforming Growth Factor Beta 1 (Tgfβ1) (a), S100 Calcium Binding Protein A9 (S100a9) (b), and Interferon Regulatory Factor 8 (Irf8) (c) mRNA expression was measured for all cultured neutrophils taken from the bone marrow from either wild-type (C57BL/6J) or Erα null mice. Neutrophils were cultured in either DF8 media or tumor-conditioned media (TCM). For wild-type mice, n = 6, while for Erα null mice, n = 3. For each point, three biological replicates were used and two technical replicates were run. The data plotted is represented as means ± SEM. Unpaired t-test determined the significance between conditions with and without the presence of estrogen receptor α. ns $p > 0.05$, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

### Real-Time Quantitative PCR

Total RNA was extracted using the RNeasy Plus Mini Kit (74134, QIAGEN, Hilden, Germany). RT-qPCR reactions used qScript RT-qPCR Tough Mix (89236-672, Quantabio, Beverly, MA) and TaqMan primers (Applied Biosystems, Waltham, MA). The StepOne plus Real-Time PCR system (Applied Biosystems) was used. The Taqman Primers used were mouse GAPDH (loading control, Mm99999915_g1), mouse ELANE (Mm00469310_m1), mouse CXCR2 (Mm00438258_m1), mouse Nos2 (Mm00440502_m1), mouse IRF8 (Mm00492567_m1), mouse Cd274 (Mm03048248_m1), mouse S100a9 (Mm00656925_m1), and mouse Tgfb1 (Mm01178820_m1). Samples were tested in duplicates for the target gene expression. After, mRNA threshold cycle (ΔΔCT) values were normalized to GAPDH levels and represented the relative mRNA.

### Statistical Analysis

GraphPad PRISM 10 software was then used to express these results. Each probe was normalized to GAPDH levels for the specific day it was run, and then relative values were graphed. Unpaired t-tests were performed to determine the significance between the mean CT values, and differences in the means were compared using Tukey's multiple comparisons test. Significance was denoted by ns ($p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

## Results

### Neutrophil Elastase (Elane) and Nitric Oxide Synthase 2 (Nos2) expression in neutrophils from the bone marrow are enhanced by ERα signaling.

Prior work has shown that estradiol stimulation in uterine tissues increased the expression of Neutrophil Elastase (Elane), which has pro-tumorigenic properties [6, 11, 12]. Additionally, Nitric Oxide Synthase 2 (Nos2) expression in cancer cells predicts poor outcomes, as it increases chemoresistance, metastasis and has immunosuppressive functions [17]. Both markers are predicted to be expressed by neutrophils in the presence of estrogen signaling in LAM. In this study, we used real-time quantitative polymerase chain reaction (RT-qPCR) to measure the mRNA expression of the genes Elane and Nos2 in both wild-type and ERα knockout mice in two conditions: DF8 media and tumor-conditioned media (TCM). Our results indicate that the expression of both Elane (Figure 3a) and Nos2 (Figure 3b) was dependent on ERα signaling in neutrophils. For Elane expression (Figure 3a), significant difference was observed between the WT and KO mice in DF8 conditions (*$p < 0.05$), while the difference in TCM was trending towards significance ($p = 0.0807$). For Nos2 expression (Figure 3b), there were significant differences between the baseline expression in WT and KO mice in both the DF8 and TCM conditions (*$p < 0.05$). These baseline differences in Elane and Nos2 indicate that ERα signaling increases the expression of these genes, potentially promoting a pro-tumorigenic neutrophil phenotype.

### Programmed Cell Death Ligand 1 (PD-L1, Cd274) expression is mediated by the presence of tumor-conditioned media, rather than ERα signaling.

Programmed Cell Death Ligand 1 (PD-L1), also known as Cd274, is a checkpoint inhibitor that binds to PD-1 receptors on T cells, preventing T cells from carrying out anti-tumor functions [18, 20]. Prior studies have shown that Cd274 is expressed by immune cells, including neutrophils in tumor microenvironments, and inhibits anti-tumor T cell functions to promote tumor progression [18]. In this study, our results demonstrate that Cd274 mRNA expression (Figure 2a) increased in the presence of TCM, regardless of whether the cells were isolated from WT or ERα-null mice. The difference in expression between DF8 and TCM conditions for the WT mice was trending towards significance ($p = 0.0787$).In ERa-null mice, Cd274 expression was significantly elevated in the presence of TCM (*$p < 0.05$). There was also a slight but significant increase in the expression of Cd274 mRNA in the

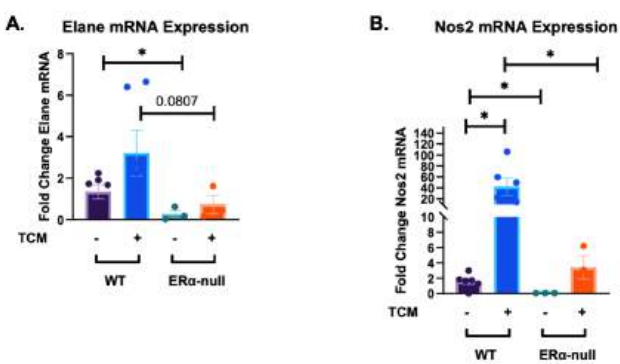


**Figure 3. Neutrophil Elastase (Elane) and Nitric Oxide Synthase 2 (Nos2) mRNA expression are influenced by estrogen signaling in both DF8 media and tumor-conditioned media (TCM).**

Neutrophil Elastase (Elane) mRNA expression (a) and Nitric Oxide Synthase 2 (Nos2) mRNA expression (b) were measured in cultured neutrophils taken from the bone marrow of either either wild-type (C57BL/6J) or Erα null mice. Neutrophils were cultured in either DF8 media or tumor-conditioned media (TCM). For wild-type mice, n = 6, and for Erα null mice, n = 3. Each data point represents the mean of three biological replicates, with two technical replicates run for each. Data are represented as means ± SEM. Unpaired t-tests determined the significance between conditions. ns $p > 0.05$, *$p < 0.05$.

KO mice in DF8 media, indicating that estrogen signaling may decrease the expression of Cd274. However, there was no significant difference in the TCM condition between both mice. ($p > 0.05$).

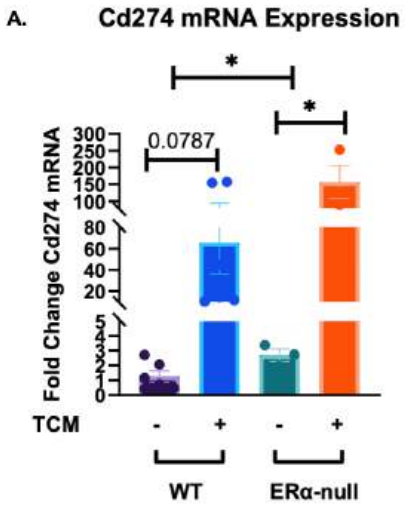


**Figure 4. Increases in Programmed Cell Death Ligand 1 (PD-L1), also known as Cd274 mRNA expression is tumor-conditioned media mediated in both wild-type and ERα-null (KO) mice.**

PD-L1 mRNA expression (a), was measured for all cultured neutrophils taken from the bone marrow from either wild-type (C57BL/6J) (n = 6) or Erα null mice (n = 3). Neutrophils were cultured in either DF8 media or tumor-conditioned media (TCM). Each data point represents the mean of three biological replicates, with two technical replicates performed for each.The data plotted is represented as mean ± SEM. Unpaired t-test determined the significance between conditions with and without the presence of estrogen receptor α. ns $p > 0.05$, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

mice. There was no significant difference in Cxcr2 expression between the DF8 and TCM conditions in either mouse model, suggesting that mRNA expression is not affected by TCM but is inhibited by ERα signaling possibly.

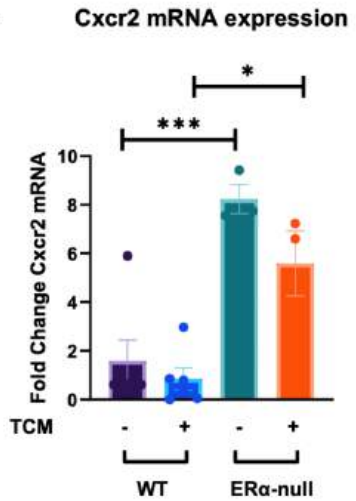


**Figure 5. C-X-C Motif Chemokine Receptor 2 (Cxcr2) mRNA expression is not decreased in the presence of ERα signaling in both wild-type and knockout mice.**

C-X-C Motif Chemokine Receptor 2 (Cxcr2) mRNA expression (a) was measured for all cultured neutrophils taken from the bone marrow from either wild-type (C57BL/6J) or Erα null mice. Neutrophils were cultured in either DF8 media or tumor-conditioned media (TCM). For wild-type mice, n = 6, while for Erα null mice, n = 3. For each point, three biological replicates were used and two technical replicates were run. The data plotted is represented as means ± SEM. Unpaired t-test determined the significance between conditions with and without the presence of estrogen receptor α. ns $p > 0.05$, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

### C-X-C Motif Chemokine Receptor 2 (Cxcr2) mRNA expression is decreased in the presence of ERα signaling.

Previous studies have shown that C-X-C Motif Chemokine Receptor 2 (Cxcr2) is upregulated in various cancers and is associated with poorer prognosis, indicating some pro-tumorigenic effects such as promoting tumor cell proliferation [21]. Cxcr2 affects neutrophil maturation and function and is expressed by neutrophils in both mice and humans [22]. In addition, previous work from our lab has shown that Cxcr2 inhibition decreases uterine weight in the uterine-specific TSC2-null mouse model [11]. In this study, our results indicated that Cxcr2 expression is greatly decreased in the presence of ERα signaling in both DF8 and TCM conditions. There was a significant decrease in expression between the neutrophils cultured in DF8 media (***$p < 0.001$), as well as between the neutrophils cultured in TCM (*$p < 0.05$) in the wild-type

### Transforming Growth Factor Beta 1 (Tgfb1), S100 Calcium Binding Protein A9 (S100a9), and Interferon Regulatory Factor 8 (Irf8) mRNA expression are not affected by the presence of ERα or tumor-conditioned media (TCM).

PAll of these markers: Tgfb1, S100a9, and Irf8, are known to be pro-tumorigenic or immunosuppressive markers. Tgfb1 is highly expressed in the setting of some cancers and supports the growth and metastasis of tumor cells [23]. S100a9 is involved in the inflammatory pathways and migration in multiple types of myeloid cells [24]. Finally, Irf8 is a transcription factor that contributes to the development of myeloid cells and is involved in the inflammatory response [25]. Our results indicated that ERα signaling does not affect the mRNA expression of any of these genes. There were no significant increases or decreases between the mouse models or culture conditions for Tgfb1 (Figure 6a), S100a9 (Figure 6b), or Irf8 (Figure 6c).

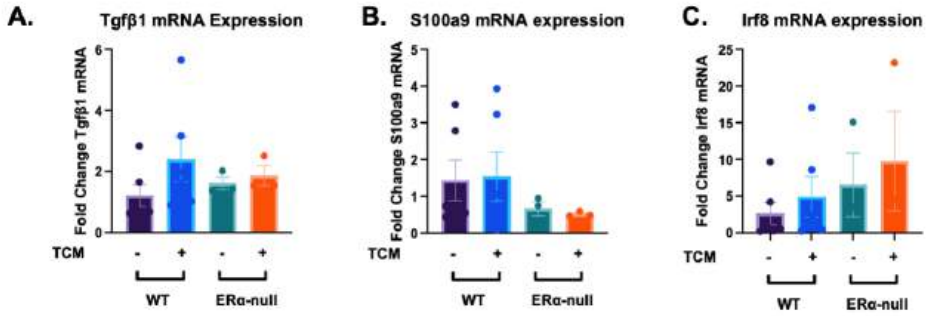


**Figure 6. Transforming Growth Factor Beta 1 (Tgfβ1), S100 Calcium Binding Protein A9 (S100a9), and Interferon Regulatory Factor 8 (Irf8) mRNA expression does not change in both WT and KO mouse models cultured in DF8 or TCM.**

Transforming Growth Factor Beta 1 (Tgfβ1) (a), S100 Calcium Binding Protein A9 (S100a9) (b), and Interferon Regulatory Factor 8 (Irf8) (c) mRNA expression was measured for all cultured neutrophils taken from the bone marrow from either wild-type (C57BL/6J) or Erα null mice. Neutrophils were cultured in either DF8 media or tumor-conditioned media (TCM). For wild-type mice, n = 6, while for Erα null mice, n = 3. For each point, three biological replicates were used and two technical replicates were run. The data plotted is represented as means ± SEM. Unpaired t-test determined the significance between conditions with and without the presence of estrogen receptor α. ns $p > 0.05$, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

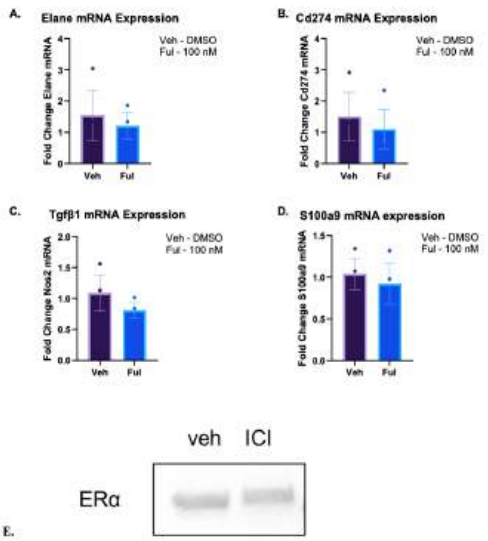


**Figure 7. mRNA expression is not affected by Estradiol Antagonist Fulvestrant in Elane, Cd274, Tgfb1, and S100a9.**

Neutrophils in DF8 were cultured for 24 hours with either DMSO or Fulvestrant. RNA was collected and qPCR was run. For each experiment, 3 biological replicates were done and 2 technical replicates were run for qPCR. The data plotted is represented as ± SEM for Elane (a), Cd274 (b), Tgfb1 (c), and S100a9 (d). Unpaired t-test determined the significance between conditions with and without the presence of estrogen receptor α. A western blot was also run to see whether the expression of ERα decreased in the presence of Fulvestrant (e).

**Treating neutrophils with Fulvestrant does not significantly alter mRNA expression in Neutrophil Elastase (Elane), in Programmed Cell Death Ligand 1 (PD-L1), also known as Cd274, Transforming Growth Factor Beta 1 (Tgfβ1), S100 Calcium Binding Protein A9 (S100a9).**

All of the markers mentioned above possess either pro-tumorigenic or immunosuppressive properties. Neutrophils were cultured in either Veh (DMSO) or Ful (Fulvestrant) for 24 hours and RNA was harvested. Our results showed that there was no significant difference in mRNA expression of Elane (Figure 7a), Cd274 (Figure 7b), Tgfb1 (Figure 7c), and S100a9 (Figure 7d) ($p > 0.05$ for all). Some of the mRNAs showed a non-significant downward trend in the presence of Fulvestrant, an estradiol antagonist. Western blot showed a slight decrease in expression of ERα in the the presence of Fulvestrant, an estradiol antagonist. Western blot showed a slight decrease in expression of ERα in the presence of Fulvestrant, however, these results lack a loading control (Figure 7e).

## Discussion

Estrogen is a well-known stimulatory signal for some cancers. Prior studies have shown that estrogen is involved in the progression of multiple types of cancers including prostate, breast, and endometrial cancer in vivo and in vitro [26-30]. For example, cervical cancer patients with elevated estradiol levels were shown to have shorter progression-free survival rates and lower overall survival rates [26]. As mentioned earlier, LAM is an established estrogen-sensitive disease as demonstrated through multiple in vivo and in vitro studies.

Estrogen has been shown to enhance lung colonization of TSC2-null ELT3 cells when mice were injected with tumor cells through the tail vein [5]. Furthermore, estrogen removal in a uterine-specific TSC2-null mouse model caused LAM-like tumors to shrink [6].

While in vivo studies involving TSC2-null cell lines have shown some transcriptional changes with estrogen, estrogen had minimal effects on cell proliferation, migration, and invasion, indicating that estrogen does not have as much of an effect in vitro as in the in vivo studies mentioned above [3, 6, 31]. To explain these observations, it is hypothesized that estrogen supports LAM disease progression by changing the function of immune cells in the disease setting. Our previous work showed that estrogen treatment in C57BL/6J mice increased both the presence of neutrophils and neutrophil elastase, a serine protease that promotes tumor growth and pro-inflammatory activities [32]. Furthermore, immunotyping of WT and uterine-specific TSC2-null mice revealed increased neutrophil accumulation in blood, bone marrow, lungs, spleen, and uteri, suggesting the LAM microenvironment is filled with neutrophils [3, 11]. Finally, our lab showed that estrogen alters the neutrophil phenotype and changes gene expression to promote tumor progression in LAM. [3]. These findings emphasize the importance of studying how estrogen signaling can influence the LAM microenvironment, and as a result, tumor growth and metastasis.

In this study, we proposed ERα signaling and tumor-derived factors promote a pro-tumorigenic, immunosuppressive neutrophil phenotype in the setting of LAM. We chose to measure specific mRNA markers because the proteins they encode have both immunosuppressive and pro-tumorigenic properties, and their expression in neutrophils would support our hypothesisUltimately, we found that Elane (Neutrophil Elastase), and Nos2 (Nitric Oxide Synthase 2) expression could depend on signaling through ERα. Since Elane and Nos2 are pro-inflammatory enzymes, this study shows that estradiol through ERα signaling could promote neutrophils to express inflammatory enzymes. Tumor-derived factors may have an effect as well, but this effect was not as prominent as ERα signaling for these two markers.

Cd274/PD-L1 (Programmed Cell Death Ligand 1) was found to be dependent on the tumor-derived factors in TCM. This dependence was further observed when we compared the values between tumor-conditioned media in the wild type and ERα-null mice. There was a clear difference in the levels of expression with and without estrogen receptors. Since PD-L1 is an immunosuppressive marker, immunosuppression could be better regulated by tumor-derived factors rather than ERα signaling.

Interestingly, the marker Cxcr2 indicates that estrogen signaling suppresses the full expression of these markers in both DF8 and TCM conditions. Estradiol may suppress neutrophil migration in response to chemokines as an activated neutrophil does not need to migrate since it is differentiated.

## Acknowledgments

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## About the Author

Katie is a Take5 student majoring in Biology and East Asian Studies. She is interested in the intersection of cancer biology and immunology. Katie finds it fascinating how environmental factors can influence the immune system and how its responses vary from person to person. She has been working in the Hammes Lab (URMC, Endocrinology and Metabolism) since junior year, and spent a summer in working Rowe Lab (URMC, Pediatric Infectious Disease) as well.

# Exploring Predictive Factors for Heart Disease: A Comprehensive Analysis Using Logistic Regression

**Haolin Wang '25**

Advised by Dr. Aruni Jayathilaka, *Department of Mathematics*

## Abstract

This study investigates the predictive factors for heart disease utilizing logistic regression analysis on a dataset containing various health indicators. By using a backward elimination approach and Akaike Information Criterion (AIC) for model selection, significant predictors such as sex, chest pain type, blood pressure, cholesterol level were identified. Assumption checks confirmed the model's validity. Visualizations and statistical summaries provided insights into the relationships between heart disease occurrence and key predictors. The final logistic regression model, validated by ROC curve and a confusion matrix, demonstrates a robust predictive capability. The primary contribution of this study lies in demonstrating logistic regression techniques and model validation through tools like ROC curves and confusion matrices. This research thus serves as a tutorial on statistical modeling of heart disease risk factors.

## Introduction

Heart disease is still a significant issue in the global health field. Inside the US, more than 690,000 people die of heart disease each year and more than 800,000 people will experience a heart attack. Thus, understanding predictive factors is crucial for effective prevention and diagnosis [6]. In this study, we analyze a comprehensive dataset relating to heart disease from UCI Machine Learning Repository [8]. It comprises records from four databases collected from Cleveland, Hungary, Switzerland, and the VA Long Beach. It includes 13 features, focusing on attributes such as age, sex, chest pain type, and cholesterol levels, among others. These features are represented in categorical and integer. Logistic regression is used to explore the impact of various factors on heart disease. The methods include data preprocessing, model selection using Akaike Information Criterion (AIC), and model interpretation.

In developing a logistic regression model to predict heart disease, our approach aligns with Zhang, Diao, and Ma, who demonstrated logistic regression's effectiveness in heart disease prediction among the elderly, emphasizing the model's potential in clinical settings [5]. Furthermore, Anshori and Haris supported our findings by highlighting logistic regression's high accuracy in diagnosing heart disease using comprehensive patient medical records [4]. Beyond heart disease, logistic regression has been effectively applied in other medical fields. For example, Nusinovici compared logistic regression with machine learning algorithms in predicting chronic diseases such as diabetes and hypertension, showing that logistic regression often performs well [3]. These references collectively reinforce the applicability and efficacy of logistic regression models in the field of medical prediction and diagnostics, providing a strong foundation for our study.

The article identifies some key features related to heart disease, including sex, max. HR, type of chest pain, blood pressure, cholesterol level, the number of vessels shown in coronary angiography, ST depression, and slopes of ST and Thallium were identified as key factors that significantly influence heart disease occurrence. The logistic regression model constructed through a backward elimination approach allowed us to quantify the relative impact of each variable on the probability of heart disease.

In the model evaluation section, we introduced the AIC as a powerful tool for model selection, balancing goodness of fit and model complexity[7]. Furthermore, the concept of the confusion matrix was explained, along with the calculation of metrics such as accuracy and precision[1]. These metrics comprehensively assessed the performance of the logistic regression model in predicting heart disease.

## 1. Logistic Regression

### 1.1 Introduction to the Binary Logistic Regression Model

Logistic regression is a powerful statistical learning model used for binary classification problems. The fundamental idea of the model is to map the output of a linear regression model through a logistic function, transforming predictive values into probability values, which are then used to make classification decisions [1]. In this section, the main purpose is to introduce the core concepts of logistic regression, including the definition of the model and its basic assumptions, and we will derive the estimation process of the model parameters in detail using the maximum likelihood estimation method.

**Definition of Logistic Regression:**

Logistic regression is a statistical model used to depict the probability of a binary outcome, often coded as 0 or 1, based on one or more predictor variables. The main goal of logistic regression is to determine the likelihood that a particular event will occur, as a function of the input variables. The probability that an event occurs, represented as P (Y = 1), is given by the

formula:

$$P(Y=1)=\frac{1}{1+e^{-(\beta_0+\beta_1X_1+\beta_2X_2+\ldots+\beta_kX_k)}}$$

P(Y = 1) is the probability of the event occurring.

e is the base of the natural logarithm.

β0, β1, . . . , βk are the coefficients of the model, indicating the impact of each input variable on the probability of the event occurring.

X1, X2, . . . , Xk are the values of the input variables.

**Derivation of Maximum Likelihood Estimation:**

To estimate the parameters of the logistic regression model, this paper uses the maximum likelihood estimation method[1]. The objective of maximum likelihood estimation is to find a set of parameters that maximize the likelihood function for the observed sample data. For logistic regression, the likelihood function is:

$$L(\beta_0,\beta_1,\ldots,\beta_n)=\prod_{i=1}^{N}P(Y_i=y_i)^{y_i}\cdot(1-P(Y_i=y_i))^{1-y_i}$$

$i=1$

N is the number of samples.

Yi is the actual category of the ith observed sample (0 or 1).

yi is the probability of the predicted category for the ith sample, given by the logistic regression model.

P(Yi = yi) is the predicted probability that the outcome Yi is equal to yi.

The goal of the likelihood function is to maximize this probability, or more specifically, to find the model parameters β0, β1, . . . , βk, that make the probability of observing the data the highest. Usually, for the convenience in calculation, the natural logarithm of the likelihood function is taken, resulting in the log-likelihood function:

$$\mathrm{L}(\beta_0,\beta_1,\ldots,\beta_k)=\sum_{i=1}^{K}[y_i\log(P(Y_i=1))+(1-y_i)\log(1-P(Y_i=1))]$$

Logistic regression usually uses an iterative method (such as gradient descent) to initiate maximum likelihood estimation to obtain the coefficients of the model.

## 2. Interpretation of the Fitted Logistic Regression Model

### 2.1 Akaike Information Criterion (AIC)

The AIC is a statistical criterion for model selection, proposed by the Japanese statistician Hirotugu Akaike. The core idea of AIC is to balance the evaluation of model fit and complexity. It considers the fit of the model to the data and complexity of the model to avoid overfitting.

The formula for calculating AIC:

AIC = 2 × log-likelihood + 2 × number of parameters

Here, the log-likelihood is the value of the log-likelihood function for the model fitted to the observed data, and the number of parameters is the count of parameters in the model. The goal of AIC is to minimize its value, as it takes into account both the goodness of fit of the model and its complexity.

### 2.2 Confusion Matrix and Accuracy

The confusion matrix is a table used to evaluate the performance of models, particularly suitable for binary classification problems. Through the confusion matrix, we can calculate the following metrics:

$$\text{Accuracy}=\frac{\text{True Positive}+\text{True Negative}}{\text{True Positive}+\text{True Negative}+\text{False Positive}+\text{False Negative}}$$

$$\text{Precision}=\frac{\text{True Positive}}{\text{True Positive}+\text{False Positive}}$$

$$\text{Recall}=\frac{\text{True Positive}}{\text{True Positive}+\text{False Negative}}$$

$$\text{F1 Score}=\frac{2\times\text{Precision}\times\text{Recall}}{\text{Precision}+\text{Recall}}$$

## 3. Dataset

### 3.1 Summary of the Dataset

This study uses a dataset on heart disease to investigate factors associated with the risk of heart disease. The dataset contains multiple key variables, which are believed to be potentially related to the occurrence of heart disease.

**Variable Definition:**

1. Sex: Represents the individual's biological sex, categorized as male or female (0: female, 1: male).

2. Age: Records the age information of each individual (29 - 77 years).

3. Chest Pain Type: Describes the type of angina pectoris (1: typical angina, 2: atypical angina, 3: non-anginal pain, 4: asymptomatic).

4. Blood Pressure (BP): Measures the individual's blood pressure level (94 - 200 mmHg).

5. Cholesterol Level: Records the individual's cholesterol level (126 - 564 mg/dl).

6. Fasting Blood Sugar (FBS): Indicates whether the individual's blood sugar level exceeds 120 mg/dl when fasting (0: No, 1: Yes).

7. EKG Results: Describes the findings of the electrocardiogram, (0: normal, 1: ST-T wave abnormality, 2: probable or definite left ventricular hypertrophy).

8. Maximum Heart Rate (Max HR): Records the maximum

heart rate achieved by the individual (71 - 202 beats/minute).

9. Exercise Angina: Indicates whether the individual experiences angina during exercise (0: No, 1: Yes).

10. ST Depression: Measures the level of ST segment depression (0 - 6.2).

11. Slope of ST: Describes the slope characteristics of the ST segment (1: up-sloping, 2: flat, 3: down-sloping).

12. Number of Vessels in angiography: Records the number of vessels shown in coronary angiography (0 - 3).

13. Thallium Test Results: Describes the results of the Thallium test (3: normal, 6: fixed defect, 7: reversible defect).

14. Heart Disease: Whether a person has heart disease (0: No, 1: Yes).

Through the analysis of these variables, this paper aims to establish a model for predicting the probability of heart disease occurrence, to gain a deeper understanding of the impact of various factors on the risk of heart disease.

## 3.2 Data Visualization

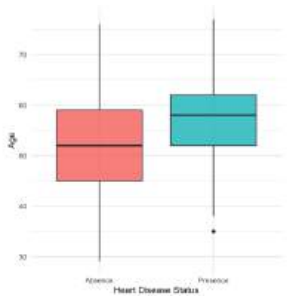


**Figure 1.** Boxplot of Age Distribution

**1. Age and the occurrence of heart disease: (Fig.1)**

Visualization results: The boxplot of age versus the occurrence of heart disease shows that the median, first, and third quartile of individuals with heart disease are higher than individuals without heart disease, and individuals with heart disease exhibit a left-skewed distribution.

Statistical results: The average age of individuals without heart disease is 52.7, with a median age of 52; for individuals with heart disease, the average age is 56.6, with a median age of 58. Based on the results above, it suggests that older people might have a higher chance to gain heart disease.

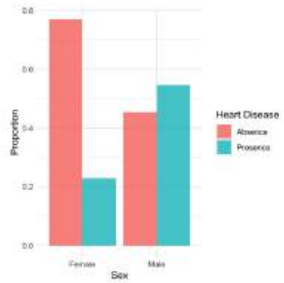


**Figure 2.** Sex Barplot

**2. Sex and the occurrence of heart disease: (Fig.2)**

Visualization results: The bar chart of sex versus the occurrence of heart disease shows that there are more cases of heart disease among males than females.

Statistical results: The probability of males having heart disease in our sample is 54.64%, compared to females (22.99%).

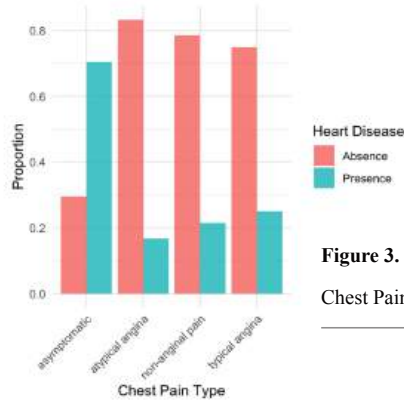


**Figure 3.** Chest Pain Type Barplot

**3. Chest pain type and the occurrence of heart disease: (Fig.3)**

Visualization results: The bar chart of chest pain type versus the occurrence of heart disease shows that among individuals with chest pain type 4(asymptomatic), the rate of having heart disease is apparently higher than the other three types.

Statistical results: The probability of heart disease cases with chest pain type 4 is 70.54%, compared to other three types (25.00%, 16.67%, 21.52%).

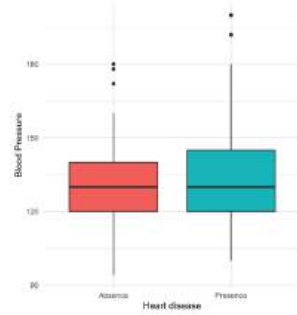


**Figure 4.** Boxplot of Blood Pressure

**4. Blood pressure and the occurrence of heart disease: (Fig.4)**

Visualization results: The boxplot of blood pressure versus the occurrence of heart disease shows that there is no significant difference between individuals with and without heart disease.

Statistical results: The average blood pressure of individuals without heart disease is 128.87, with a median age of 130; for individuals with heart disease, the average age is 134.442, with a median age of 130. Based on the results above, it suggests that blood pressure might have no impact on the occurrence of heart disease.

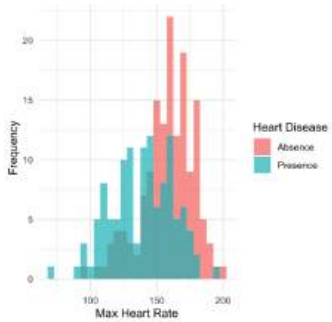


**Figure 5.** Max Heart Rate Distribution Plot

**5. Maximum heart rate and the occurrence of heart disease: (Fig.5)**

Visualization results: The histogram of maximum heart rate versus the occurrence of heart disease shows that individuals with heart disease tend to have a relatively lower maximum heart rate.

Statistical results: The average maximum heart rate for individuals without heart disease is 158, with a median of 161; for individuals with heart disease, the average maximum heart rate is 139, with a median of 142.

## 4. Model Selection

### 4.1 Exploring the Full Model

In the process of fitting the full model, this paper adopts a binary logistic regression model, aiming to gain a deeper understanding of the characteristics of the heart disease dataset. The full model includes multiple predictive variables, among Sex, age, chest pain type, and other key factors. This section will detail the steps of fitting the full model, model selection by backward elimination, and various aspects of reduced model.

### 4.2 Binary Logistic Regression Model

First, the paper reviews the definition and basic assumptions of the binary logistic regression model. The logistic regression model is a statistical learning model for binary classification problems, used to predict the probability of occurrence of a certain event. The basic form of the model is as follows:

$$\text{logit}(p) = \beta 0 + \beta 1X1 + \beta 2X2 + \ldots + \beta kXk$$

p is the probability of the presence of the characteristic of interest (e.g. having heart disease).

1-p is the odds ratio (OR) of the characteristic of interest occurring or not. Xj represents the independent variables (predictors).

βk represents the coefficients that measure the impact of predictors.

| coefficients | | | | |
|---|---|---|---|---|
| | estimate | std. error | z value | p value |
| (intercept) | -6.970 | 3.150 | -2.213 | 0.027 |
| Male | 1.763 | 0.581 | 3.036 | 0.002 |
| Age | -0.016 | 0.026 | -0.606 | 0.544 |
| Atypical Angina | 1.389 | 0.893 | 1.555 | 0.119 |
| Non-Anginal Pain | 0.553 | 0.747 | 0.739 | 0.459 |
| Asymptomatic | 2.386 | 0.757 | 3.154 | 0.002 |
| Blood Pressure | 0.026 | 0.012 | 2.153 | 0.032 |
| Cholesterol | 0.007 | 0.004 | 1.566 | 0.117 |
| Fasting Blood Sugar | -0.370 | 0.626 | -0.591 | 0.555 |
| EKG.abnormality | 0.648 | 3.185 | 0.203 | 0.839 |
| EKG.left ventricular hypertrophy | 0.634 | 0.412 | 1.538 | 0.124 |
| Max.HR | -0.019 | 0.011 | -1.683 | 0.092 |
| Exercise.angina | 0.597 | 0.461 | 1.296 | 0.195 |
| ST.depression | 0.449 | 0.245 | 1.836 | 0.06 |
| Flat ST | 0.950 | 0.500 | 1.898 | 0.058 |
| Down-sloping ST | 0.123 | 1.042 | 0.118 | 0.906 |
| Number of Vessels | 1.200 | 0.281 | 4.271 | 0.000 |
| Thallium.fixed | -0.146 | 0.846 | -0.173 | 0.863 |
| Thallium.reversible | 1.432 | 0.450 | 3.183 | 0.001 |

**Table 1.** Logistic Regression Coefficient Summary

### 4.3 Model Generated by Backward Selection

**Method of Backward Selection**

For the model selection process, this paper uses the method of backward selection, evaluating the model's performance through the Akaike Information Criterion (AIC). Backward selection improves the model's simplicity by progressively eliminating variables that contribute less to the model.

**Model Selection Results**

The results of backward elimination show that the final model includes key variables such as Sex, Chest pain type, Blood Pressure (BP), Cholesterol level, Max heart rate, ST.depression, Slope of ST, Number of vessels shown in coronary an-

| coefficients | | | | |
|---|---|---|---|---|
| | estimate | std. error | z-value | p-value |
| (intercept) | -7.193 | 2.648 | -2.717 | 0.007 |
| Male | 1.851 | 0.562 | 3.295 | 0.001 |
| Atypical Angina | 1.264 | 0.883 | 1.432 | 0.152 |
| Non-Anginal Pain | 0.446 | 0.748 | 0.596 | 0.551 |
| Asymptomatic | 2.532 | 0.749 | 3.381 | 0.001 |
| Blood Pressure | 0.024 | 0.011 | 2.178 | 0.029 |
| Cholesterol | 0.007 | 0.004 | 1.812 | 0.070 |
| Max.HR | -0.020 | 0.011 | -1.925 | 0.054 |
| ST.depression | 0.467 | 0.233 | 2.002 | 0.045 |
| Flat ST | 1.025 | 0.493 | 2.078 | 0.038 |
| Down-sloping ST | 0.166 | 0.991 | 0.167 | 0.867 |
| Number of Vessels | 1.135 | 0.262 | 4.338 | 0.000 |
| Thallium.fixed | -0.324 | 0.813 | -0.398 | 0.691 |
| Thallium.reversible | 1.377 | 0.428 | 3.215 | 0.001 |

**Table 2.** Backward Regression Coefficient Summary

giography and Thallium. These variables are considered to have a significant impact on the occurrence of heart disease.

### 4.4 Model Analysis

Using the glm function in R, this paper successfully fitted a binary logistic regression model. The fitting results of the model are displayed using the summary function, which includes the coefficients, standard errors, z-values, p-values, etc., for each predictive variable. This paper focuses on the significance of each coefficient to determine whether the variables have a statistically significant impact on the occurrence of events.

Furthermore, the paper conducted model diagnostics, including using the Box-Tidwell test to check the model's linearity, the Durbin-Watson test to verify the independence of observations, VIF to examine multicollinearity, and MC distance to check the extreme outliers. This series of diagnostic processes helps to ensure the model's reasonableness and accuracy.

**Assumption:**

1. Multicollinearity checking: VIF (Variance Inflation Factor) values are used to test for multicollinearity, and most variables have VIF values within a reasonable range (less than 2), suggesting that the influence of multicollinearity among variables is minimal. Since no VIF value exceeds 2, the multicollinearity may not be considered as an issue.

2. Independence checking: The Durbin-Watson statistic is 2.137, with a p-value of 0.262. The statistic being close to 2 and the relatively large p-value suggest that there is no significant auto-correlation between different variables, indicating that the variables are independent.

3. Linearity checking: Check the linearity of logistic regression by Box-Tidwell. The Box-Tidwell test is for checking the linearity between continuous predictors and the logit of the dependent variable. The significance of Box-Tidwell means the non-linear relationship between predictors and the logit of the dependent variable. From the result of the Box-Tidwell test, we can find out that the p-value of all continuous variables is larger than 0.05, which indicates no violation of linearity needs to be considered.

4. Outliers checking by Cook's distance: By calculating Cook's Distance, it is possible to identify outliers that may have a significant impact on the model. Based on the plot, potential outliers can be identified - those whose Cook's Distance exceeds a predetermined threshold - and these outlier points can be removed. Although some observations' Cook's Distance might be much higher than other points (2, 88, and 265), they can not be considered as influential points since their Cook's Distances are all less than 0.5, indicating that they do not have significant impacts on the model results after removing them.

### 4.5 Model Explanation

**Influence of Parameters**

By interpreting the model parameters, this paper focuses on the relative contributions of various variables to the probability of occurrence of heart disease, holding all other variables constant. In logistic regression, the coefficients represent the change in the log odds of the outcome for a one-unit increase in the predictor variable or different types, with all other variables held constant. Exponentiate coefficient for translating the coefficient into an odds ratio. If the coefficient is

positive, indicating that the odds of having heart disease will time some value (exp(coefficient)) higher than 1, which will raise the occurrence of heart disease, in contrast, the negative coefficient will decrease the occurrence of heart disease.

1. Sex: When a patient's sex is male, the odds of a patient having heart disease are exp(1.851) =

6.366 times the odds of a female patient having heart disease, holding all other variables constant.

2. Chest Pain Type: When a patient's chest pain type is Atypical Angina, Non-Anginal Pain and Asymptomatic, the odds of a patient having heart disease are exp(1.264) = 3.540, exp(0.446) = 1.562, exp(2.532) = 12.579 times the odds of patient's chest pain type is typical angina, holding all other variables constant.

3. Blood Pressure (BP): For each 1 mmHg increase in the patient's Blood Pressure, the odds of having heart disease multiply by exp(0.024) = 1.024, holding all other variables constant.

4. Number of Vessels Shown in Coronary Angiography: For each number increase in patient's vessels shown in Coronary Angiography, the odds of having heart disease multiply by exp(1.1346) = 3.110, holding all other variables constant.

5. ST depression: For each 1 unit increase in ST segment depression, the odds of having heart disease multiply by exp(0.467) = 1.595, holding all other variables constant.

6. Slopes of ST: When a patient's slope of ST is flat and downsloping, the odds of a patient having heart disease are exp(1.025) = 2.787, exp(0.166) = 1.181 times the odds of the patient's slope of ST is up-sloping, holding all other variables constant.

7. Thallium: When a patient's thallium test result is a fixed defect and reversible defect, the odds of a patient having heart disease are exp(−0.324) = 0.723, exp(1.377) = 3.963 times the odds of the patient's thallium test result is normal, holding all other variables constant.

8. Cholesterol: For each 1 mg/dl increase in the patient's cholesterol level, the odds of having heart disease multiply by exp(0.007) = 1.007, holding all other variables constant.

9. Max.HR: For each 1 unit of heart rate increase for patients, the odds of having heart disease multiply by exp(−0.020) = 0.980, holding all other variables constant.

**Overall Performance**

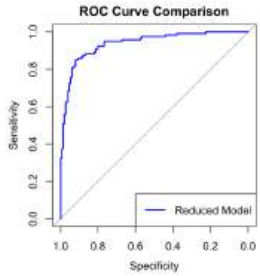


**Figure 6.** ROC Curve

The overall performance of the Backward reduced model is evaluated using a confusion matrix and ROC curve (Fig.6). An ROC curve with an AUC of 0.8692 indicates that the model has a high accuracy in predicting heart disease. The model's accuracy is 87.41%, and the confusion matrix shows the model's predictive performance.

**Confusion Matrix of Reduced Model:**

| | | Predicted | |
|---|---|---|---|
| | | 0 | 1 |
| True | 0 | 137 | 13 |
| | 1 | 21 | 99 |

**Likelihood Ratio Test**

Comparing the reduced model with the full model, by using the Likelihood Ratio Test (LRT) for model comparison. The full model (Model 1) includes all predictor variables, while the backward regression model (Model 2) achieves model simplification by gradually removing some variables. During the model comparison process, differences between Model 1 and Model 2 were observed and the corresponding p-value was calculated. The results show that the reduced model is adequate (p-value = 0.457), indicating that the additional variables in Model 1 might not provide sufficient explanatory power and the simpler Model 2 is, the more preferable due to its parsimony.

## Conclusion

In summary, our research comprehensively analyzed the predictive factors for heart disease using logistic regression. Through a rigorous process of model selection and validation, we successfully reduced the variables from 13 to 9, and identified key variables such as sex, max. HR, cholesterol, type of chest pain, blood pressure, number of vessels, ST slopes, ST depression, and Thallium levels as significant predictors for the occurrence of heart disease. The logistic regression model, selected based on the AIC, provides a quantifiable understanding of each variable's influence on heart disease risk. This study's findings underscore the importance of these predictors in clinical settings, offering healthcare professionals a robust tool for early identification and management of individuals at high risk for heart disease. The model's high accuracy (0.874), as evidenced by the ROC curve and confusion matrix, demonstrates its potential utility in real-world applications, particularly in preventative health strategies and personalized medicine.

Furthermore, assumption checks for multicollinearity, independence, linearity, and outliers confirmed the model's reliability, ensuring that the results are both statistically and clinically valid. Data visualization techniques were used to enhance the interpretability of key variables, providing clear insights into their relationship with heart disease occurrence.

However, this research is not without limitations. The dataset used, although comprehensive, may not capture all potential risk factors for heart disease. Future research could benefit from incorporating additional variables such as genetic markers, lifestyle factors, and other biomarkers to improve the model's predictive power. Additionally, the model's applicability to diverse populations needs further investigation to ensure its generalizability across different demographic groups. Furthermore, we acknowledge that the dataset used in this study is relatively dated and may not fully capture the current trends and patterns in heart disease risk factors. To enhance the relevance and accuracy of future predictions, it would be beneficial to utilize updated datasets, potentially sourced from electronic medical records (EMRs) and other modern data collection techniques. Access to current data would allow for the identification of contemporary risk factors that are more reflective of the present-day patient population, thereby improving the model's applicability and accuracy.

Overall, this research provides a valuable meaning for understanding the predictive factors of heart disease, offering significant implications for both clinical practice and public health strategies and contributing to the ongoing fight against heart disease on a global scale.

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## About the Author

My name is Haolin Wang, a senior student major in statistics and business information system. My interest is merging statistics with healthcare, channeling my analytical expertise and entrepreneurial spirit to develop innovative solutions for chronic patient care and early disease detection. I have develop a smoke cessation app and Antibiotic- Resistant bacterial Strain database. My scientific interest is using of advanced statistical model to creating a smart wristband to avoid stroke patients miss the critical window from onset to treatment.

# *Where Do We Put Saint Thecla?* An Examination of Saint Thecla's Place in Time, Theology, and Femininity

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Advised by Dr. Laura Smoller, *Department of History*

## Abstract

Saint Thecla is a prominent early Christian martyr. Drawing from *Acts of Thecla,* a second-century text, and the *Life of Thecla,* a fifth-century account, this paper examines the evolution of Thecla's narrative. The earlier Acts of Thecla leads contemporaries to portray her as a radical and autonomous figure who baptizes herself without the permission of the Apostle Paul, challenging early Christian patriarchal norms. The *Life of Thecla*, on the other hand, frames her more conservatively, aligning her actions with male authority while maintaining her status as a revered virgin and martyr. There are, of course, scholarly and religious debates surrounding Thecla's apostolic status, her representation of female piety, and her role in Christian tradition. This paper highlights how her story resonated with female audiences over the centuries since her death, especially in the context of her shrine and cult in Seleucia where Thecla was revered. This paper argues that Thecla's complex narrative reveals her to be an ultimately autonomous character whose existence reflects broader tensions in early Christian society regarding gender, religious authority, and feminine autonomy.

*Acts of Thecla*, from *The Apocryphal Acts of The Apostles*, is a text about the life and martyrdom of Thecla, an early Christian figure and the first female martyr (second only to Stephen). The author, an anonymous presbyter from Asia Minor, wrote this text in the apostolic style in the second century alongside *Acts of Paul.* The two texts were often grouped together as the *Acts of Paul and Thecla*, though Thecla's section was much more popular, while *Acts of Paul* gradually disappeared in Late Antiquity.

Around the same period, a second text, a collection of twenty-eight chapters on Thecla's life, appeared in Seleucia, the home of Thecla's shrine, cult, and adulthood. Known as the *Life of Thecla* and written somewhere between 444 and 448 AD, the fifth-century text is much longer and based directly on the *Acts.* While initially attributed to Archbishop Basil of Seleucia, this assumption has been debunked due to internal textual evidence[1]. Both the *Acts* and the *Life* portray Thecla as a devout virgin who, inspired by the teachings of the Apostle Paul, embarks on a faith-filled journey. Along the way, she defends her chastity, survives near-executions, and famously performs an act of self-baptism. Although these texts share thematic elements, these texts differ significantly in cultural perspective, religious ideology, and dramatic flair. They both (but more significantly *Acts*) are the subject of fierce debate between scholars of female piety and apostolic texts, usually over the origin of the narrative and the radical nature of Thecla. Thecla was certainly radical and feminine, and she faced her share of male pious critics over the ages. However, we cannot assign to her story a total rebellion of patriarchal piety, as her texts survived (in some respect or another) every era of masculine hierarchy. A comparison of the two texts, *Acts* and *Life*, allows for further insight into this thin line that Thecla tows into late antique society.

*Acts of Thecla* (translated by J. K. Elliot[2]) closely mirrors the scriptural style of the New Testament, a point that has generated significant controversy. The scriptural composition style garnered the negative attention of the theologian Tertullian (160-240 AD), as Thecla's feminine ability to baptize is given (in his opinion, unfounded) validity by her apostolic portrayal. More modern scholars further argue that the historical and theological accuracy of the text is not consistent with apostolic and first-century Christianity, contrasting the writing style. Naturally, these theologians resent the implication that Thecla originated at the time of the Apostles (1st Century). The fifth-century text, *Life of Thecla*, however, does classify

[1] Stephen J. Davis, The Cult of Saint Thecla: A Tradition of Women's Piety in Late Antiquity (Oxford: OUP Oxford, 2008), 40.
[2] J.K. Elliot, trans., "The Acts of Paul and Thecla," in The Apocryphal New Testament: A Collection of Apocryphal Christian Literature in an English Translation

Thecla as an Apostle (as well as a martyr[3]). This argument over the possibility of a female Apostle no doubt holds serious weight for feminist scholars and theologians, as well as for the Catholic church that canonized Thecla.

*Life of Thecla* is certainly not in the scriptural style. It uniquely embellishes lofty and dramatic speeches and monologues of the characters, both pious and pagan, and is the first half of a broader text by the same author, *Life and Miracles of Thecla*. *Miracles* portrays Thecla's miracles (mostly in life and surrounding healing, some posthumous) in Seleucia, where her cult and shrine were located in the 5th Century, but is not fully explored in this analysis. This particular English translation by Andrew S. Jacobs is based upon the French translation from Greek by Gilbert Dagron and covers only *Life*. The differences between the two original texts, *Acts* (from the 2nd Century), and *Life* (from the 5th Century), can be largely accounted for by the difference in cultural and generational perspective, especially considering the large cult already amassed by Saint Thecla in the town where the authorship of *Life* took place.

Though both follow a certain glorifying and romantic pattern of adventure (which some scholars argue is Hellenistic and unchristian due to the abundance of earthly rewards), *Life* is lengthy enough to include the drama of interpersonal relationships. The text is littered with personal laments about Thecla's aesthetics and her decision to remain chaste, such as characters exclaiming over her beauty or giving dramatic and self-important speeches. Thecla's mother began a public speech with the words, "Hear of this calamity from someone who doesn't want to speak of it!"[4] with all the narrative drama of a layman's work of fiction. These speeches often give (arguably too much) characterization and reason to the opposers of Thecla and Christianity that were not there in the 2nd Century text. Perhaps the author uses these arguments to highlight and comment on the debates more relevant to fifth-century Seleucia. A particularly compelling case for resurrection through marriage rather than chastity is expressed by a town leader in *Life*, as he states, "the true and accurate 'resurrection' is preserved and accomplished every day in human nature itself: for the succession of children born from us is what exists and is desired, with the image of those sowing and reproducing being renewed again in their children, and so in this way 'resurrecting,' such that we seem to see those who long ago passed away in the living people who are once more with us".[5] This is uniquely eloquent, especially for a character branded as an enemy of Christianity in a religious text. This is not an isolated example in 5th Century *Life*, but there are no examples of such developed debates in the 2nd Century *Acts*.

*Acts* does, however, afford Thecla a more clear and autonomous Baptism, which is the heart of much of the scholarly debate and radical sentiment surrounding *Acts*. *Acts*'s Thecla states clearly, after surviving multiple beasts, harnessing a Lioness, and before jumping into a pit of "savage seals"[6], "In the name of Christ I baptize myself on my last day."[7] This baptism is not preceded by the permission of the Apostle Paul; she baptizes with full autonomy from all but Christ. The 5th Century text *Life*[8], however, does require such permission, which leads many to believe that Thecla had more religious autonomy in the original *Acts*. The baptism in *Life* is still somewhat autonomous and transpired under similar circumstances, with Thecla stating, "resolve this fear for me through baptism of death."[9] This version gives less power to the secular, antemortem self, and has more suicidal undertones. This certainly aligns with the antiquitous and medieval move into a pious rejection of the body out of ultimate respect for the spirit.

Whether or not this scene grants Thecla the power to baptize *others* after surviving this attempt is another matter of debate surrounding both texts, and usually comes down to differences in translation from ancient Greek. In any case, these differences illuminate the stark and aggressive controversies in a few scholarly questions. When and how did the story of Thecla originate? Was Thecla an Apostle? Was she an example of the possibility of radical femininity in Christian piety, or just a submissive follower of a masculine religion with exemplary chastity?

The first question, of when and how the story of Thecla came to be, is not nearly as straightforward as one might hope. Of course, *Acts* came first, and its writer was deposed in the latter second century. But some scholars, such as Stephen Davis, argue that the text was a written account of an earlier oral history, passed down by women.[10] Evidence for this, beyond the story's female protagonist and working knowledge of female struggle (marriage, sexual assault, travel), lies a particular chastisement in the Old Testament that some believe is a direct attack on Thecla (which would imply her existence in the original world of Christianity and apostles). The tendencies of patriarchal New Testament texts to chastise gossip and "old wive's tales"[11] had, according to J.K. Elliot, substantial evidence implying a reference to the Thecla specifically. Several recent scholars, such as Lynne C. Broughton and Esther Yue Lo Ng, deny this claim of oral sources and pre-2nd Century origin (and many other popular claims about *Acts*) with fervor. Broughton goes so far as to claim that most of Thecla's

[3] Andrew S. Jacobs, "Life of Thecla (Ps.-Basil of Seleucia)," accessed December 2023, https://andrewjacobs.org/translations/thecla.html. Chapter 9.
[4] Jacobs, "Life of Thecla." Chapter 3
[5] Jacobs, "Life of Thecla." Chapter 5
[6] Most scholars agree that this is a mistranslation, with the original intent being "sharks" rather than "seals"
[7] "The Acts of Paul and Thecla," Ch. 34, p181
[8] Jacobs, "Life of Thecla." Chapter 14
[9] Jacobs, "Life of Thecla." Chapter 20
[10] Davis, The Cult of Saint Thecla, 41
[11] "The Acts of Paul and Thecla," 177

scholarly concern is riddled with "Catholic affiliation and feminist concerns,"[12] and states that claims attributing Thecla to early apostolic texts or assigning her religious accolades are full of "self-fulfilling hypotheses and questionable selection of evidence."[13] Broughton suggests that specific moral elements of *Acts* (the earlier text) are direct misalignments with the Hebrew teachings that ruled scripture during the time of the apostles[14], and the scriptural composition of the text is "dangerous"[15] for that reason. Lo Ng's analysis aligns with Broughton's and adds further evidence to her claims against the apostolic classification of Thecla. She adds locational inaccuracies regarding a "1st Century" character in *Acts*, the Queen Tryphaena.[16] Lo Ng spends most of her analysis on the defensive against scholars like Stephen Davis and D. R. MacDonald. She specifically attacks the latter on their claim that Thecla was "initially accepted as scripture until its pro-woman stance was seen as heretical by a patriarchal church."[17]

D. R. MacDonald's claim may not have enough evidence to hold such a lofty implication. However, Broughton and Lo Ng's classification of *Acts* as "anti-scriptural" in content, and Thecla as too uneducated to be an apostle[18], are ignorant of a couple of important points (one of which is illuminated by the 5th Century text, *Life*). The first, which Broughton weakly acknowledges, is the possibility that certain pieces of apostolic evidence were naturally or intentionally stifled by the years of patriarchal clergy and record-keeping. This would not be an implausible or isolated situation if it were the case. The second, which neither analyses confront, is far more relevant. The fifth-century text, *Life*, outright classifies Thecla as an Apostle. In this text, it is said by the (quite revered and influential) apostle Paul, directly to Thecla, "Apostles, among whom also you will be numbered," in a manner which I am sure is not light-hearted.[19]

The extent to which Saint Thecla represents the radically feminine in Christianity goes beyond (though is very much intertwined with) her status as an Apostle. The controversial idea was that her story was not only *about* women but also *by* women through oral tradition. It furthers Thecla's message of autonomy and creates another example of female authorship in scripture/hagiography. This theory that the tale of Thecla was originally an oral history, *by and for* women, is championed by Dr. Stephen Davis. While the *by* is highly contested, there is reasonable evidence that the story of Thecla was *for* women. *Acts*, the original text, was just a short part of the much larger text, *Acts of Paul and Thecla*. According to Davis, this implies that Thecla was for a specifically female audience.[20] The success of *Acts of Thecla* compared to the lengthy irrelevancy and disappearance of *Acts of Paul* suggests that the particular style and thematic content of the author's texts were better received by women than men. For *Acts of Thecla* in particular, the adventurous and autonomous Thecla represents the literary and scriptural rarity of a charismatic feminine leader. Not only is Thecla feminine, but her story accurately represents a few uniquely female struggles surrounding early Christian faith and late Antique culture.

Thecla (in both *Acts* and *Life*) was subjected to pressure from a legally binding marriage proposal to a Pagan man, as well as social obligations to procreate for her town. Throughout this pressure, Thecla derived her faith from a male Apostle, rebelling against her mother and local government by deciding to keep her virginity, rather than simply save it for her future husband. The complexity of this social and religious pressure would not resonate well with male crowds.

Such is true for Thecla's trials in Antioch, as she is faced with an attempted rape by a socially powerful man and leans on the support of a female community to retain her chastity. An older woman and a gossiping female chorus support Thecla, while Paul simply flees the situation.[21] There is evidence that the author of *Life* is aware of the success of this story amongst women, as this latter text (which was authored by a theologian who would have been well aware of Thecla's female cult) depicts a more intense description of the sexual assault. The earlier text, *Acts*, simply states that the man "embraced her in the street,"[22] while *Life* describes the man "entwining himself with her and feverishly pushing against her."[23] In the latter text, Thecla responds with a defiant and courageous speech and physically humiliates the man, and the survival of her subsequent execution by beasts leads the women of the town to follow Thecla socially and in her faith in God. This happens (albeit less dramatically) in the earlier text as well, omitting the speech. In the words of Stephen Davis, "such charismatic figures do not typically subsist on their own…they tend to exist in symbiosis with settled communities that offer subsistence and material support."[24] Among the women in support of Thecla is Trypheana, a queen who recently lost her daughter, and took immediate responsibility for the young Thecla because of it. This is yet another example of the uniquely feminine thematic elements in the story of Thecla.

[12] Lynne C. Boughton, "From Pious Legend to Feminist Fantasy: Distinguishing Hagiographical License from Apostolic Practice in the 'Acts of Paul/Acts of Thecla,'" The Journal of Religion 71, no. 3 (1991): 376.
[13] "From Pious Legend to Feminist Fantasy," 370.
[14] "From Pious Legend to Feminist Fantasy," 372.
[15] "From Pious Legend to Feminist Fantasy," 383.
[16] Esther Yue L. Ng, "'ACTS OF PAUL AND THECLA'. WOMEN'S STORIES AND PRECEDENT?" The Journal of Theological Studies 55, no. 1 (2004): 19
[17] Ng, "'ACTS OF PAUL AND THECLA'," 26.
[18] "From Pious Legend to Feminist Fantasy," 380.
[19] Jacobs, "Life of Thecla." Chapter 9
[20] Davis, The Cult of Saint Thecla, 39.
[21] Jacobs, "Life of Thecla." Chapter 15
[22] "The Acts of Paul and Thecla," Ch. 26, p180.
[23] Jacobs, "Life of Thecla." Chapter 15
[24] Davis, The Cult of Saint Thecla, 34.

Conversely, Lo Ng claims that the story of Thecla is not explicitly empowered by gender and is instead meant to "demonstrate the power of God to the helpless."[25] This "helplessness" could be based upon Thecla's subjection to execution (multiple times), and the unlikely resilience of the young virgin. However, if this were the intention, Thecla would be a flawed example of the helpless. Her wealth and ability to travel safely (although she did have to dress like a man for protection) would hinder this theme, as would her reliance on other women, as well as God, for mercy. Another issue with this claim that Thecla's helplessness was more central than her femininity is the success of her cult, which was dominated by monastic women at her shrine, *Hagia Thecla*.[26] There were many pilgrimages to this shrine, most notably by the female pilgrim Elgeria in the 4th Century. There is certainly a gendered element to Thecla's appeal beyond "helplessness".

*Life*, the fifth-century text, was authored in the same town as *Hagia Thecla*, and the author most likely used their insight to shape and alter the story of Thecla from the original Acts. This shrine was located in Seleucia (in modern-day Turkey), and centralized the cave where the text claimed that "still living, she [Thecla] sank and entered secretly into the earth."[27] This strange fate for the mortal Thecla (being absorbed by rock) is unprecedented but may have had a motive. With intimate knowledge of *Hagia Thecla* and the culture surrounding it, the author of *Life* explains the lack of relics or bones in the cave.[28] Another, slightly humorous, display of the author's social locality to *Hagia Thecla* is his snarky remark about who Thecla belongs to. The author states that Thecla traveled in *Syrian* Antioch, and *not* Pisidian Antioch, "even if the Pisidians should wish it!"[29] It seems that the author's insight into the area and cult in Seleucia also modifies key details in his depiction of Thecla. These modifications create yet another scholarly debate: the fifth-century author's alleged "domestication" of Saint Thecla.[30] It is believed that the latter text tones down Thecla's original power and subversion, especially because of the differences in the baptism scene.

In response to the idea that the fifth-century text "domesticates" the Thecla of the original text, Susan E. Hylan represents the opposition. In her analysis, the fifth-century text may even radicalize her. Hylan does not believe that Thecla's self-baptism is toned down, and she asserts that fifth-century *Life* assigns Thecla more modes of power (such as ultimate forgiveness of sin and baptism of others). This would imply that the audience in fifth-century Seleucia would relate to female power, as the text "expects that readers will not be surprised by a woman undertaking such actions."[31] From Thecla's agency and the author's classification of Thecla as a martyr and an apostle, the fifth-century Thecla is not "domesticated" or watered down. Unfortunately, the analysis that this insinuates an egalitarian culture or faith does not account for certain crucial factors.

There are over fifteen examples throughout the fifth-century *Life* of the author asserting in great detail the subservient and diminished role of women, either directly or through a character, in words that were *not* present in the original text. Not only this, but he attributes some of Thecla's power to her adherence to these roles. To account for Thecla's silence in court (which was attributed to her attraction to Paul in the original text), the author states that there is, to a woman, "nothing so fitting as silence and keeping quiet."[32] These remarks are interspersed with Thecla's radical and powerful actions, some of which directly attributed her power to Paul, rather than herself, in ways not considered by 2nd Century *Acts*. Paul was, overall, a much stronger character in the fifth-century text, and is given an almost prophetic role, as he warns Thecla of her future trials "through fire and beasts,"[33] long before the events of her martyrdom transpire. Once again, this detail was not present in *Acts*. Davis accounts for this disparity, stating that "the work portrays a cult that is strongly patronized by women, and yet at the same time gripped by elements of patriarchal bias."[34] It is possible, however, that these changes are a little more deliberate than simply products of their time. Perhaps the author meant to channel Thecla's power for an agenda rather than diminish it.

*Life* contains more elements of explanation and religious instruction than the scriptural bluntness of the second-century text. Usually through Paul, the author instructs chastity, arguing in favor of marriage (though also of virginity), and often touches on points completely irrelevant to the narrative of Thecla, or the spirit of *Acts*. In a long monologue, Paul speaks on matters such as the "second coming of Christ."[35] It would not be far-fetched to assume that the author wanted this text to instruct Thecla's unruly cult and other Christian monastics and laymans. This is revealed quite directly in *Life*, as the governor of a town within the narrative exclaims at Thecla's female popularity; "she taught the women among us not to consider anything more honorable than temperance!"[36] In the story, Thecla was a tool for the governor to tame the women of his town. Perhaps the author of *Life* meant to do the same for his retelling of Thecla.

[25] Ng, "'ACTS OF PAUL AND THECLA'," 29.
[26] Davis, The Cult of Saint Thecla, 43.
[27] Jacobs, "Life of Thecla." Chapter 28.
[28] Davis, The Cult of Saint Thecla, 42.
[29] Jacobs, "Life of Thecla." Chapter 15.
[30] "The 'Domestication' of Saint Thecla: Characterization of Thecla in the Life and Miracles of Saint Thecla," Journal of Feminist Studies in Religion 30, no. 2 (2014): 5–21.
[31] Hylen, "The 'Domestication' of Saint Thecla," 21.
[32] Jacobs, "Life of Thecla." Chapter 12.
[33] Jacobs, "Life of Thecla." Chapter 12.
[34] Davis, The Cult of Saint Thecla, 48.
[35] Jacobs, "Life of Thecla." Chapter 26.
[36] Jacobs, "Life of Thecla." Chapter 23.

The entire story of Saint Thecla, as portrayed in both *Acts of Thecla* and *Life of Thecla* is a romantic adventure, often criticized for its deviation from the expected monotony and humility of monastic life and scripture. Yet, Thecla remains a woman revered for her chastity and her martyrdom (though she is said to have survived both execution attempts). The total classification of Thecla as a martyr, apostle, Saint, or heretic is a heavy and treacherous scholarly liability. Perhaps this is because of the tendency of the original hagiographers to differ and embellish. A certain fourth-century account of Thecla by Eusebius mentions her so briefly that she only has time to die a martyr's death accompanied by two men, which is a complete deviation from all other accounts. *Acts* portrayed her as an Apostle and *Life* used this status for political and religious messaging. Modern scholars sort through Thecla's texts without agreeing upon their context. Now, the Catholic Church largely ignores this Saint and original Martyr. Perhaps it is so difficult to fit Thecla somewhere simply because she does not fit anywhere, and miraculously existed unto herself in Seleucia into and beyond Late Antiquity. Still, the differences between the origin of the cult, *Acts of Thecla*, and the text influenced by it, *Life of Thecla*, assist the scholar in her burden of classification of the radically autonomous Saint Thecla.

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## About the Author

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# Bilingual Children: How Do Their Two Languages Talk to Each Other?

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## Abstract

In the United States, English is the formal language of society and schooling instruction. Therefore, English language proficiency—in speaking, reading, and writing—is critical to academic and career success. For children from immigrant families, transitioning to English-only instruction can be challenging. This challenge raises a conflict: should one focus solely on English skills or should they also maintain their home or heritage language? This presents a logical paradox. On one hand, mastering any skill requires practice. Therefore, more practice in English should lead to better proficiency. On the other hand, bilingualism theories posit that bilinguals can transfer their language skills across both languages (Chung et al., 2019). Thus, practicing heritage language skills may also support English proficiency.

**Keywords:** language comprehension, cross-linguistic or cross-language transfer, semantics, morphology, language proficiency

## Background

In the United States, where English is the primary language of instruction, English proficiency is crucial for academic and career success. For children from immigrant families, this creates a dilemma: should they focus exclusively on developing English skills, or maintain their heritage language? Bilingualism theories suggest that skills in one language can enhance abilities in another, indicating that practicing a heritage language, like Spanish, may support English proficiency (Chung et al., 2019). This study explores this dynamic by examining how Spanish-English bilingualism influences language comprehension, highlighting the interconnectedness of language proficiency and comprehension. Understanding how skills transfer between languages can provide valuable insights for supporting bilingual students in educational settings.

Language skills permeate many aspects of daily life. Language comprehension, a skill unique to humans, enables understanding of spoken or written language, including word meaning and sentence structure (Gibbs, 2021). Adding onto that, language proficiency is described as the degree of accuracy in speaking and language comprehension (Jaros-White, 2023). These two literacy concepts are interlinked and related to language use and learning because comprehension is a key indicator and facilitator of overall language proficiency (Al Quanayeer, 2021). For example, a study by Al Quanayeer (2021) examines the connection between vocabulary knowledge and reading comprehension in EFL (English as a Foreign Language) students, showing that a larger vocabulary size substantially improves comprehension and demonstrates more proficiency than those with a smaller vocabulary size. Additionally, various languages have different word structures, which influences how learners develop comprehension and proficiency, and cross-linguistic differences might interfere with or support language use. In this study, we explore the relationship between English comprehension and Spanish language proficiency, discussing how skills in one language can influence and enhance abilities in another for Spanish-English bilingual speakers.

Bilingualism is defined as the ability to speak more than one language or dialect (Spitzer, 2016). This definition encompasses a wide range of experiences, from individuals who grew up bilingual to those who became proficient through higher education at an older age. In the United States, approximately 68 million people speak a language other than English at home (United States Census Bureau, 2019). As the Latino population in the US has increased in recent years, Spanish has been one of the fastest-growing languages (Krogstad et al., 2023). In the fall of 2021, Spanish was the most often reported home language of EL (English Learner) pupils enrolled in public schools (4 million students), accounting for 76.4 percent of all ELs (National Center for Education Statistics, 2021).

The current study focuses on Heritage Language Learners (HLLs) and early bilinguals. HLLs are individuals who speak a home (minority) and a community (majority) language while early bilinguals are those who acquire both languages before puberty (Montrul, 2012). Bilingual children are at risk of low literacy rates when they lack the linguistic foundations necessary to access academic content, which can lead to long-term consequences such as an increased likelihood of dropping out of high school (Peña et al., 2020). Consequently, teachers in the United States face significant challenges in supporting bilingual learners. Therefore, our study focuses on this bilingual population to inform both theory and educational practice for bilinguals.

## Bilingualism Theories

Within the brain, the two languages are thought to communicate with each other. This communication process is typically termed "cross-linguistic transfer." Cross-language transfer is key to bilingual development, as skills in one language can aid learning in another (Yang et al., 2017; Chung et al., 2019). Therefore, the bilingual brain sustains an interaction between L1 (first language) and L2 (second language). L1 morphology, which studies morphemes–the smallest unit of meaning combined to create complex words (Levesque et al., 2020), and phonology, the study of patterns of sounds or phonemes, the smallest units of sound in a language (University of Sheffield, 2022), have been developed and can positively impact the learning development of those elements in L2. Various frameworks, like the transfer facilitation model and the interdependence hypothesis, explain cross-language transfer (Koda, 2008; Cummins, 1981). In HLLs the two languages are often acquired simultaneously during early childhood. This phenomenon is better known as "dual first language acquisition." Instead of labeling one language as the first (L1) and the other as the second (L2), researchers often refer to them as the "home language" and the "school language." This is because children are learning both languages during a critical brain developmental period ideal for language learning. As they grow, their skill and preference for each language might change at different developmental stages t. This phenomenon is better known as "dual first language acquisition." Rather than using L1 and L2, researchers term those as, e.g., home and school languages, as children are acquiring both languages within the optimal brain developmental periods for language learning and their proficiency and dominance in these two languages may vary across different developmental stages. Chung et al. (2019) proposed an interactive framework to capture the complexity of dual-language interaction, and suggested that multiple factors contribute to this intricate process, necessitating further research into their interactions.

A recent study by Sun-Alperin (2011) found that among second and third grade Spanish-English bilingual children, Spanish phonological processing predicted English real word and pseudoword reading and spelling. Additionally, Spanish orthographic processing also predicted English word reading. These findings demonstrate that the heritage language (Spanish) supports the second language (English), validating the cross-language transfer theory, which posits that phonological and orthographic skills in Spanish (L1) are strongly linked to English (L2) reading. Further research on cross-language transfer has shown that English Language Learners (ELL) kindergarteners with a high level of knowledge in Spanish letters and sounds tend to exhibit high levels of knowledge in English letters and sounds (Cardenas-Hagan et al., 2007). This highlights the transferability of phonology and morphology in children who have a strong base in Spanish and implement it into English. Similarly, Bedore et al. (2023) discovered a robust relationship between oral language and reading measures in both languages, particularly in English, showing that having a good foundation in Spanish vocabulary helps one learn English vocabulary, which further helps one learn English morphosyntax and pre-reading abilities. This indicates that children's early readiness in Spanish facilitates English acquisition. For instance, Spanish vocabulary knowledge supports English vocabulary, which then aids in learning English pre-reading skills and morphosyntax (Bedore et al., 2023). These findings underscore the role of native Spanish skills in developing English proficiency, further supporting the cross-language transfer theory.

The acquisition of a second language is influenced by Age of Exposure to English (AoEE) and the amount of language exposure (input/output). Bedore et al. (2016) found that earlier AoEE improves English skills in bilingual children, especially first graders, but diminishes in importance as English exposure increases. However, early English exposure also correlates with lower Spanish performance, suggesting a trade-off in bilingual proficiency. The authors propose a usage-based approach, emphasizing the continuous use of Spanish to mitigate this trade-off. These findings align with other studies showing that as young children's English skills improve, their Spanish grammatical accuracy declines (Castilla-Earls et al., 2019), suggesting that while the age of acquisition is important, the amount of usage plays a crucial role. As these children use Spanish less frequently when they start school, their Spanish proficiency becomes limited. These studies highlight the importance of Spanish or heritage language maintenance and necessitate a closer look at the potential benefits of heritage language maintenance for children's dual-language proficiency, including listening comprehension in English, as addressed in our work.

## Bilingual Language Assessment Tools

This early language exposure also influences the semantics and morphosyntax of bilingual children. Exploring the semantics, or the meanings of words and sentences(Yule, 2005, p. 100), and morphosyntax, the rules governing word formation and sentence structure (Ardila, 2021), in bilingual children unveils a compelling narrative of linguistic agility, cognitive flexibility, and the delicate interplay between two vibrant languages. Various measures have been created to evaluate bilingual children's language development in terms of semantics and morphosyntax. Tests like the Bilingual Spanish English Oral Screener (BESOS) and the Test of Narrative Language (TNL) are commonly used for bilingual Spanish-English kids to detect impaired language development. To identify the classification accuracy of these measures, Peña et al. (2020) studied the effectiveness of three tools (BESA-ME morphosyntax, BESA-ME semantics, and TNL) and sought to find the best combination of scores across all three measures for the highest classification accuracy in each grade. Their study focused on 175 Spanish-English bilingual children in the second and fourth grades in Central Texas. Among these children, only 26 (15%) had previously met the criteria for developmental language disorder (DLD). The age range of the children in the

sample was 7;2 to 11;6 (M = 8;7, SD = 1;0), with 49% female participants (Peña et al., 2020).

The study used reference measures (BESOS screener data, teacher concerns, ITALK) and index measures (BESA-ME morphosyntax and semantics, TNL). The BESA-ME morphosyntax assesses children's grammatical knowledge with "cloze and sentence repetition items." Meanwhile, BESA-ME semantics measures both semantic breadth and depth with receptive and expressive items assessing "naming words and categories, functions, definitions, analogies, associations, and similarities/differences" (Peña et al., 2020). The investigation revealed that the highest language scores, regardless of whether in Spanish or English, produced strong classification accuracy. Specifically, BESA-ME morphosyntax was the most accurate for second graders, while both BESA-ME morphosyntax and TNL were the most accurate for fourth graders. These findings suggest that bilingual children with DLD struggle with morphosyntactic structures and that English proficiency tends to increase with prolonged exposure, often impacting Spanish proficiency (Peña et al., 2020).

Additionally, only 25% of second graders and 9% of fourth graders scored higher in Spanish across all three domains. These results are consistent with Bedore et al. (2016), which found that third graders scored significantly lower on the Spanish BESOS exam than the first graders. This suggests that extended exposure to English leads to increased English proficiency, while Spanish skills either decline or remain stable in comparison.

Recent studies highlight the importance of studying bilingual development to support those at risk of language difficulties. Bedore et al. (2023) found that low semantic performance in first graders increased reading risks, emphasizing the need for a strong semantic foundation. Early readiness in Spanish aids English acquisition, as supported by Sun-Alperin (2011), who found that Spanish orthographic and phonological processing aid English reading. Chung (2019) proposed an interactive framework for cross-language transfer. Bedore et al. (2016) showed that earlier English exposure improves English performance but leads to variable Spanish proficiency. Castilla-Earls et al. (2019) found that increased English proficiency decreases Spanish grammatical accuracy in young children. Pena et al. (2020) demonstrated that BESA-ME morphosyntax measurements effectively predict developmental language disorder (DLD). These findings suggest that having knowledge in Spanish aids English learning, and greater English exposure enhances English competency while possibly reducing Spanish proficiency, emphasizing the need for effective diagnostic tools and a better understanding of bilingual children's language development.

## The Present Study & Methods

The current study aims to explore the relationship between English comprehension and Spanish language proficiency among bilingual Spanish-English children in Southeast Michigan. Given the growing number of Spanish-English bilingual children, understanding their language development is crucial for improving educational practices and dispelling negative perceptions of bilingualism. This study aimed to answer the following question: Is there a significant correlation between English comprehension and Spanish proficiency among early Spanish-English bilinguals? Based on existing literature (Bedore et al., 2016; Peña et al., 2020), we hypothesize a positive linear correlation between children's Spanish proficiency and their levels of English comprehension.

In this work, we recruited and tested 17 children. However, five of these children had incomplete assessments. Some children were unable to finish the tasks, while others frequently responded with "I don't know" on both the Spanish and English assessments. Such responses were scored as zero, and consistent use of them often indicates a lack of interest in participating. To respect the child's autonomy, we discontinued the assessments in these cases. As a result, only 12 of the 17 children fully completed all the assessments. Participants were all Latinx children: 66.67% Mexican, 16.67% Venezuelan, 8.33% Cuban, and 8.33% Colombian, with grade levels ranging from 1 to 6 (average grade level = 3.67).

To measure children's core linguistic competence in each of their languages, I used BESA-ME, which assesses a child's proficiency in semantics (language meaning) and morphosyntax (grammar and sentence structure)). It consists of two parts: morphosyntax and semantics. BESA-ME Morphosyntax evaluates the child's ability to construct sentences correctly, including sentence repetition and construction based on possessives, regular and irregular past tenses, plural nouns, and relative clauses. To assess a child's ability to conjugate regular past tense verbs, one question is: "Today he is talking to his friend. Yesterday, he did it too. What did he do yesterday?" The correct answer is: "Today he talked to his friend." BESA-ME Semantics examines the child's understanding of word meaning through tasks such as associations, comparisons, analogies, and categorization. For example, to assess the child's association with different words about their meanings, a question is: "If I say 'healthy,' what do you say?" Some possible answers are nutritious, carrots, and exercise.

To measure children's English comprehension, I used the Test of Narrative Language (TNL), which evaluates a child's language production and comprehension abilities through storytelling. During this task, the child answers comprehension questions based on a set story read by a research assistant, as well as produces and retells their own story based on set images shown to them. These specific measures were employed because both BESA-ME and TNL complement each other: BESA-ME provides insights into the child's processing of word meanings and grammaticality, while TNL offers an understanding of the child's vocabulary complexity.

To answer my research question, "Is there a significant correlation between English comprehension and Spanish proficiency among early Spanish-English bilinguals?", I used a correlational analysis approach to investigate the relationship

between Spanish proficiency and English comprehension. We anticipated a positive linear correlation between children's Spanish proficiency and their levels of English comprehension, as indicated by their BESA-ME and TNL scores. If a positive correlation is found, it will support the cross-language transfer theory, demonstrating that Spanish proficiency enhances English comprehension. This study aims to contribute to the existing literature by providing empirical evidence on how Spanish proficiency benefits English language development. The findings will guide the development of improved teaching strategies to better support Spanish-English bilingual children. This is particularly important in regions with limited access to a Spanish-speaking community, which may otherwise impede the continuous enrichment of Spanish language skills.

## Results

| Variable | N | Mean (M) | Standard Deviation (SD) | Minimum | Maximum |
|---|---|---|---|---|---|
| English Morphology | 12 | 29.75 | 3.49 | 22 | 33 |
| English Semantics | 12 | 17.08 | 2.11 | 13 | 21 |
| English Comprehension | 12 | 36.33 | 5.73 | 23 | 43 |
| Spanish Morphology | 12 | 15.33 | 4.75 | 9 | 21 |
| Spanish Semantics | 12 | 17 | 4.97 | 7 | 23 |
| Spanish Comprehension | 12 | 34.42 | 5.68 | 21 | 39 |

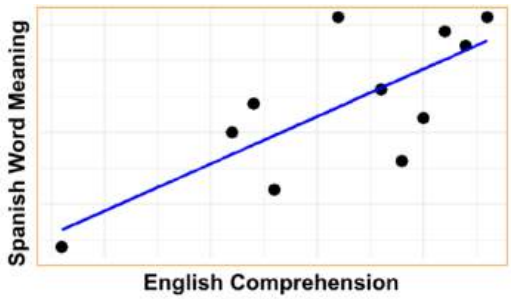
**Table 1.** Descriptive Statistics for English and Spanish Proficiency Variables

| Variables | English Morphology | English Semantics | English Comprehension | Spanish Morphology | Spanish Semantics | Spanish Comprehension |
|---|---|---|---|---|---|---|
| English Morphology | | | | | | |
| English Semantics | 0.73* | | | | | |
| English Comprehension | 0.84*** | 0.74** | | | | |
| Spanish Morphology | 0.79*** | 0.68** | 0.63* | | | |
| Spanish Semantics | 0.82*** | 0.79*** | 0.76*** | 0.85*** | | |
| Spanish Comprehension | 0.68** | 0.62* | 0.71** | 0.68** | 0.82*** | |

*The correlation is significant at the 0.05*, 0.01**, and 0.001*** levels.*

**Table 2.** Pearson Correlation Coefficients for English and Spanish Language Measures

Table 2 presents the Pearson correlation coefficients between English and Spanish language measures. Significant positive correlations were found between most measures of language proficiency within and across languages. The Pearson correlation analysis revealed a significant positive correlation between all Spanish proficiency variables (morphology, semantics, and comprehension) and English comprehension. Morphology showcased a significant correlation of $r(10) = 0.63$, $p < .05$, while semantics indicated a correlation of $r(10) = 0.73$, $p < .001$, and comprehension indicated a correlation of $r(10) = 0.71$, $p < .01$. This indicates that higher Spanish proficiency is associated with better English comprehension among early Spanish-English bilinguals. Table 2, along with Graph 3, show




**Graph 3.** Spanish semantics (meaning) is significantly correlated with English comprehension.

-cases that the most significant correlation between Spanish proficiency variables was Spanish meaning-based skills, or semantics, to English comprehension. This evidence suggests that meaning-based skills are more readily transferable between languages than morphosyntax or grammatical skills.

## Discussion

The purpose of this study was to explore how the home language of heritage (L1) or early bilingual learners impacts their school language comprehension (L2). Usingdual-language proficiency tests such as BESA-ME and TNL, we assessed 12 Spanish-English bilingual children's language proficiency. We found significant positive correlations between English comprehension and Spanish language skills, suggesting that proficiency in one language is positively associated with comprehension in another. Semantics was the Spanish variable most strongly associated with English comprehension, suggesting that this skill can transfer easily between both languages. These findings support the cross-language transfer theory, which posits that skills in one language can aid the development of those skills in another language. The strong correlations suggest that bilingual individuals who excel in one language are likely to perform well in the other.

It is essential to highlight an interesting finding in this study, that one child's scores were significantly lower in both languages compared to the others. Given the larger project aims to identify language proficiency and diagnose potential developmental language disorders (DLD), this particular child could be a candidate for further evaluation. Children with DLD often perform poorly on screening tests like BESA-ME. However, upon reviewing the parent's survey, which detailed the child's language development and the amount of English and Spanish they hear (input) and produce (output), there were no notable concerns. The child may have been less motivated during the test, which could have affected their performance. Notably, this child was the youngest in the group, having just turned 7 before testing. This raises the question of how the age of acquisition and length of exposure impact language proficiency, as they had the least English ex-

posure in school. Further testing is needed to determine whether the child has DLD or if other factors caused the low scores.

These findings have important implications for bilingual education strategies. Understanding this cross-transfer of skills between languages validates the development of curricula that support a child's proficiency in their home language while simultaneously fostering skills in the second language. This approach can be particularly beneficial for younger children, such as the ones in this study, who may have had less exposure to English but still demonstrate potential for strong bilingual development. By promoting balanced instruction, educators can harness the power of cross-linguistic transfer to promote overall language comprehension and academic success in bilingual students.

## Implications

The results underscore the importance of establishing support for bilingual children at various levels. At the educational level, educational policy should be developed to create bilingual programs to better support proficiency in both languages. Educational policy should advocate for curricula that integrate and strengthen skills in both languages. By considering bilingual experiences, schools can foster a more balanced and comprehensive language education.

At the teaching level, teachers should consider finding ways to capitalize on the child's first language to help them learn English, as well as incorporate strategies that reinforce both languages. For instance, activities that use vocabulary and comprehension in both languages simultaneously can support children's overall language development. Utilizing bilingual instruction methodologies can further enhance language skills and academic achievements, creating more competent future bilingual professionals.

At the family level, parents should encourage and support Spanish development at home and in surrounding communities, especially because these children are constantly enhancing their English skills at school. For instance, parents could enroll children in after-school programs that focus on building Spanish language skills or only communicating with the child back and forth in Spanish. By ensuring that their children are continuously practicing their Spanish skills, families can foster a comfortable environment where children feel encouraged despite making mistakes and motivate them to continue learning for themselves.

## Limitations

Since this was a summer project, there are various limitations. First, our small sample size may limit the generalizability of these findings. A larger and more diverse sample may provide more insights into this relationship. Second, the population comes only from a state where Latinx families are smaller in proportion compared to states like New York or California, which shows more constancy, but can lack diversity in children's bilingual experience. Additionally, the sample lacks diversity among Latinx groups, as more than half of the participants were Mexican. Third, using standardized testing may not capture all aspects of bilingual skills. Future research could incorporate alternative assessment methods to gain a more comprehensive understanding. Fourth, the study didn't consider the difference in the amount of Spanish exposure among the children, which can impact their scores. Future research should consider this and potentially compare those with more exposure to those with less exposure. Finally, given the nature of this study, causation cannot be drawn; further research is needed to find out how these skills transfer between Spanish and English. Lastly, since this study focused only on early bilinguals, future research should determine whether the correlational patterns found in this study can also apply to sequential and late bilinguals.

## Acknowledgments

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## About the Author

Albenys Diaz Hernandez, originally from the Dominican Republic and now residing in the Bronx, NY, studies Psychology and Spanish with a minor in Latin American Studies. She is completing her honors' thesis in the Families, Academics, Motivation, and Identity (FAM&I) lab at the UofR, exploring how racial, ethnic, and academic socialization affect STEM career choices among minoritized students, and how discrimination may impact these decisions. Albenys aims to become a bilingual therapist and researcher to support the Latino community.

# An Experience-Based Sampling Approach to Examining Prior Experience in Adaptive Speech Perception

**Seth H. Cutler '24**
Advised by Dr. Chigusa Kurumada, *Department of Brain and Cognitive Sciences*

## Abstract

Prior experience is a powerful mechanism for many types of learning. In speech, listeners draw on their prior experience to perceive sounds, predict words, and determine the likely sentence structure intended by the speaker. However, the impact of listeners' long-term linguistic experiences understanding novel talkers' speech and their accents remains unexplored. Researchers have attempted to quantify and assess prior experience, but their efforts are tainted by shortcomings related to recall and self-reporting biases. The goal of this thesis is to develop a means of assessing prior experience, as it relates to accents, by combining multiple different metrics to provide a holistic view on prior experience. Specifically, this paper proposes and validates a new mobile-based experience-based sampling survey of nonnative accent perception (SNAP). Combined with a perceptual experiment, the SNAP survey provides an individualized predictor of listeners' accent-comprehension capabilities. The paper discusses how multiple research instruments including SNAP can provide sufficient evidence to be able to conclude that prior experience does play a role in facilitating accent adaptation.

## 1. Introduction

Human speech perception is amazingly fast and accurate. We map a continuous speech stream into discrete units of meaning as quickly as 2.5 words/second (Griffiths et al., 1990). What's more, these mappings are probabilistic and change depending on the speaker and their accent. For example, a /z/ sound (e.g., zip) produced by a nonnative speaker with a Spanish accent often sounds like an /s/ (e.g., sip) to native English listeners (Núñez-Méndez, 2022). We must therefore adapt our speech perception to various speakers and accents. This ability is becoming ever more important considering the increasing linguistic diversity in the United States (Tamasi et al., 2014).

Research over the past 20 years has shown that speech adaptation occurs rapidly and subconsciously. For example, Clarke & Garrett (2004) showed that significant adaptation and learning occurred rapidly when native English speakers heard English spoken in a foreign accent. This effect persists in the face of language related disorders (e.g. dyslexia) (Hazan et al., 2009) and through perceptual and cognitive aging. For example, Gordon-Salant et al., (2010) demonstrated that both younger and older adults can conduct rapid adaptation to non-native speech, despite significant hearing differences. The capacity of the human brain is thus malleable, readily accommodating novel speech patterns and accents.

Our adaptivity to language is trainable and can be enhanced by environmental exposure, in both the short and long term. In addition, long-term experiences or "familiarity" with any language variant facilitate general adaptation (Porretta et al., 2020; Witteman et al., 2013). Xie & Kurumada (2024) have demonstrated that there are recognition benefits stretching across three weeks, when integrating foreign accented English. However, questions remain open about the types and amounts of linguistic experience that impact adaptivity of speech perception.

The current thesis aims to extend this literature by testing the hypothesis that linguistic diversity in everyday language experiences can predict the adaptivity listeners show when they encounter a new nonnative accented talker. For instance, Marisa, who lives in a multicultural city with a large immigrant population, may be adept at accommodating a previously unfamiliar accent. In contrast, Sarah, who lives in an English-dominant environment, might struggle to understand an unfamiliar accent. Although intuitive, this hypothesis has not been tested with empirical data. By investigating this hypothesis, we hope to better understand the way everyday linguistic experiences foster perceptual adaptivity and linguistic comprehension.

The work reported here is couched in a larger research project led by Dr. Chigusa Kurumada (University of Rochester) and Dr. Xin Xie (University of California, Irvine) (Gu, Cutler, Xie & Kurumada, 2023). This research intends to quantify the diversity of linguistic experiences in the U.S. and investigate its links to listeners' perceptual adaptivity in nonnative speech perception. To do so, our team has been developing a composite measure of Socio-Linguistic Diversity (SOLID). As described below, SOLID will assess listeners' daily exposure to various accents of English through an application compiling subjective ratings, daily surveys, and census-based statistics on their geographic location of residence.

### 1.1 SOLID measures

Quantifying one's linguistic experience can be challenging since our integration of spoken language is pervasive and mostly automatic. This means that conscious recall of specific events (e.g., how many non-native speakers have you spoken

to in the past week?) is easily clouded by errors in memory encoding and retrieval. The difficulty is intensified by a significant amount of individual variation; even in a single geographic location or household, linguistic experiences can differ widely from person to person. Previous studies on language acquisition have addressed this by conducting dense (and sometimes "around-the-clock") recording of speech input to infants (Gilkerson et al., 2017; Richards et al., 2017). However, this approach can be invasive of privacy and social interactions when applied to older children and adults.

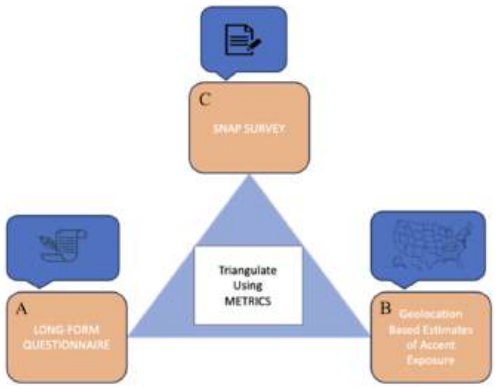


**Figure 1.** The SOLID measures of accent perception: a three-pronged approach to quantifying prior accent experience. This approach utilizes a long form questionnaire, geolocation-based assessments of accent exposure, and the Experience Sampling Model (ESM) based SNAP survey.

Composed of three parts (Figure 1), SOLID was developed to circumvent these issues and paint a holistic and accurate picture of one's linguistic experiences. The long form questionnaire in part (A) is a tool that asks participants to reflect on their prior experiences with accents, including their own. Participants are asked to recall experiences up to 40 years in the past (for our oldest participants). Additionally, the long form questionnaire considers many demographic factors, as well as information about a participant's surroundings. Some of the most crucial and informative questions are oriented toward the individual's housemates, family, and friends' language backgrounds (e.g., Where did your parent(s) grow up? Where did your roommate(s) live previously?).

Part (B) of the SOLID measures is a geolocation-based assessment using the American Community Survey data (the United States Census Bureau, Table# B16001). In contrast to the participant's subjective assessments in A, this measure provides an objective estimate of their daily accent exposure. By collecting information about zip code and county of residence, we can identify the number of non-native speakers in close proximity to the participant. This provides a better idea of how likely a participant is to come into contact with someone speaking with a given accent.

Lastly, part (C) of the SOLID measures is the daily mobile survey (the Survey of Nonnative Accent Perception, "SNAP") which takes a snapshot of one's daily linguistic experience through frequent and repeated sampling of linguistic experiences. The current implementation of SNAP uses a periodic survey that samples linguistic exposure, delivered via text message three times daily over a week. This approach complements traditional language background questionnaires, which are prone to memory and recall biases. Through the simplicity, immediacy, and frequency of sampling, SNAP will accurately capture an individual's daily linguistic experience.

The novelty and significance of SOLID lies in the fact that it is a composite measure, addressing weaknesses associated with each of the three individual parts. The long form questionnaire alone presents a recall bias and a propensity for participants to overestimate their amount of exposure throughout such a broad timescale. In a geolocation-based approach, the scarcity of individualized data is traded for a more holistic view on the subject's environment. The SNAP surveys allow for a closer look at the daily experiences of individuals, confined within a precise scope and time window. By using the three parts as interlocking measures, we can better collect accurate, individualized, and time/location-locked information about socio-linguistic experiences.

## 1.2 Thesis Overview

This study validates that the SNAP survey protocol allows for three dimensional assessments of SOLID. This protocol was delivered via a mobile application three times daily for seven days. The current thesis describes the development of the survey (Section 2), accompanied by a perceptual experiment that evaluates participants' adaptivity to a nonnative accent (Mandarin accented English, Section 3). The design and the data in this section are from a larger study partially described in Xie et al., (2023). The specifics of the study conducted by the primary author will be discussed (Section 4) before providing a general discussion and future directions (Section 5).

This study observed 20 subjects at two sites: Rochester (NY) and Irvine (CA), representing starkly different demographics and linguistic landscapes. The protocol for assessing adaptive perception of nonnative accent was validated based on the survey responses, after administering the SNAP and the perceptual experiment.The research compiled in this thesis was based on this experiment, although subsequent studies may be conducted to further support the evidence. In this light, the current protocol aims to provide a critical initial step towards clarifying the way daily accent exposure might induce a rapid adaptation to accented speech. If daily experience promotes adaptivity, then subjects with more exposure to a certain accent (e.g., Mandarin-accented English) should better comprehend and adapt to a Mandarin-accented speaker presented in our perceptual experiment. Alternatively, if experience is not a strong predictor, the measures will not explain the variance in the experimental results.

Thus, SNAP and perceptual data will provide insights into the sources of perceptual adaptivity. By proposing and refining the protocol, this thesis will ultimately contribute to a better understanding of how our life-long experiences may interact with the mechanisms of robust and effective human speech perception.

## 2. Developing the SNAP survey

### 2.1 Experience sampling model background

In recent years, several social, clinical, and cognitive science studies used the Experience Sampling Model (ESM) to assess everyday behaviors. The ESM typically consists of regular assessments of a behavior or exposure variable, repeated throughout multiple days to paint a picture of recent, natural participant experience. The main advantage of the ESM over alternatives is its immediacy. Respondents do not need to recall and reconstruct past events; instead, they can report on their most recent experiences, providing more accurate and detailed responses. Using an ESM, Goodman et al. (2021) quantified social anxiety "with a clinical sample of adults diagnosed with social anxiety disorder (SAD) and a psychologically healthy comparison group." They gathered significant amounts of information about the participants' immediate experience and thoughts regarding socially stressful situations, which are less likely to be recalled later.

More recently, Arndt et al. (2023) utilized ESM to quantify language experience, making it a compelling starting point for validating my research and study design. This study analyzed second language (L2) acquisition. Their reporting was done through a similar application, gauging a participant's use of a language with regard to both qualitative assessments and frequency. They found that "[M]any studies report no more than moderate correlations between aggregated ESM and retrospective survey data […] which indicates that these methods provide substantially different pictures of an individual's typical behavior and experiences " (p. 46). Arndt's protocol served as the most relevant precursor that helped inform the development of the SNAP survey.

### 2.2 Protocol

Under the current protocol, the SNAP survey is administered over seven consecutive days. Participants receive surveys in three different time windows: "morning" (9:00am until 1:00pm), "afternoon" (1:00pm until 5:00pm), and "evening" (5:00pm until 9:00pm). During each of these time windows, one survey is sent out at a random time. This makes it difficult for participants to anticipate the exact timing of a notification, resulting in the most naturalistic and unbiased responses. The survey remains active for one hour, after which it expires. No replacement survey is provided. In order to receive full compensation at the culmination of the study, each participant must respond to 19 out of 21 (90%) of the surveys. This threshold is set to encourage responses and to collect an adequate number of datapoints to analyze across all participants.

SNAP Surveys were administered through ExpiWell (https://www.expiwell.com/), an application designed for ESM and ecological momentary assessments. Participants downloaded the ExpiWell app receiving notifications and reminders (Figure 2A). To log in, each participant entered an individualized four-digit ID provided by the research team (Figure 2B). This increased privacy, by eliminating the need for participants to share their name or email address. The app interface (Figure 2C) and usage of pop-up notifications made it both easier and more likely for participants to respond, in comparison to, for example, generic email notifications.


**Figure 2.** ExpiWell app notifications trigger participant responses as they show up on participant's phones at randomized times throughout the day. Notifications will show up on participant's mobile devices when the survey is made available on the app (this will include a notification picture in 1-2 days)

### 2.3 Survey flow and questions

In each survey, participants answered up to 10 questions intended to assess participants' daily exposure to different linguistic inputs, including nonnative-accented English. Each question targets a specific component of the participant's experience, providing researchers with data about the types and amounts of linguistic input, as well as the context for these inputs.[1] There is branching at Question 3, where the "Yes" and "No" options lead to different sets of questions (Figure 3). Both options resulted in participants answering a similar number of questions overall. If they answer "No", they receive a series of questions about their general linguistic behavior.

However, their answers to these questions are not of interest here. Each of the following questions, and their intended

[1] There is a separate branch of the SNAP survey not discussed below, which ensures honest completion of the study. This branch of the SNAP survey stems from the "No" response at Question 3. If a participant answers "No," they are directed to questions asking about their language experience throughout the whole day. Additionally, there are further questions about the participant's language environment with reference to their surroundings and social network, (i.e., how many people have you heard speak (in conversation, via social media, listening in a lecture, etc.) since waking up?)

purpose, as described below, with focus on the path where participants answered "Yes" to Q3 ("In the past hour, have you listened to any speech?").


**Figure 3.** A logic tree of the branching involved in the SNAP survey questions on ExpiWell. The important branching node occurs at Question 3, where participants are directed to a "Yes" branch or a "No" branch.

**Question 2: Where are you taking this survey?**

This question asks about the context of linguistic interactions. Participants have three options: home, indoor public place, and outdoor public place. This gains information about where and in what kind of environment participants may be encountering a given type of linguistic input. For example, "home" suggests the input is provided by a family member or through media being viewed at home, such as a movie. On the other hand, "indoor public place" and "outdoor public place" suggest that the input is likely associated with different types of social functions (e.g., visiting a hospital or attending a lecture vs. overhearing a conversation on the street or meeting a friend in a park). This is primarily an introductory question that requires a simple, straightforward answer and that remains constant across all the surveys.

**Question 3: In the past hour, did you consciously listen to any speech?**

This question is the "trigger" for the rest of the survey. If the participant answers "Yes" to this question, they then continue to the next part of the survey, which asks about their most recent language experience. If they answer "No", they will be directed to 7 different questions.

**Question 4: Was your experience with the speech interactive in-person (participating in conversation, in person), interactive online (remote, over the phone, FaceTime), just listening in-person (sitting in a lecture, bystander to conversations), or just listening online (social media, movies, songs)? Choose all that apply.**

This question asks about the mode of linguistic interactions participants engaged in. This is based on the assumption that linguistic input might have different effects on participants' perceptual abilities depending on if it occurs in interaction or in passive exposure.

**Question 5: Of the speech that you heard in the last hour, whose speech were you listening to? Choose all that apply. (Family member, friend, somebody that I know and have met before, colleague, stranger).**

This question is a checkbox question, indicating that participants can answer with multiple responses. It assesses both the types of talkers they interact with and the diversity of the interactions.

**Question 6: Which language did you primarily communicate in/listen to in the past hour?**

This question is included primarily for the future extension of the SNAP survey to bilingual and multilingual individuals and communities. As the survey participants were native English speakers, it was expected that the predominant answer would be English, however, it is possible that some of the participants have more exposure to linguistic input in a language other than English.

**Question 7: In the past 1 hour, how many people did you talk to or listen to? (Include non-personal interaction such as watching TV, listening to podcasts, attending a lecture).**

This question is another quantitative assessment of speaker diversity, related to the existing literature on the effect of social network size on adaptive speech perception. For example, people with a larger social network are found to be better at vowel perception in noise (Lev-Ari, 2018), but worse at speech recognition, presumably due to the crowded memory traces (Lev-Ari, 2018; 2022). In the past, a social network size has been estimated based on subjective estimates (e.g., how many adult individuals do you regularly interact with for at least 5 minutes each week?), but this question provides more precise estimates of the number of interaction partners as well as the contexts of interaction.

**Question 8, 9, and 10: In the past 1 hour, did you talk with or listen to anybody in English who has a Chinese/Spanish/ other non-native accent? (Include all personal interactions, phone calls, and TV/movie watching experiences).**

These questions ask if there is any input from a Chinese accent, Spanish accent, or other non-native accent. In this study, we expected that participants in Irvine would be more likely to answer "yes" overall than participants in Rochester. However, within each location, there is expected to be a substantial individual variance. This will be used to predict the behavioral results of the perceptual experiment.

## 3. Background on perceptual experiment

The SNAP survey can be combined with a multitude of experiments. This work has used a cross-modal word matching task developed to examine rapid adaptation to

accented speech and speech in noise (Clarke & Garrett, 2004; Xie et al., 2018). Because the task is short and relatively straightforward to implement online, it is suitable for the SNAP survey.

In this task, participants are instructed to listen to spoken sentences and provide judgements as to whether the last word of a sentence matches a printed prompt on the screen (Figure 4A). These sentences are meant to be "low predictability", meaning the preceding context in a sentence is not informative about the identity of the final word (e.g., "Dad pointed at the beaver"). The basic idea is that the accuracy and response times would reflect the listener's current level of adaptation. The more exposure a participant gets, the more accurately and rapidly one can make the "match" vs. "mismatch" judgments.

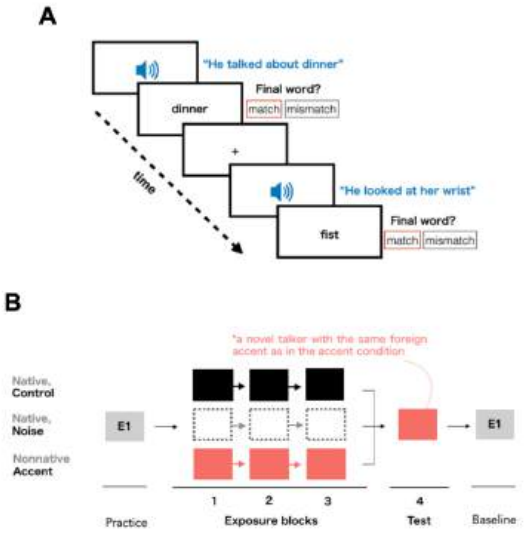


**Figure 4.** A) Trial layout for all trials in the perceptual experiment. B) Outline of a participant's full experience when participating in the perceptual experiment

In Xie et al., (2018), the main experimental portion of this task consists of three exposure blocks and one test block (Figure 4B). Using a between-subject design, participants were randomly assigned to one of the three exposure conditions: a) native, clear speech (Control); b) native speech embedded in speech shaped white noise (Noise); and c) Mandarin-accented speech (Accent). After 6 trials of practice items produced by a native talker, participants received 18 exposure items and 6 test items. The design shown in Figure 5 is from Experiment 2 of Xie et al. (2018), in which the test talker is a novel talker of Mandarin-accented English to equate the talker-switch costs for all conditions. Finally, participants receive 6 more baseline trials, information from which is used to normalize baseline differences in response times.

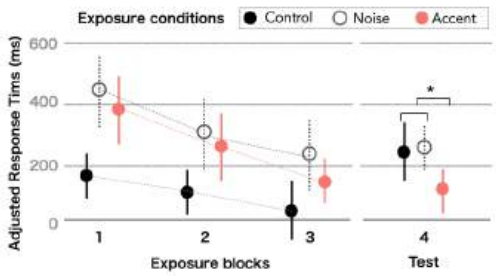


**Figure 5.** Depicting the results for participants in Xie et al., (2018)'s cross modal priming paradigm, adapted for the current study. Each experimental group is shown in a different color and undergoes three exposure blocks along with one test block

The original study found a significant difference between the Accent vs. the other conditions in both error rates and response times (Figure 5). Overall, the response times decrease for all groups as they proceed through the exposure blocks as they adapt to the task and stimuli. In the test block, however, only those exposed to the same nonnative accent in the exposure block were able to respond much more quickly than other groups. This has been taken to suggest that the mere 18 items of exposure led to adaptation to the Mandarin-accented speech. These findings also suggest that exposure effects generalize across speakers with the same accent (Xie et al. 2018). The paradigm has been extended in two ways for this study, in which the Noise and the Accent conditions are administered in a within-subject manner. Each participant is first exposed to the accent condition before the SNAP survey and returns to the noise condition. Between the two conditions, the practice and baseline trials remain identical.

## 4. Study Overview

### 4.1 Participants

20 participants were recruited in Rochester, NY, and Irvine, CA (n=10 each, Figure 6). These two locations were chosen for their contrasting language landscapes,[2] particularly their representation of languages other than English and the diversity of nonnative accents. For feasibility, the current study targeted college-aged individuals on the campuses of the sociolinguistic profiles of campus populations are expected to differ from those of off-campus populations, we believed that participants at the two sites would have different language input because of their surroundings. Recruiting these participants was done using flyers distributed at both the UR and UCI. This distribution happened via word of mouth and various online platforms.

[2] The 2021 American Community Survey data (the United States Census Bureau, Table# B16001) was used to obtain objective estimates of participants' ambient exposure to nonnative-accented speech. For instance, the numbers of foreign-born immigrants who speak Spanish at the county level were starkly different: The estimates will be ~25% in Orange County, where Irvine is located, as compared to <5% in Monroe County, where Rochester is located. The distributions, however, are likely more comparable between the campus populations at the University of Rochester and UC Irvine. We will come back to this point in General Discussion.

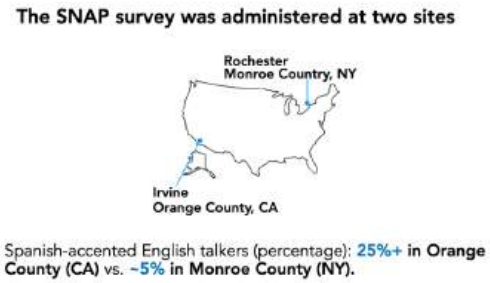


**Figure 6.** The current study took place in two geographical locations with starkly different linguistic diversity profiles: Rochester (Upstate NY) and Irvine (Southern California)

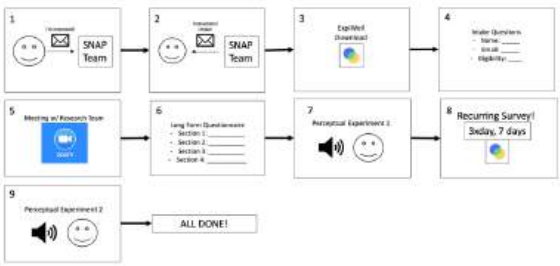


**Figure 7.** Full participant experience outline, showing each of the steps that a participant undergoes as they proceed through the study. Each of these nodes may take a different amount of time for different participants.

We began with two exclusion criteria: (1) Participants must be between the ages of 18 and 40; and (2) participants must be native, monolingual speakers of English. Exclusion criteria (1) was established because we wanted to constrain our subject pool to adults who had not experienced significant hearing loss yet. Exclusion criteria (2) was created to ensure that participants would not have prior experience with other languages, which may increase their prior accent experience. Furthermore, individuals who speak another language may also have more input from other bilingual speakers, even in English. This may cause bilingual individuals to receive more accented-English input. Due to time constraints on recruitment, we modified criterion (2). As a result, two of the participants at UCI did not meet this criterion.. The average age of participants across both cities was 21 and the gender distribution was 11 males and 9 females.

## 4.2 Methods

The participant experience is shown in Figure 7. It begins when a participant sends an email to the research team, which triggers an automatic email response containing onboarding instructions about (a) downloading the ExpiWell app, (b) the intake questionnaire on the app, and (c) scheduling a Zoom meeting with the team for some further onboarding processes. The Zoom meeting consists of three parts. The first part verifies that the participant is set up on the app and ready for the bulk of the surveys to begin. The second part is a Long Form Questionnaire about their demographic information and past language experience (see Appendix I). The last part of the Zoom meeting entails a Perceptual Experiment (Section 3), where participants received the stimuli from the noise condition. Upon completion of the perceptual experiment, the SNAP survey begins on the next day.

After finishing the bulk of the experiment, participants proceed to the second Perceptual Experiment (the Accent condition), which marks the end of their time as a participant in the study, and they receive a $50 Amazon Gift Card via email.

## 4.3 Results

In this section, the responses given to the questions described in Section 2.4 are summarized. The 20 participants responded to 351 surveys out of the 420 surveys for which they were notified (which is a 84% response rate). All notifications were sent out to all participants, and the app had no issues, meaning that all "missed" notifications were due to participant negligence. The rates of missed notifications were higher in the morning (13%) as compared to the other two time-windows (9% in the afternoon and 9% in the evening). Nonetheless, the overall high rate of responses supports the validity of the current protocol.

### Q2. In the past hour, did you listen to any speech?

284 out of 351 (81%) of the responses indicated that participants had received some linguistic exposure (Figure 8). The remainder of the data report will be focused on the cases where participants responded "Yes" to this question.

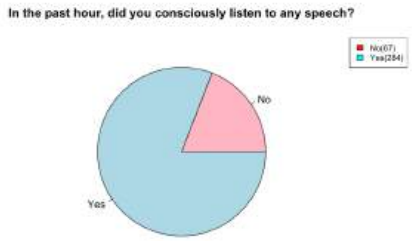


**Figure 8.** Conscious speech reporting: showing a significantly higher number of participants who were coming into contact with speech in the past hour, when prompted with the survey.

### Q3. Interactive vs. passive / In-person vs. online?

The four options (shown in Figure 9) were generated in a 2x2 manner. One of the distinctions was between "just listening"

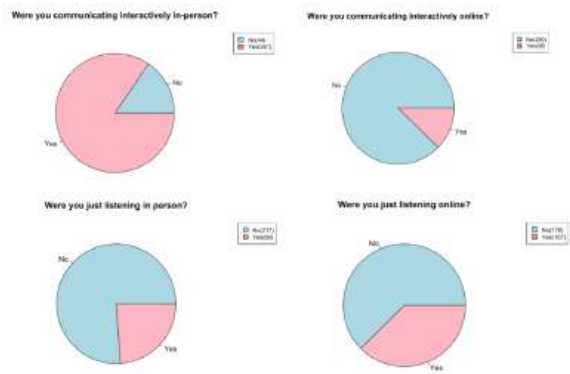


**Figure 9.** Responses to the questions regarding whether individuals were engaging with speech passively, just listening.

and "interactive", which outlines the quality of speech interaction, and whether there would have been room for hands-on engagement with an accent, rather than just passive listening. The other distinction occurs between "in-person" and "online" speech. This distinction is necessary because of prior research outlining the differences in language acquisition and proficiency when exposed to stimuli through media versus in person (Lytle et al., 2018). The responses suggested that when participants were using language interactively, they were more likely to do so in person than online (including a phone call). On the other hand, when they were passively listening to language, they were likely doing so online rather than in person.

**Q5. Whose speech did you listen to?**

Figure 10 summarizes the relationships between our participants and their primary conversational partners. Participants were permitted to respond "Yes" in a checkbox manner (i.e., allowed to select multiple answers during the survey). The results display that the participants responded to friends and strangers the most,which gives valuable insight regarding their linguistic experiences in a naturalistic and time-locked manner.

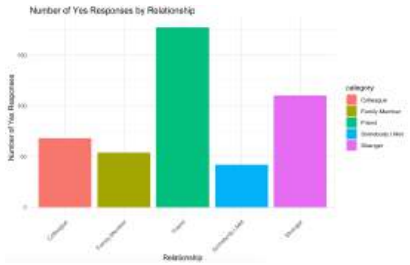


**Figure 10.** Breakdown of the people with whom our participants interacted throughout all surveys. Participants could respond that they conversed with more than one of these individuals in each survey response.

**Q6. What language did you communicate in?**

This question, in other circumstances with more resources and subject selection abilities, could be used to ensure that participants were conversing only in English. For us, it is used to showcase whether there is any linguistic diversity in the participant's speech. The results show that participants were conversing mainly in English (97%), with some individuals speaking in Cambodian, Vietnamese, and Chinese.

**Q7. In the past hour, how many people did you speak with?**

The number of speakers varied from 1 to 50 (mean = 5.26) (Figure 11). In general, participants were interacting with a larger number of speakers during the afternoon (mean = 5.96) as compared to in the morning or the evening.

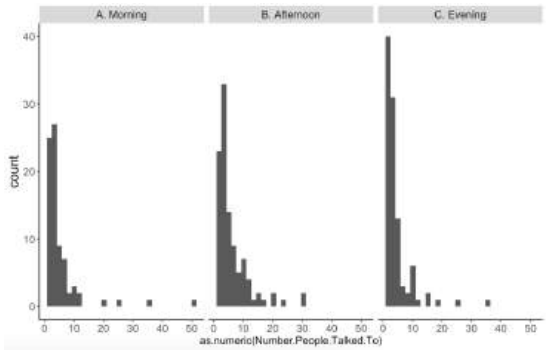


**Figure 11.** The number of speakers that participants have listened to in the past hour, split up by the different timeframes in which surveys were distributed

**Q8,9,10. In the past hour, did you speak to anyone with a Chinese, Spanish, or other accent?**

It was expected that participants at the Irvine site would be more likely to answer "yes" to these questions due to the more diverse linguistic representation in Southern California. On the contrary, overall data were similar across the two sites. The "yes" responses for a Chinese accent were slightly more frequent at the Irvine site (31/135, 23%) than at the Rochester site (22/145, 15%) (Figure 12). However, the proportions were reversed for a Spanish accent (9% vs. 19%) (Figure 13) and comparable for any other accent (24% vs. 25%) (Figure 14). This was likely due to the fact that the current participants were college students, whose daily linguistic interactions occur on campus with large bodies of international students.

To better understand the differences between the two sites, as well as individual differences in accent exposure, I have summarized the participant' responses. Figure 15 represents the "yes" responses to question 8. This is shown to provide an example of the individual variation for one of the questions, showcasing the power of the SNAP survey. Next, Figure 16 is a combination of questions 8-10, highlighting the individual variation that occurs throughout these three questions. There


**Figure 12.** The number of respondents that heard a Chinese accent in the past hour (Left = Rochester, Right = Irvine)

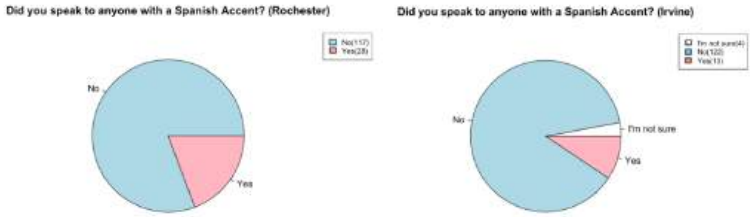


**Figure 13.** The number of respondents that heard a Spanish accent in the past hour(Left = Rochester, Right = Irvine)

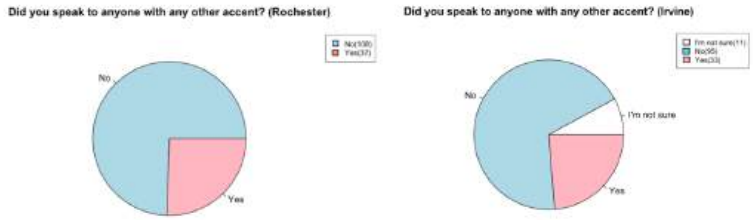


**Figure 14.** The number of respondents that heard any type of OTHER accent in the past hour, excluding Chinese and Spanish accents

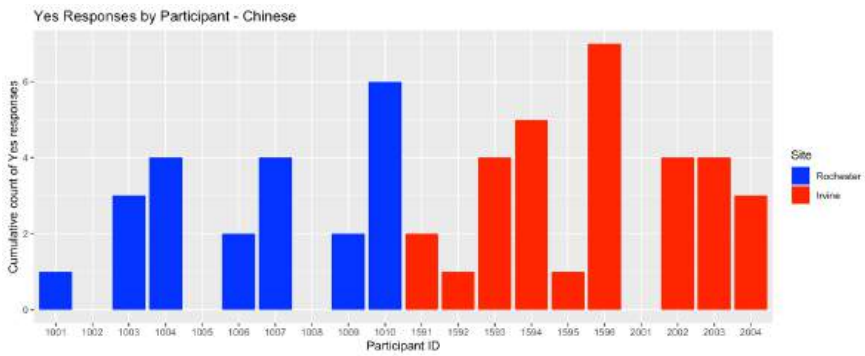


**Figure 15.** The individual breakdown for Chinese of how participants respond throughout the survey, and how many times they reported hearing Chinese accents in the past hour. The participant ID 1001 – 1010 were from the Rochester site and 1501-2004 were from the Irvine site.

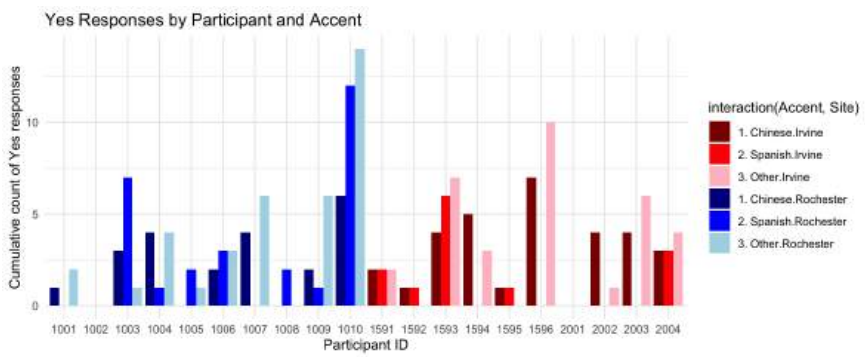


**Figure 16.** The individual breakdown of how participants respond throughout the survey questions 8-10, and how many times they reported hearing Spanish accents in the past hour. The participant ID 1001 – 1010 were from the Rochester site and 1501-2004 were from the Irvine site.

are some underlying trends that appear when viewing Figure 16. First, there were a few individuals at both sites who never answered "yes" to any of these questions (e.g., participant 1002 at Rochester and participant 2001 at Irvine). Second, and in contrast, a few subjects answered “yes” at a much higher rate than others. For example, participant 1010 consistently answered “yes” to all three of these questions, suggesting that they were likely interacting with speakers from different linguistic backgrounds. Finally, there are a few others who answered “yes” to a particular question (i.e., a particular non-native accent) but not to others. For example, participant 1596 (at Irvine) frequently answered "yes" to questions 8 (a Chinese accent) and 10 (any other non-native accent), but not to question 9 (a Spanish accent). These patterns support the idea that individuals vary significantly in their exposure to accents, even in the same geolocation, and that SNAP surveys can effectively capture these individual differences.

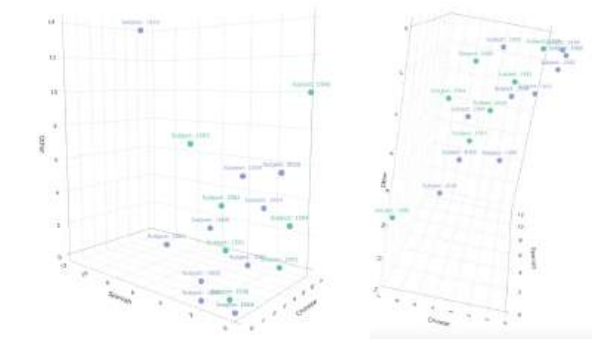

**Figure 17.** These graphs show a 3-dimensional plot of the data collected by the SNAP survey. These points are all representations of where each individual lies within the 3-dimensional space.

**Comparisons Across Participants**

The SNAP survey questions, as they stand, are able to generate three dimensions of accent experience information,including Chinese, Spanish, and other accented English. These dimensions are represented by the quantity of “yes” responses and can be plotted as such.

This plot is effective at capturing the diverse nature of the relationship between different types of accent experience. Naturally, individuals will not have the same experience levels with all accents, but it does seem to hold that high exposure to one accent is an adequate indicator of high exposure to other accents, as well.

What remains to be explored, though, is the relationship between this exposure at different sites, which is discussed below.

**Comparisons Across Locations**

The nature of exposure across diverse and homogenous locations is a core tenet to this thesis. Hence, a matrix of graphs is useful to showcase the differences between these locations. The lower level of the below graph (Figure 18) is a linear model representation of each of the three relationships between accents. This model helps us bolster our above argument. It furthers the argument that as one type of accent experience increases, so do the others. The linear model is especially indicative of a linear relationship between Chinese and Spanish accent experience.

Next, the middle layer helps us answer the question of location differences in responses. As the two shadings are primarily shown in overlap, we are able to determine that the densities of responses which indicate that accent experience over-

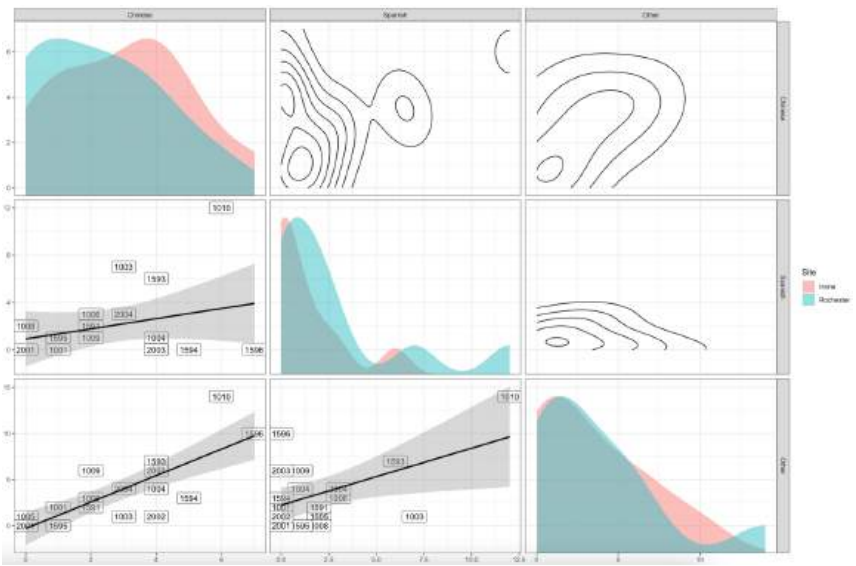

**Figure 18.** These graphs show three levels of comparison, discussed above. They are useful in disambiguating the differences in location accent experience (especially the second layer). The layers are: lower = linear model of participant accent experience reporting, middle = density plot of responses across both locations, upper = 2-dimensional plot of the density of responses across each 2-dimensional space.

lap . This suggests that there is very little difference in accent experience between the two sites.

The overall density, across both sites, displayed in the 2-dimensional graph in the upper layer, is powerful in showing where the responses primarily clump for each accent experience relationship.

All of these layers allow us to conclude that there is significant difference in individual variation, but that this difference does not appear across location (diverse vs. homogeneous).

**Perceptual Experiment Results**

Figure 19 summarizes the perceptual experiment results from both sites. Irvine is represented as the "diverse" location due to the heterogeneous nature of its language profile compared to Rochester. Overall, Rochester participants responded similarly to participants in Clarke and Garrett (2004) and Xie et al. (2018) in that their RTs (response times) decreased steadily throughout the exposure phase in both the accent and noise conditions. Their RTs at test did not differ between conditions, which may be due in part to the small sample size. In contrast, the Irvine participants showed a different trend which showed much more variability in the accent condition and they performed better (numerically) on the accented test speaker after exposure to native speech in noise.

The lack of significant effect of the condition on RT in Rochester, may be due to within-participant carryover from the accent condition to the noise condition. Despite the 7-day interval, participants may have been able to recall the experiences in the accent condition and apply them in the noise condition. As for the increased response variability in the accent condition in Irvine, this could be due to the greater heterogeneity among the participants (e.g., 2/10 were not monolingual English speakers). We will continue to examine their long-form questionnaire responses to explore possible sources of the response variability. More generally, the results suggest that researchers should exercise caution and significant randomization protocols when conducting a within-subjects design and using this paradigm.

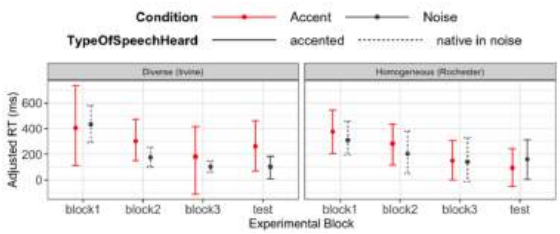


**Figure 19.** The progression of participants through the experimental paradigm: the three exposure blocks into the test block. Each site has 10 participants, who participated in the accent vs, noise condition before and after the 7 day SNAP survey.

A next step to investigate is this paradigm's interaction with the SNAP data. As it stands, the most effective way to achieve this would be appending test block reaction time data to the SNAP data. Specifically, the SNAP data should outline the percentage of surveys in which each individual responded "yes" to each accent. By analyzing these percentages alongside the reaction times, and discussing whether lower reaction times correspond to higher percentages (especially for Chinese accents), we would be able to effectively combine these measures.

## 5. General Discussion

While effects of long-term linguistic experience on accent adaptation have been widely assumed, few empirical tests are currently available. This thesis put forward a new experience-based sampling method implemented as a mobile-based survey (SNAP). Participants responded to the SNAP survey over seven days, and participated in the perceptual experiment from Xie et al. (2018) twice in two conditions, "accent" and "noise". By involving participants at two different sites, Rochester, NY and Irvine, CA, I provided a proof-of-concept validation of the SNAP survey in multiple areas in the United States. Encouragingly, the survey delivery was seamless with no technical issues, and the response rate was high (84%). This supports the feasibility of the current SNAP survey protocol. This data will provide additional insights into what future iteration of the SNAP protocol can tell us about the long-term effects of accent exposure.

In the study, we found that participants routinely experience language exposure throughout the day, with a larger number of talkers during the day than in the morning or evening. However, it is important to keep in mind that there was a greater instance of missed notifications in the morning. This could mean that the samples in the morning were noisier than those in the afternoon or in the evening. The most prominent type of interaction was communicating interactively in-person at 85% (241/285). The least prominent mode of interaction was communicating interactively online at 12% (35/285).

Importantly, and contrary to our original expectations, the accent exposure data at the two sites were largely comparable with each other. For example, participants in Irvine reported hearing Chinese-accented English in 23% (31/135) of the surveys they received while participants in Rochester reported hearing Chinese-accented English in 15% (22/145) of their surveys. These proportions were reversed for Spanish-accented English, while they were equivalent for other accents. The by-participant analysis suggested that the observed similarity emerged from interesting individual differences. Some participants (e.g., 1010) reported on a much higher rate of exposure to multiple accents than some others who reported having none. Our results(Figures 8-19), indicate that high exposure in one accent likely suggests high exposure across all accents. These points yield support to the idea that individuals vary widely in terms of their accent exposure and famil-

iarity. Individualized measures like SNAP will be a useful and instructive supplement for geolocation-based estimates of SOLID.

The biggest hurdle was to integrate the SNAP survey into a larger study workflow including the subject intake, the long-form questionnaire, and perceptual experiments. In the current implementation, the study team must be very hands-on with participant registration and progress monitoring. We met with each participant via Zoom to go over the study procedure as well as to provide instructions for the surveys and the perceptual experiments. Whereas this was effective in terms of ensuring task compliance and ensuring eligibility criteria (e.g., monolingual status of the participant), it will be prohibitively expensive and therefore unattainable as the study scales up in size. We have created an instruction video (https://vimeo.com/924986229) to aid this process. In the future, we should implement a self-paced registration process with eligibility check questions to streamline this process and eliminate the need for individual zoom sessions.

As discussed in Section 1.1, one of the instruments uses the US census data of speakers of non-English languages. One previously tested hypothesis is that listeners who are in a region of the US with a more diverse linguistic profile would be better at adapting to a talker with an unfamiliar accent than those who are in a more linguistically homogeneous region (Xie et al., 2023).

These results could support two mutually compatible possibilities. The first is that adaptation through short-term exposure is so powerful that it overrides whatever individual differences may exist at the start of the experiment. The second is that a zip code area, let alone a state, is too coarse a unit of analysis. Individuals within each area/state represent highly heterogeneous linguistic experiences, and grouping like this dilutes individual differences in accent familiarity. The results of the study reported above shed light on such heterogeneity. If this is the case, the SNAP survey may be able to provide a more fine-grained, individualized measure of accent exposure. In a future iteration, we plan to administer SNAP with a larger group of participants recruited from different regions across the US. In doing so, we aim to provide a more rigorous test of the hypothesis about prior linguistic exposure and its effects on short-term, rapid accent adaptation.

In conclusion, the SNAP survey provides a unique predictor of the data collected in the perceptual experiment (e.g., cross-modal priming). From the simple, time-locked surveys administered throughout the day, researchers are able to accurately track the amount of accent exposure to infer the individual participant's accent familiarity. Combined with the other SOLID measures, the SNAP survey will fill in the blanks in terms of an individualized estimate of accent experience and its impacts on adaptive speech perception.

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## About the Author

Seth Cutler is a 2024 graduate of the University of Rochester. He majored in Brain and Cognitive Sciences, Linguistics, and Spanish and conducted this work, his honors thesis, in BCS. Since graduation, Seth has gone on to become a research fellow at the National Institutes of Health, working with intellectual disability through the lens of neuropsychological methods. He plans on continuing his academic journey through a Ph.D. at the end of his fellowship.

# Retrospective Analysis of the Relationship Between Obstructive Sleep Apnea and Nocturnal Bradycardia

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## Introduction

Obstructive sleep apnea (OSA) is a highly prevalent respiratory sleep disorder characterized by recurring apneic episodes during which the upper airway is obstructed (Mayo Clinic, 2023). Globally, approximately 900 million adults - ages 30 to 69 - have been diagnosed with OSA; 44% of whom have moderate to severe OSA (Teo et al., 2022). OSA patients show a relaxation of the pharyngeal muscles, a collection of skeletal muscles located posterior to the oral cavity, that are essential for airway patency and support of the upper airway (Oliven et al., 2008). As a result, the airway narrows and may temporarily collapse, restricting ventilation while increasing respiratory drive as a compensatory mechanism (Jordan & White, 2007). Transient disturbances in airflow may be classified as either hypopnea or apnea. Hypopnea refers to airflow reduction of at least 30% and/or decrease in oxygen saturation of at least 3% for ten or more seconds. Apnea is a complete cessation in breathing for ten or more seconds (Berry et al., 2022). OSA symptoms may include but are not limited to: daytime fatigue, loud snoring episodes, abnormal breathing patterns, excessive night sweating, insomnia, headaches, and frequently waking up gasping for breath (Slowik, 2022).

OSA can be classified as mild, moderate, or severe based on the apnea-hypopnea index (AHI) system. The AHI is calculated as the average number of apneas and hypopneas per hour of sleep, and is one of several metrics reported during a polysomnography test (sleep study) in addition to brain activity, heart rate, oxygen saturation, and muscle movements (Somers et al., 2008). The American Academy of Sleep Apnea delineates mild apnea as 5-15 events per hour, moderate apnea as 15-30 events per hour, and severe apnea as 30 or more events per hour (Figure 2). Treatment of OSA varies from lifestyle modifications with an emphasis on weight loss, to oral appliances such as continuous positive airway pressure (CPAP) machines, surgeries such as removal of tonsils and adenoids through Uvulopalatoplasty, and hypoglossal nerve stimulation (Pavwoski & Shelgikar, 2017)

The AHI has been the standard metric to quantify OSA severity for several decades. However, recent studies suggest that the AHI severity grading system may have inherent pitfalls (Malhotra et al., 2021). The system classifies apnea and hypopnea as equal events, despite significant differences in their physiological effects. Variation in definitions of hypopnea may also give rise to inconsistent measurements, and therefore cause discrepant calculations of final AHI scores. (Ro

TABLE. APNEA-HYPOPNEA INDEX SCORE CLASSIFICATION FOR ADULTS

| APNEA SEVERITY | APNEA-HYPOPNEA INDEX (AHI) (EVENTS/HOUR OF SLEEP) |
|---|---|
| Normal | <5 |
| Mild | 5 ≤ AHI < 15 |
| Moderate | 15 ≤ AHI <30 |
| Severe | ≥30 |

**Figure 2.** The AHI scoring system

bards, n.d.). The AHI system also fails to account for body position duration, how long someone remains in a position like sitting or laying down, during apneic events, which may reduce overall scoring accuracy (Soori et al., 2022). The aforementioned findings collectively express that AHI as a standalone for severity classification may lack nuance and oversimplify the categorization of OSA. Consequently, OSA may be misclassified, causing key variables influencing patient health status to be overlooked which impacts clinical outcomes as well as treatment strategy choices

As emerging evidence suggests limitations to the AHI system, more attention is being focused toward broader clinical complications and conditions comorbid with OSA. Physical obstruction of the upper airway hinders the flow of oxygen during sleep, resulting in OSA becoming an associated underlying co-morbidity in numerous non-communicable, chronic conditions such as cardiovascular disease (CVD), hypertension, stroke, cardiac arrhythmias, and mental health-related disorders such as depression and anxiety (DiCaro et al., 2024). The association between OSA and cardiovascular disease is widely known, with growing evidence suggesting that OSA may exacerbate cardiovascular conditions through a variety of physiological mechanisms (Tietjens et al., 2019). The incidence and prevalence of OSA are of epidemic proportions among patients at risk for Sudden Cardiac Death or CVD, which Blackwell et al. in 2019 attributed to several shared risk factors (Figure 1).

Despite its high comorbidity with cardiovascular disease, OSA remains alboth underdiagnosed and undertreated (Yeghiazarians, 2021). Though studies continue to connect CVD and OSA while others reject the AHI system as the sole standard for OSA categorization, it is essential to develop classification mechanisms that are not only accurate, but also multidimensional. The limitations of the AHI system may contribute to t-

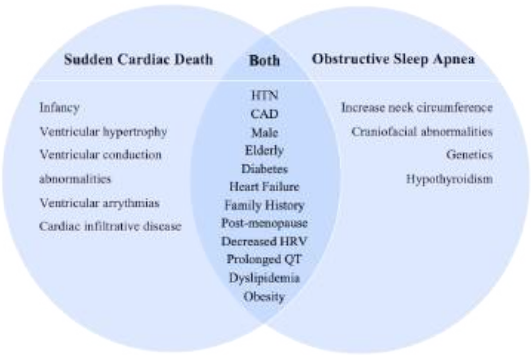


**Figure 1.** Individual and shared risk factors of sudden cardiac death and obstructive sleep apnea

he underdiagnosis and undertreatment of OSA within patients who have CVD. For instance, Suen et al. in 2020 found that there are "significant knowledge gaps regarding the effect of treatment and OSA severity" on cardiovascular outcomes due to existing scientific literature only comparing the prevalence of CVD in OSA patients and vice versa.

Given that OSA is a multifaceted condition, this study aims to identify another quantifiable surrogate that serves as an adjunct for the current AHI system. Existing literature across diverse medical fields within internal, cardiovascular, and sleep medicine commonly acknowledge that there is a high prevalence of OSA within patients who have nocturnal bradycardia. A notable meta-analysis in 2022 quantified the occurrence of OSA in patients with nocturnal bradycardia, and vice versa, as well as the effectiveness of the CPAP (Continuous Positive Airway Pressure) machine in addressing the bradyarrhythmia. Cumulative incidence rates and prevalence were tracked through several statistical analysis platforms. Among the 34 articles used, 4852 patients were identified and used for the analysis. The prevalence of both daytime and nocturnal bradycardia was approximately 25% and 69.8% respectively. Within the patients who had bradyarrhythmia, the prevalence of OSA was approximately 56.8%. CPAP treatment did not seem to significantly impact the prevalence/occurrence of daytime or nocturnal bradycardia (Teo et al., 2022). Researchers have connected the two conditions together and conducted studies to display how sleep apnea gives rise to bradycardia in the night. Zwillich et al. (1982) investigated the potential mechanisms eliciting bradycardia during sleep apnea, and how vagal efferent activity was a common trigger for bradycardia amongst enrolled subjects. This seminal study was the first to evaluate heart rate variability amongst patients with sleep apnea. Along with heightened vagal activity, it has been discovered that sleep apnea gives rise to nocturnal bradycardia through the snowball effect, beginning with the blocking of lung expansion. Rossi et al. (2013) evaluated the "Effects of Sleep Apnea and Heart Rhythm", further affirming that the upper airway prevents lung expansion and stretching of vagolytic fibers in the lung.This elicits the diving reflex, which then increases sympathetic vasoconstriction to muscles and viscera to maintain perfusion to the vital organs. The result of the cascade is a rise in blood pressure and vagally-induced reflex bradycardia. With clear scientific connections to OSA — as well as a high prevalence within OSA patients — nocturnal bradycardia may be an appropriate and useful cardiovascular condition to potentially contribute to earlier and more accurate OSA diagnosis and classification.

Patients diagnosed with daytime or nocturnal bradycardia may receive a loop recorder, a device inserted under the skin that monitors heart rhythm and rate. Cardiac pacemakers may also be implanted to pace the heart if the rhythm reaches a rate that is below the set threshold rate. As of yet, current research does not establish a correlation between pacemaker therapy and alleviation of OSA symptoms.

It was hypothesized that the degree of nocturnal bradycardia coupled with the AHI system increases the specificity of severity grading for OSA. It was also expected that patients who benefit clinically and physiologically have less frequency and duration of nocturnal bradycardia with optimal therapy for OSA.

## Research Design and Method

### Study Design

The purpose of this study was to assess nocturnal bradycardia as a potential indicator for OSA severity grading within cardiac patients. A retrospective pilot observational study was conducted to assess the association between nocturnal bradycardia and OSA in cardiac patients who meet established criteria for OSA treatment and event monitoring (Medicaid Coverage Database, n.d.).

### Data Acquisition

Pre-existing data was extracted and analyzed from AthenaHealth electronic medical records (EMRs) to minimize risk to subjects and to efficiently assess any trends pertaining to nocturnal bradycardia events and OSA pathogenesis. Data abstraction took place after obtaining informed consent from the cardiology clinic and respective patients enrolled. Printed informed consent forms were provided to all enrolled patients. Each form provided a description of the study, the data being acquired, and how the data will be used to establish conclusions or relationships between OSA and nocturnal bradycardia. All patient data abstraction processes de-identified personal health information to maintain patient confidentiality.

Through chart abstraction, relevant data (socio-demographic, clinical characteristics, patient characteristics, event monitor and sleep study results) were analyzed and extracted for the purpose of this study (Figure 3). Race, gender, age, comorbidities, current medications, functional status (New York Heart Association Classification), alcohol and tobacco use, and family history were all reported as patient factors. AHI

scores, and for some subjects, oxygen saturations, and heart rate were extrapolated from sleep study data. Additional metrics for certain patients were reported on the sleep study such as time spent under 90% Oxygen saturation, and were therefore listed in this study. Nocturnal bradycardia was defined according to the American Heart Association position statement as a heart rate of less than 60 beats per minute (Kusumoto et al., 2018). To determine the prevalence of nocturnal bradycardia, event monitor data in the form of device interrogations were utilized to display continuous monitoring of the patient's rhythm during a specified time period.

The process of care for patients with event monitors and OSA was quantified by concurrent formal guidelines based on the American College of Cardiology (Kusumoto et al., 2018) and American Association of Sleep Medicine (Kapur, 2017) for the diagnosis of Nocturnal bradycardia and obstructive sleep apnea.

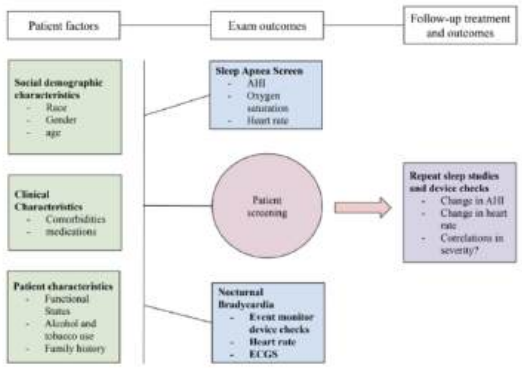


**Figure 3.** Flowchart illustrating the relationship between various subject factors and exam outcomes in sleep apnea assessment. Subsequent evaluations, such as repeat sleep studies and device interrogations, were used to monitor changes in apnea severity, heart rate, and oxygen saturation to evaluate potential relationships between nocturnal bradycardia and OSA.

### Participant Selection

This study enrolled all consecutive patients who have a formal diagnosis of OSA (n=5), and fulfill other inclusion criteria such as an event monitor implanted from an outpatient cardiology office setting. Given that this study is a pilot study, the association between OSA and nocturnal bradycardia is still being explored experimentally and the inclusion criteria of both conditions are highly specific. Five cases were chosen to be analyzed. Although sample size was limited, the purpose of this study was to establish and assess baseline trends among patients with both OSA and nocturnal bradycardia, which could be tested in larger cohort studies.

**The inclusion criteria for this study is as follows:**

- The patient is diagnosed with OSA and currently being monitored with a 30-day event monitor (AHI > 5.0)

- The patient is willing and able to give informed consent for their clinical and background data to be examined

Exclusion criteria included non-English speaking patients to maximize efficient communication as well as patients who could not give informed consent to participating in this study. No expectation for early withdrawal was anticipated, given the observational nature of this study. However, a patient was able to withdraw from the study at any time.

## Results

Of the five selected subjects for the study, three underwent facility-structured sleep studies, and the remaining two subjects participated in an in-home sleep study. All patients were diagnosed with OSA based on having a minimum AHI of 5.0. Each subject also had an implantable loop recorder, which permitted the analysis of device checks including bradycardia events. A de-identified description of each subject's clinical profile is presented, containing information such as major cardiac events, medications, family history, and compliance. Loop recorder implantation dates and sleep study dates were recorded to serve as baseline patient data. Device checks on loop recorders are displayed over time to convey potential relationships between OSA and Nocturnal bradycardia. Milestone tables and supporting visuals are also included to convey possible trends.

### Subject A

Subject A is a 72-year-old Caucasian male with comorbidities including paroxysmal atrial fibrillation, bilateral carotid disease status post left carotid endarterectomy, essential hypertension, severe aortic valve stenosis, status post transcatheter aortic valve replacement, and embolic stroke. The subject had an in-home sleep study on May 19, 2015 and was subsequently diagnosed with OSA (AHI of 6.5). The patient refused OSA therapy, but received a loop recorder implantation on January 28, 2022, which detected several bradycardic episodes.

An asystolic episode of 4.4 seconds occurred on February 4, 2023, at 5:03 AM during sleep. The loop recorder started to detect atrial fibrillation in July of 2022. The patient had a repeat in-home study in early March, which demonstrated an AHI of 18 (nearly a three-fold increase in AHI since diagnosis). The patient has been prescribed the following medications: Sotalol (80 mg twice daily), Losartan (100 mg daily), Atorvastatin (80 mg at night), Amlodipine (10 mg daily), Pantoprazole (40 mg daily), and Eliquis ( 5mg twice daily). The patient's mother has a pacemaker and a history of hypertension and atrial fibrillation, and his father had a coronary artery bypass graft to treat coronary artery disease. His functional status has no limitations, and he does not smoke or drink alcohol.

| Date | Event |
|---|---|
| 01/04/2022 | Sinus rhythm (63 bpm) without any abnormalities |
| 02/28/22 | Sinus rhythm (no nocturnal bradycardia or atrial fibrillation |
| 07/09/2022 (device check) | 4 days of intermittent atrial fibrillation with a mean heart rate of 78 bpm (no nocturnal bradycardia |
| 07/20/2022 | Atrial fibrillation detected |
| 08/05/2022 (device check) | Atrial fibrillation recorded at 5 AM with a heart rate of 26 bpm |
| 01/25/2023 | 6 nocturnal bradycardia events recorded with 140 pauses. An atrial fibrillation episode of 1 hour and 3 minutes results in an atrial fibrillation |
| 02/04/2023 | At 5:03 PM (while the subject naps) there is a 4.4 second long pause |
| 02/05/2023 - 03/07/2023 | Over 40 asystolic events are detected |
| Early March (no specific date provided) | An AHI of 18 is recorded |

**Table 1.** Subject A's Milestone table

| | |
|---|---|
| AHI | 65 |
| ODI (1/hr) | 3.9 |
| Mean Oxygen Saturation | 95% |
| Min Oxygen Saturation | 79% |
| Mean Heart Rate (bpm) | 59 |
| Minimum Heart Rate | 54 |
| Maximum Heart Rate | 115 |
| Time spent under 90% Oxygen Saturation | 2.6% |

**Table 2.** Subject A's initial sleep study data

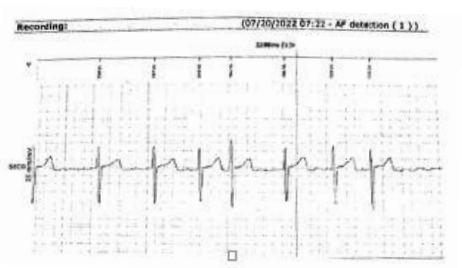


**Figure 4.** An EKG on 07/20/2022 displays a baseline rhythm of atrial fibrillation

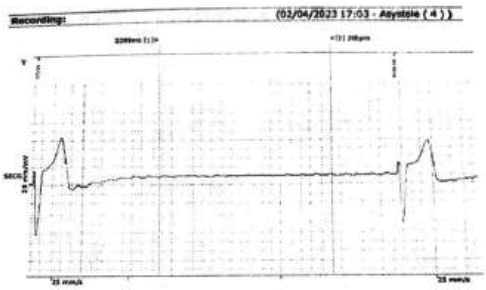


**Figure 5.** The patient has a 4.4 second pause on 02/04/2023 at 5:03 PM (during a nap)


**Figure 6.** A list of 40+ asystolic events detected from 02/05/2023 to 03/07/2023

## Subject B

Subject B is a 63-year-old Caucasian male with comorbidities including coronary artery disease, coronary stent placement involving the left anterior descending (LAD) artery, permanent atrial fibrillation, essential hypertension, obesity (BMI of 37), hyperlipidemia, and congestive heart failure (Diastolic) Class II. The patient had a facility-structured polysomnography test on August 28th, 2011. He was subsequently diagnosed with OSA (AHI of 14). The patient was prescribed a CPAP machine and was initially noncompliant with therapy. The patient received a loop recorder implantation on December 7th, 2021. After March 10th, 2022, the patient became

compliant with CPAP therapy and medicines. The patient has been prescribed the following medications: Metoprolol Tartrate (75 mg twice daily), Losartan ( 25 mg daily), Brilinta (90 mg twice daily), Xarelto (20 mg nightly), Atorvastatin (20 mg nightly), and Glimepiride (2 mg twice daily). The patient's grandmother has a heart disease history and a pacemaker. His functional status has no limitation, and he does not smoke or drink alcohol.

| Date | Event |
|---|---|
| 12/07/2021 | The subject's baseline rhythm of chronic atrial fibrillation is recorded |
| 12/20/2021 | Permanent atrial fibrillation is detected with a ventricular response of 80 bpm |
| 02/27/2022 | At 5:43, the subject is napping and experiences a sudden rate drop with a 4.5 and 6.4 second pause |
| 03/10/2022 (device check) | A heart rate of 27 bpm is recorded |
| 03/03/2023 | At 8:30 PM a heart rate of 60 bpm is detected. The rate decreases from 60 bpm to 48 bpm without any asystolic events |
| 03/09/2023 | No pauses are detected |

**Table 3.** Subject B's Milestone table

| | |
|---|---|
| AHI | 14 |
| Min Oxygen Saturation | 88% |

**Table 4.** Subject B's initial sleep study data

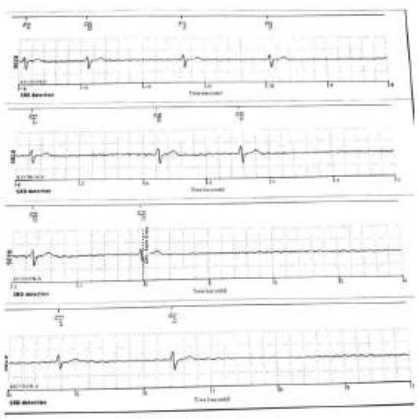

**Figure 7.** Lowest heart rates for Subject B

## Subject C

Subject C is a 53 year old Caucasian male with comorbidities including diabetes mellitus type I, morbid obesity (BMI of 46), essential hypertension, chronic kidney disease stage III, and chronic diastolic heart failure. The patient had an in-home sleep study on April 6th, 2021. He was subsequently diagnosed with OSA (AHI of 44). After being diagnosed with OSA, the patient was prescribed an oral appliance. Due to jaw pain, the patient abandoned therapy at three months of use and switched to CPAP therapy. The patient was also compliant with medicines but non-compliant with his CPAP. He also received a loop recorder implantation on November 10th, 2021. The patient has been prescribed the following medications: Brilinta (90 mg twice daily), Aspirin (81 mg daily), Fenofibrate (160 mg nightly), Bumetanide (1mg daily), Amlodipine (10mg daily), Hydralazine (100 mg thrice daily), and Oxybutynin Chloride (5mg nightly). The patient's mother had coronary artery disease and a coronary stent; his father has a history of coronary artery disease with a coronary stent and has had atrial fibrillation ablation. His functional status is Class II - III, and he does not smoke or drink alcohol.

| Date | Event |
|---|---|
| 11/22/2021 | Nocturnal bradycardia is detected at 4 AM with a heart rate of 47 bpm. At 4:27 AM, the patient has a 2.2 second asystolic event. |
| 02/02/2022 | At 4 AM the patient has a 2 second pause and a 5.4 second pause at 2:07 AM. |
| 07/28/2022 | The patient had a heart rate of 20 bpm for 20 seconds at 3:31 pm. At this time, the patient was napping. |
| 01/12/2023 | At 2:53 PM the patient had an asystolic episode of 4.5 seconds. |
| 02/20/2023 | The patient has another sleep study. An AHI of 88.8 is recorded. The subject spent 55.6 minutes below 88% oxygen saturation and had a mean heart rate of 44. The patient was prescribed a CPAP machine. |
| 03/21/2023 | No nocturnal bradycardia or asystole is detected. AHI is recorded to be 5 from a repeat sleep study test. |

**Table 5.** Subject C's Milestone Table

| | |
|---|---|
| AHI | 44 |
| ODI (1/hr) | 61.4 |
| Mean Oxygen Saturation | 92% |
| Min Oxygen Saturation | 67% |
| Mean Heart Rate (bpm) | 63 |
| Min Heart Rate | 45 |
| Max Heart Rate | 83 |
| Time spent under 90% Oxygen Saturation | 18% |

**Table 6.** Subject C's initial sleep study data

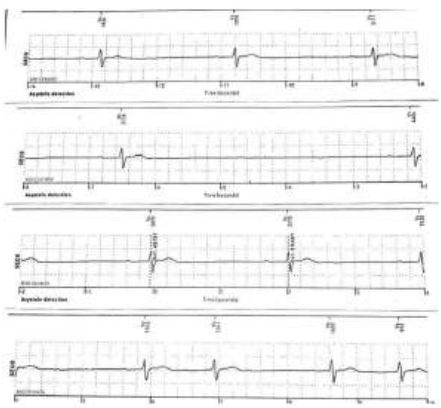

**Figure 8.** On 01/12/2023 2:53 PM, Subject C has a 4.5 second pause

## Subject D

Subject D is a 64-year-old Caucasian male with comorbidities including paroxysmal atrial fibrillation, morbid obesity (BMI of 31), chronic diastolic heart failure Class II, syncope, hyperlipidemia, essential hypertension, and diabetes mellitus type II. The patient had a facility polysomnography test on December 26th, 2016. He was subsequently diagnosed with OSA (AHI of 69) and due to his cleft palate, was prescribed a CPAP machine over an oral appliance. The patient received a loop recorder implantation on December 1st, 2022. The patient has been prescribed the following medications: Xarelto (20 mg nightly), Doxepin (50mg daily), Metoprolol Tartrate (12.5 mg daily), Propafenone (225 mg thrice daily), Fenofibrate (134 mg daily), Lexapro (20mg daily), Metformin (500mg twice daily), and Atorvastatin (20 mg daily). The patient's mother has a history of terminal lung cancer. His functional status is Class II, and according to his record, quit smoking in 2013.

| Date | Event |
|---|---|
| 12/01/2022 | In office check: At 2:24 PM, the patient has a baseline heart rate of 63 bpm (baseline) |
| 12/02/2022 | At 7 AM, the patient has a heart rhythm of atrial fibrillation with a heart rate of 147 bpm. He suddenly drops to 27 bpm while sleeping. |
| 02/21/2023 | A remote device check detects a long atrial fibrillation episode of approximately two hours, with the maximum heart rate being 152 bpm. |
| 04/06/2023 | At 4:54 AM, atrial fibrillation was detected at approximately 160 bpm for an hour and 54 minutes. |
| 04/08/2023 | At 8:44 PM, atrial fibrillation was detected at approximately 140 bpm. |

**Table 7.** Subject D's Milestone table

| Date | Event |
|---|---|
| 08/03/2022 | At 11:10 PM, the patient has a heart rate of 82 bpm in sinus rhythm. At 4:18 PM, the heart rate decreases to 54 bpm, and at 1:46 AM, the heart rate again decreases to 36 bpm. |
| 03/16/2023 | A baseline rhythm of sinus bradycardia is detected with a heart rate in the 50s. No asystolic events were recorded. |
| 07/22/2022 | The patient has a cardiac arrest from ventricular tachycardia |
| 03/30/2023 | No bradycardia is detected, with the minimum heart rate reaching 50 bpm. Patient reports feeling well. |

**Table 8.** Subject E's Milestone Chart

### Subject E

Subject 5 is a 74 year old Caucasian male with comorbidities including ventricular tachycardia, cardiac arrest, COVID-19 infection, overweight (BMI of 28), essential hypertension, paroxysmal atrial fibrillation, and palpitations. The patient had a facility-structured polysomnography test on September 10th, 2019. He was subsequently diagnosed with OSA (AHI of 10.5) and prescribed a CPAP machine that he was only intermittently compliant with. The patient was admitted to a local hospital on July 21st, 2023 and was resuscitated from a cardiac arrest on July 22nd, 2023. The patient had a left coronary angiogram and a drug-eluting stent was deployed within the ramus coronary artery. The subject had a loop implantation on March 2nd, 2023. The patient has been prescribed the following medications: Amiodarone (200 mg daily), Atorvastatin (40 mg nightly), Ticagrelor (90 mg twice daily), Eliquis (5mg twice daily), Metoprolol Tartrate (12.5 twice daily), and Lisinopril (10 mg daily). The patient's father has a history of congestive heart failure, heart disease, and diabetes. His functional status is Class II, and he has a moderate alcohol intake.

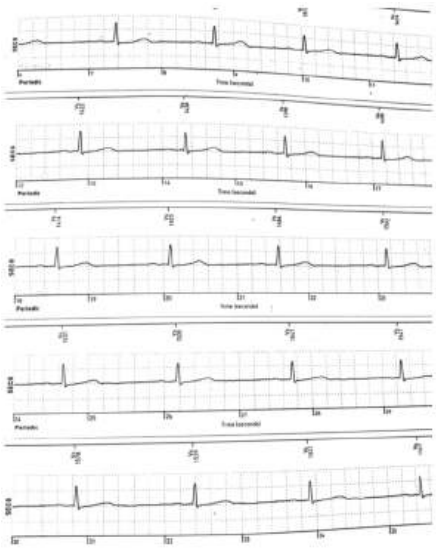

**Figure 9.** The subject's heart rates get progressively slower (50 bpm to 30 bpm)

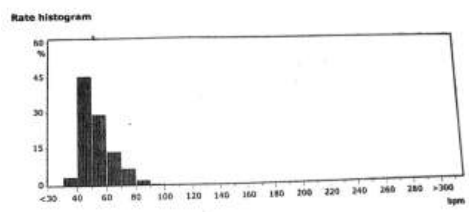


**Figure 10.** Subject E's rate histogram on 03/16/2023 shows bradycardia around 50 bpm and no asystole events

## Discussion

The present study is a retrospective observational evaluation designed to elucidate the relationship between the severity of OSA and Nocturnal bradycardia. This study demonstrates the occurrences of bradycardia during periods of apnea amongst the five subjects that were studied. All patients had a formal sleep study with the lowest AHI of 6.5 (Subject A) and lowest oxygen saturation of 79% to the most severe apneic patients with an AHI of 88 (Subject D) with the lowest oxygen saturation of 67%. This study demonstrates several interesting relationships:

- The degree of bradycardia - as recorded with loop technology - appeared to have an association with OSA severity as observed in Subjects A and C. As OSA increased in severity, their heart rates decreased over time.
- There is a potential relationship between severity of bradycardia and OSA treatment compliance as noted in Subjects B and C. Nocturnal bradycardia burden decreased with CPAP adherence (>4 hrs/night). However the degree of CPAP compliance to offset bradycardia was not studied and must be further investigated among larger cohorts to establish clinical significance.
- There is a possible relationship between the presence of atrial fibrillation and OSA severity as observed in Subject A, B, D and E. Unlike other subjects, Subject D had tachycardia (heart rate > 60 bpm) in the setting of atrial fibrillation which is a rhythm that reflects a conduction abnormality. As bradycardia in subjects D and E persisted (noncompliant with therapy), their atrial fibrillation appeared to worsen.
- Improvement in AHI with reduction in nocturnal bradycardia burden was observed among subjects who had experienced more frequent and prolonged episodes of nocturnal bradycardia burden during the evaluation period, which suggests that the effect of OSA therapy on AHI reduction might be dependent on bodyweight, frequency of and duration of nocturnal bradycardia.

## Limitations

This retrospective study had a variety of limitations:

1. Up to date, there are no randomized trials developed to study the potential effects of nocturnal bradycardia on severity of Sleep Apnea based on AHI.

2. The sample size was insufficient for robust statistical analysis, and the study demographic lacked females or subjects from other racial groups.

3. Medication induced bradycardia may have confounded the data retrieved from Loop interrogation

4. Patient's had different types of sleep studies with only two patients having repeat sleep studies.

5. Patients had a variety of OSA therapies which may present additional confounding variables.

6. Timing of Loop recorder interrogation was not standardized across all five subjects.

7. There were discrepancies between data provided for each patient among sleep studies and device checks due to lack of data available in the EMR.

## Conclusion

The primary objective of this study was to identify a potential association between the severity of nocturnal bradycardia and AHI. The AHI system is thought to have inherent pitfalls, however, scores may become more accurate and standardized among patients if additional metrics such as nocturnal bradycardia or atrial fibrillation is considered.

Additional randomized studies - with larger sample sizes - using nocturnal bradycardia as a metric in the AHI scoring system may suggest relationships between nocturnal bradycardia and OSA. A randomized study with a larger sample size and demographic that aims to investigate the relationship between nocturnal bradycardia and FDA approved treatments for OSA is another future area for research. In addition, one that highlights the potential effects of the therapy on weight loss, glucose tolerance, heart failure readmission and medical compliance would be beneficial findings.

Supplementing the AHI system with other criteria may lead to earlier and more accurate diagnosis of OSA in the United States, which in turn may lead to earlier intervention and therapy. Earlier treatment can reduce the chances of patients obtaining other cardiovascular comorbidities such as heart disease,therefore reducing the risk of sudden cardiac death. As emerging evidence continues to associate OSA to heart health, it is critical to explore relationships in depth to bridge gaps between dental sleep medicine and cardiovascular practice to potentially reduce mortality.

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## About the Author

I am a sophomore studying neuroscience with scientific interests revolving around heart health, neurodegenerative disease, and sleep. Sudden Cardiac Arrest is one of the leading causes of death in the United States, and is most likely to occur between the hours of 10 PM and 6 AM. I am fascinated by the emerging evidence that our sleep is deeply connected to our heart health, and look forward to researching specific associations between arrhythmias and sleep disorders as I progress throughout my higher education.


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## Featured in This Issue

**Engineering Invasion: Do Canopy Gaps Created by the North American Beaver (*castor canadensis*) Facilitate Terrestrial Plant Invasions?** *Carlina Velicer '26 (p.13)*

North American beavers (*Castor canadensis*) create canopy gaps with increased light availability through tree felling, altering the competitive dynamics of forest understory. This study examines whether these gaps facilitate the spread of invasive herbaceous plants and woody shrubs. Upon surveying plots centered around beaver-felled trees and unfelled control trees, we found that felled plots exhibited significantly higher light levels, which were associated with a greater abundance of invasive plants. Three species—Asiatic bittersweet, honeysuckles, and Japanese barberry (*Celastrus orbiculatus, Lonicera spp., and Berberis thunbergii,* respectively)—were the primary drivers of this difference, suggesting that not all invasive species are equally skilled at exploiting canopy gaps. These findings highlight the role of increased light availability resulting from beaver-induced canopy disturbance in enabling terrestrial plant invasions.

**Investigating the Relationship of LINE-1 Expression and SIRT6 Rescue Across Rodent Species** *Natasha Sieczkiewicz '25 (p.21)*

This study investigates DNA double-strand break repair mediated by the protein SIRT6 in rodent species. SIRT6, a sirtuin protein, regulates cellular processes such as aging, DNA repair, metabolism, and inflammation. It is closely linked to the expression of transposable element LINE-1 (L1), which constitutes a significant portion of genomic DNA in mice and humans. L1 activity in somatic tissues has been linked to age-related diseases, such as neurodegeneration and cancer. SIRT6 regulates L1 expression by engaging the KAP1 protein, which facilitates packaging of L1 in heterochromatin. Mice deficient in SIRT6 exhibit elevated levels of L1 expression, leading to shortened lifespan and chronic inflammation. Comparative analysis of SIRT6 and L1 expression across rodent species with varying lifespans revealed a correlation between SIRT6 rescue efficiency and maximum lifespan. Longer-lived species exhibit lower L1 expression, indicating the effectiveness of SIRT6-mediated repression. The findings underscore the importance of understanding species-specific variations in SIRT6-mediated L1 repression. Future research will focus on expanding transfection experiments to non-rodent species and conducting additional immunofluorescence analyses.

**Exploring Poxvirus Genome Packaging Using CRISPR-Cas Systems** *Daniel Kuo '24 (p.26)*

The vaccinia virus genome is an approximately 195-kb linear double-stranded DNA genome that is AT-rich (~67%) and covalently closed at the ends. It is unclear exactly how this genome is replicated and packaged. To selectively encapsidate viral DNA/RNA over cellular DNA/RNA, many viruses rely on genomic elements called packaging signals. To identify the location of packaging signals, the vaccina virus genome can be targeted by CRISPR-Cas endonucleases that cleave DNA proceeded by a short PAM. The CRISPR-Cas system can be utilized to fragment the vaccinia virus genome and with the help of fluorescent protein reporters, it will be determined which, if any, of the fragments are packaged. The process of developing a FnCas12a system and SpCas9 system that targets the vaccinia virus genome is described here.

**The Effect of Modifications to DNA Structure on the Architecture of Bacterial DNA Condensed with a Nucleoid-Associated Protein** *Kevin Zheng '24 (p.37)*

Bacteria are adaptive organisms with various protective mechanisms to respond to environmental stress. One distinct protective mechanism in Escherichia coli that will be discussed further involves the overproduction of a nucleoid-associated protein called DNA-binding protein from starved cells, or Dps. The high concentration of Dps protects the DNA of the bacteria by binding to it and forming a Dps-DNA condensate. To better understand how altering the interaction of Dps and DNA that is not due to environmental stressors will affect the resulting morphology of the condensate in an in-vitro system, we examine the characteristics of the condensate in response to the use of different DNA features. Upon varying the length of linear DNA fragments incubated with Dps protein, we observed that the overall structure of the condensate did not change. However, when incubated with linear DNA fragments of 5k in length, it resulted in the most and largest condensate formation. When Dps was incubated with either supercoiled or relaxed DNA fragments, minimal differences were observed in the size and structure of the condensates. These findings demonstrate that Dps can condense multiple DNA conformations, helping better explain the significant and widespread function of Dps in organisms other than E. coli.

**Estrogen Receptor α (ERα) Signaling and Tumor Cell-Derived Factors Mediate Pro-Tumor Neutrophil Activation in the Setting of Lymphangioleiomyomatosis (LAM)** *Katherine O'Leary '25 (p.45)*

Lymphangioleiomyomatosis (LAM) is a rare lung disease primarily affecting biological females, characterized by the proliferation of smooth muscle-like cells. This abnormal cell growth leads to cyst formation in the lungs and deteriorating respiratory function. Recent studies indicate that estrogen stimulates the growth of TSC-null smooth muscle cells and also enhances neutrophil production. We hypothesized this is done through estrogen receptor-alpha (ERα) signaling in the bone marrow, which potentially accelerates tumor progression. Our results suggest that estrogen influences the activation and function of neutrophils within the tumor microenvironment, promoting an immunosuppressive phenotype that could aid tumor growth. This paper's findings support the potential for developing therapies targeting the estrogen signaling pathways in LAM patients and help alleviate LAM symptoms. This paper also contributes to the growing body of research examining the role of estrogen in disease progression.

**Exploring Predictive Factors for Heart Disease: A Comprehensive Analysis Using Logistic Regression** *Haolin Wang '25 (p.53)*

This study investigates the predictive factors for heart disease utilizing logistic regression analysis on a dataset contains various health indicators. By using a backward elimination approach and Akaike Information Criterion (AIC) for model selection, significant predictors including sex, chest pain type, blood pressure, cholesterol level, and others were identified. Assumption checks confirmed the model's validity. Visualizations and statistical summaries provided insights into the relationships between heart disease occurrence and key predictors. The final logistic regression model, validated by ROC curve and a confusion matrix, demonstrates a robust predictive capability. This research contributes to the field by quantifying the impact of specific factors on heart disease, offering a chance guiding people learn how to use logistic regression in the medical field and make conclusion based on test results.

***Where Do We Put Saint Thecla?* An Examination of Saint Thecla's Place in Time, Theology, and Femininity** *Erin Hess '25 (p.60)*

In this paper, I dive headfirst into the fascinating contradictions surrounding Saint Thecla, exploring how her radical life pushes boundaries in Christian tradition and challenges entrenched ideas about women, autonomy, and authority. Through a close examination of the Acts of Thecla and the later Life of Thecla, I argue that Thecla's story embodies a rare defiance of both societal and religious norms, presenting her not just as a saint but as a daring figure of resistance. In the Acts, we see Thecla as a courageous and independent woman who rejects marriage and, remarkably, baptizes herself—a subversive act that asserts her spiritual agency. Moving into the fifth-century Life of Thecla, I reveal how this second portrayal strategically reframes her, attempting to contain her rebellious spirit within more traditional religious confines. Yet, I suggest that this "domesticated" Thecla may wield even greater influence, subtly defying patriarchal control by resonating with a devoted female following. My analysis interrogates these texts not only as historical documents but as contested ideological spaces, where power, gender, and theology clash. Saint Thecla emerges as an icon whose story, whether celebrated or controlled, challenges Christian orthodoxy's very foundations.

**Bilingual Children: How Do Their Two Languages Talk to Each Other?** *Albenys Díaz Hernández '25 (p.65)*

English is the language of academic success in the United States due to its use as the medium of instruction. Improving English language and reading skills requires practice in English. For children from immigrant families, transitioning to English-only instruction can be challenging. However, does proficiency in their home language contribute to English comprehension and academic success? Theories suggest that skills in one language can transfer to another. To test these theories, we examined language proficiency in 12 early Spanish-English bilingual children, ages 7-10 (M=8.92), using the Bilingual English Spanish Assessment-Middle Extension (BESA-ME) and the Test of Narrative Language (TNL). The BESA-ME assessed grammar and word meaning in both languages, while the TNL evaluated comprehension through storytelling. Our analysis revealed a significant positive correlation between Spanish proficiency and English comprehension, with semantic (word meaning) skills in Spanish most strongly associated with English comprehension. This suggests that meaning-based skills transfer more readily across languages than grammatical skills. These findings challenge the notion that bilingualism delays language development, indicating instead that strong Spanish proficiency enhances English comprehension. These results inform bilingual development theories and offer practical implications for educators and parents.

**An Experience-Based Sampling Approach to Examining Prior Experience in Adaptive Speech Perception** *Seth H. Cutler '24 (p.71)*

My article works to identify new measures that work to understand the role of prior experience on nonnative accent perception. Through the development of this our three pronged approach, my lab and I identified objective, naturalistic, and timely ways of assessing how much prior experience an individual has. We combined these metrics with a naturalistic paradigm to assess performance when attempting to understand a nonnative accent. This combinatory approach allows us to examine how the quantity and quality of prior experience impacts subjects' performance on understanding nonnative accents.

**Retrospective Analysis of the Relationship Between Obstructive Sleep Apnea and Nocturnal Bradycardia** *Sophia Nguyen '27 (p.83)*

Obstructive Sleep Apnea (OSA) is a sleep disorder that affects 900 million adults globally, and is characterized by transient reductions in airflow due to obstruction of the upper airway by pharyngeal muscles. Previous studies have connected OSA to several cardiovascular conditions such as coronary artery disease and ventricular arrhythmia, yet OSA severity is calculated through a single metric, the Apnea Hypopnea Index (AHI). The AHI is the average number of apneas (cessation of breathing) and hypopneas (reduction in breathing) per hour of sleep, and is thought to have inherent pitfalls, one of them being that there is no quantity that accounts for cardiovascular strain. This article seeks to determine if nocturnal bradycardia, a slow heart rhythm, could be a potential metric that could improve the accuracy of OSA severity scores by supplementing the AHI system. A retrospective observational study was performed for five subjects, who each had an implantable loop recorder and were diagnosed with OSA. Through sleep studies and device interrogations, data was extrapolated and organized to determine potential associations between heart rate and OSA severity.