RJBS

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About this Issue

Statement of Purpose

The Rhodes Journal of Biological Science is a student-edited publication that recognizes the scientific achievements of Rhodes students. Volume XXXVIII marks the seventeenth year since Mark Stratton and Dr. David Kesler brought the journal back into regular publication in 2006. Founded as a scholarly forum for student research and scientific ideas, the journal aims to maintain and stimulate the tradition of independent study among Rhodes College students. We hope that in reading the journal, other students will be encouraged to pursue scientific investigations and research.

Acknowledgements

The editorial staff would like to thank Dr. Boyle of the Department of Biology for her support and guidance in preparing this publication.

Image Credits

The front cover, back cover, and section dividers for this year's edition of the *Rhodes Biological Journal of Biological Science* were created by Mia Harris, a Biology Major from Nashville, TN.

Editorial Staff

Madeline Yde ‘23 (Senior Editor) is a Biology and Environmental Science double major and Latin American and Latinx Studies minor from Racine, Wisconsin. She has been involved in research at the Memphis Zoo since Spring of 2020, studying African elephant behavior and working in their amphibian lab. Maddy is a member of the women’s soccer team at Rhodes and Morgan’s Message. She is also a part of the Kappa Alpha Omicron, Tri-Beta, Gamma Sigma Epsilon, Omicron Kappa Delta, Phi Beta Kappa, and Mortar Board honors societies. After graduation, she intends to pursue veterinary medicine.

Mia Harris ’23 (Junior Editor) is a Biology major from Nashville, Tennessee. She has been involved in research at the Memphis Zoo since 2021, studying the behavioral patterns of African elephants in Dr. Sarah Boyle’s research team. She is president of the Tri-Beta and Mortar Board Senior National honor societies and a Phi Beta Kappa member. She is also a four-year athlete of the Rhodes Swim and Dive team and involved in multiple student clubs, such as A.S.I.A., S.A.C.A., and Women in STEM among others. Upon graduating, she will take a gap year to work on an animal behavior manuscript she hopes to publish, explore scientific illustration, and eventually apply for graduate schools.

Will Sarahan ‘23 (Junior Editor) is a Biology with a concentration in Biomedical Sciences and Spanish double major from Austin, Texas. On campus, he works as a Writing Fellow, serves as a volunteer coordinator for the Laurence F. Kinney program, and is the president of the Phi Circle of the Omicron Delta Kappa honor society. He is also a member of the Beta Beta Beta, Mortar Board, and Phi Beta Kappa honor societies. He is also involved in the Sigma Nu fraternity. After graduation, Will plans to work as a Church Health Scholar in their referrals department before heading to medical school.

Kathryn Franks ’24 (Junior Editor) is a Biochemistry and Molecular Biology major from Savannah, Tennessee. She is the Senior Research Assistant in Dr. Terry Hill’s molecular mycology lab and interned at the Memphis Zoo researching in their Amphibian Laboratory. She is an active member of her sorority, Tri Delta, and holds an executive position as the VP of Community Relations among other leadership roles. Last year, she served as the Director of Recruitment for the Rhodes Panhellenic Council and oversaw the entire 2023 formal recruitment process. In connection with art, she works as the Arts Coordinator in the Kinney Service Program providing volunteering opportunities for students as well as being featured and contributing paintings to the Dredge Art Magazine. She is also a member of the Tri-Beta Biological Honor Society. For the upcoming summer, she plans to work as a Biology Teaching Assistant in the bio department at Rhodes. After graduation, she intends to pursue a career in Research Science and to earn her PhD in a specialized science field.

Izzy Wollfarth ’24 (Junior Editor) is from New Orleans, Louisiana and is currently pursuing a major in English. After college, she hopes to pursue a career in journalism or something in the publication/editing industry. Aside from her interest in writing and English, she is a part of the Catholic Student Association, Kappa Delta, and The Sou’wester. She enjoys cooking new and healthy meals, watching movies, and writing whenever she has the time. She hopes you enjoy this year’s publication of the RJBS!

Eleanor Ellsworth ’25 (Junior Editor) is a Biology and Chinese Studies double major from Memphis, Tennessee. She has been doing research in the Biology department, including research related to spiders in the summer of 2021 under the Rhodes IMPACT Scholar Summer Grant, and is currently working in the Shapes and Scales Lab with Dr. Diamond. On campus, she is the vice president of the Beekeeping Club. She most looks forward to going to summer school at Beijing University this upcoming summer to polish her Chinese skills and finally go abroad for the first time. She is currently planning on graduate school, with interests in forensic entomology and pathology.

Yihan Li '25 (Junior Editor) is a Biology major and Computer Science minor from Beijing, China. She has participated in elephant behavioral research at Memphis Zoo since the summer of 2022. She joined Dr. Moyo and other students in doing research on spider distribution and plant association that summer as well. During this school year, she has been working as a computer science tutor, been working in the Shapes and Scales Lab with Dr. Diamond, and has done research with critically endangered dusky gopher frogs at the Memphis Zoo. She plans on attending graduate school after college.

Emma Caplinger ‘26 (Junior Editor) is a Biology major, French minor from Wentzville, Missouri. At Rhodes, she is a part of the Rhodes softball team. Upon graduating, she hopes to matriculate to a graduate school program geared towards bioengineering. She hopes to work to contribute to the rapidly growing development in the genetic engineering field.

Audrey Heidbreder ’26 (Junior Editor) is a prospective Neuroscience major with the intent to double minor in Spanish and Comparative Gothic Literature. She is from Scarsdale, New York. As a St. Jude Summer Plus International Research Fellow, she is beginning mass spectrometry-based research in the Peng Lab in the Department of Structural Biology and Developmental Neurobiology at St. Jude Children’s Research Hospital. Aside from her research, Audrey volunteers as an Emergency Medical Technician (EMT) in the Scarsdale Volunteer Ambulance Corps (SVAC) as an active member. Before attending Rhodes College, Audrey interned for Scarsdale Pediatric Associates and regularly shadowed at the Lenox Hill Hospital’s Neurosurgical Department in New York City. After graduation she plans to attend medical school in her pursuit of pediatric neurosurgery.

David Jackson ’26 (Junior Editor) is a Biochemistry and Molecular Biology major and an English minor from Franklin, Tennessee. His future career interests include pursuing graduate school with a focus on molecular genetics. David is involved in several extracurricular activities, including the ASBMB Service Committee, SACA, Chemistry Club, Rhodes Sustainability Coalition, and Dungeons and Dragons Club.

Sandy Nguyen ‘26 (Junior Editor) is a neuroscience major from Memphis, Tennessee. On campus, she is in the Rhodes Orchestra and an MVP mentor. As a Clarence Day Scholar, she actively engages in campus and community events to serve the city of Memphis and participates in leadership initiatives. In the future, she aims to research schizophrenia and other neurological disorders within the scope of neurobiology.

Evan Reeder ’26 (Junior Editor) is a prospective Neuroscience major from Hoover, Alabama intending to double minor in Psychology and English. He is currently volunteering in Dr. Liam Hunt’s research lab and is set to conduct biomedical research with him in the fall. Dr. Hunt’s lab is focused on understanding fruit fly muscle function with aims at human implications that ultimately increase quality of life for aging individuals and/or those suffering from muscular diseases. Evan is a Certified Patient Care Technician (CPCT) and plans to utilize this credential by working in the Heart & Vascular Center at the University of Alabama at Birmingham (UAB) over the summer. He also plans to shadow surgical procedures of the shoulder, elbow, and knee at Andrews Sports Medicine & Orthopedic Center this summer. As a player on the football team at Rhodes, he will be entering his second season with the Lynx this fall. After graduation, Evan plans to attend medical school and obtain an M.D. degree in his pursuit of a career in orthopedic surgery.

Sarah Madison Taylor ‘26 (Junior Editor) is an intended Environmental Science/Computer Science major from Chelsea, Alabama. She hopes to use advancements in artificial intelligence and computer science to analyze large amounts of data in the environmental field. After completing a bachelor’s degree, she hopes to attend graduate school to further her education. During her time at Rhodes, she hopes to do research with professors involved in environmental research and also her own independent research.

Ashwinaa Vaithianathan ‘26 (Junior Editor) is a Neuroscience major with a double minor in Music and Chemistry from Memphis, Tennessee. She is a member of S.A.C.A, a leading tutor in Memphis Communiversity—a non-profit ACT Tutoring organization for local students— and a certified nursing assistant. She is currently conducting ophthalmological research under Dr. Monica Jablonski at the University of Tennessee Health Science Center, specifically examining the efficacy of the Jablonski Lab’s pregabalin treatment on BXD mice with primary open-angle glaucoma. After graduation, she plans to attend medical school, and pursue ophthalmology or dermatology.

Maanasa Yepuru ‘26 (Junior Editor) is a Neuroscience major with a minor in Computer Science from Memphis, Tennessee. She is the treasurer for S.A.C.A. as well as an MVP mentor. She works in the human resources department at Rhodes and hopes to attend graduate school after her time at Rhodes to pursue a degree in neuropsychology.

Bioaccumulation of Microplastics Along a Gradient of Urbanization and Potential Effects on Ribbed Mussel Respiration and Feeding Rates

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With increasing urbanization and plastic production globally, more and more plastic enters the world's oceans every day. These objects can be broken down into microplastics and potentially ingested by estuarine organisms like ribbed mussels. Increasing urbanization may lead to higher rates of microplastic ingestion which could lead to ill health effects for these organisms. To explore these possibilities, we collected ribbed mussels across an urbanization gradient on Cape Cod, dissolved their biomass, and analyzed it for microplastics. We also returned some of these mussels to the lab and exposed them to microplastics to determine the level of ingestion as well as the effect on respiration and feeding rates. All mussels across the urbanization gradient had microplastics inside their biomass, and we found that the level of microplastics increased exponentially with the level of urbanization. The mussels exposed to microplastics in the lab filtered a significant amount of these particles from the water column in just three hours. This ingestion did not have an impact on respiration rates but did slightly decrease feeding rates. Our study highlights how microplastics can have a widespread impact--infiltrating ecosystems across all levels of urbanization. These microplastics can be ingested by organisms like the ribbed mussel and have the potential to move up the food chain into their predators. Further research is needed to explore how detrimental these particles are to the health of ribbed mussels and other organisms.

Introduction

Over the last 70 years, rates of plastic production have been on par with rates of global CO_2 emissions (Figure 1). With this rise in plastic production, microplastic contamination has spread to virtually every corner of the planet (Borrelle et al. 2017). Plastics enter the ocean at a rate of eight million metric tons per year ("New Plastics Economy" 2017). These larger plastic items are broken down into microplastics through various mechanical, photochemical, and biological forms of degradation (Zhang et al. 2021).

Two main types of microplastics are fibers and fragments. Fibers originate from shedding, friction, or abrasion on synthetic clothing and textiles as well as fishing gear. Fragments mainly originate from the breakdown of larger plastic items. Fragments also include irregular-shaped items like paint chips, plastic films, and plastic pellets (Lloret et al. 2021). In coastal environments, fragments tend to be relatively local in terms of origin, while fibers can travel a long distance before reaching an ecosystem (Lloret et al. 2021). In fact, fibers have been found in remote areas like national parks and wilderness areas in the United States (Brahney et al. 2020). The rapid rates of watershed urbanization in estuaries around the globe raise concerns about the contamination of estuarine ecosystems by microplastics and their potential effects on biota.

Ribbed mussels (*Geukensia demissa*) are estuarine primary consumers that reside on intertidal shorelines–often near salt marshes. When submerged by tides, these organisms filter feed on phytoplankton and other suspended detritus. Microplastics could also be suspended in the tidal waters and are of similar size to phytoplankton. We hypothesize that *ribbed mussels may be filtering microplastics along with phytoplankton and accumulating these microplastics into their biomass*. This bioaccumulation has been demonstrated in other studies of marine filter feeders (Wu et al. 2022; Alomar et al. 2022). We hypothesize that *mussel bioaccumulation of microplastics would increase with the level of watershed urbanization in estuaries*.

Microplastics can carry harmful chemicals, diseases, and invasive species while posing other threats to marine organisms like the ribbed mussel (Khalid et al. 2021). If ingested, microplastics could impact the mussels' ability to respire and feed, as seen in other marine bivalves (X. Huang et al. 2022). With exposure to microplastics, changes in mussel respiration or feeding rates can impact the mussel's overall energy budget. A smaller energy budget means less energy can be spent on growing byssus threads, fighting off pathogens, or detoxification–impacting the mussels' overall health. Therefore, we also hypothesized that *ingestion and bioaccumulation of microplastics would lead to altered respiration and feeding rates in mussels*.

Methods

To test *whether microplastic accumulation in ribbed mussel biomass increase with levels of urbanization*, we visited estuaries in Waquoit Bay (Figure 2) with varying levels of watershed

urbanization (Table 1, (Lloret et al. 2021)). We retrieved over 15 mussels from each site, froze them, and randomly selected three to five mussels for further analysis in the lab. After thawing, soft tissues were removed from the mussel shells, and wet weight was recorded. We then analyzed these mussel soft tissues for bioaccumulation of microplastics by dissolving them in 10% KOH (w/v solution) for 48-96 hours, filtering the resulting fluid through a 10um filter, and counting the microplastics under a microscope (Alomar et al. 2022). We also sorted microplastics by type (fragments, fibers, films, pellets, etc) and color, and stored them in DI-filled scintillation vials for later analysis.

To test *whether ribbed mussels filter microplastics as they feed on phytoplankton*, we set up a series of microplastic bioaccumulation assays. We gathered mussels from Sage Lot Pond (Figure 2). We chose this site because of its low levels of microplastic accumulation in surrounding salt marsh sediments (Lloret et al. 2021). We placed 60 of these mussels in a large seawater tank and allowed them to acclimate for three days. The temperature of the seawater was 15 ℃ and the pH was 7.75. We replaced seawater daily. We fed the mussels an algal culture containing *Nannochloropsis gaditana*, *Tetraselmis sp*, *Isochrysis galbana*, *and Thalassiosira weissflogii*. We fed the mussels based on the dry weight of the algal culture as a percentage of the dry weight of a mussel. Every day, we fed the mussels the volume of algal culture required to reach ~8% of the dry weight of all mussels in the tank (Widdows and Staff 2006). After the acclimation period, we randomly placed 12 mussels into 1 L beaker containing an aeration device and 1 L of seawater filtered to 0.7 μm. We "fed" eight beakers with red high-density polyethylene microplastics. We added enough of these microplastics so that starting concentrations in the beakers were approximately five to ten items per ml. We also set up four control beakers with the same conditions but without microplastics. An additional beaker without mussels was used as a blank, and microplastics were added and later counted to ensure microplastics were not sinking, rising to the surface, or adhering to the sides of the beaker during the exposure. We fed algal culture to all 13 beakers using the same dry weight proportion as described above. Next, we took 10 ml samples of the water from each beaker and stored them in scintillation vials for later analysis. We then let the mussels feed for three hours. At the end of the exposure, we took 12 more 10 ml samples of water from each beaker. At this point, we froze four mussels for later analysis. Later, we filtered the water samples onto a 10 μm polycarbonate filter using a vacuum filter. Then, we counted the number of red microplastics under a microscope. We also dissolved and filtered the frozen mussels using the aforementioned extraction method. Then we examined the number of red microplastics the mussels had accumulated during the exposure period under a microscope.

To test *whether exposure to microplastics alters respiration in ribbed mussels*, we transferred the remaining mussels from the microplastic assays to a sealed chamber (476 ml) filled with seawater filtered to 0.7 μm. We placed the chambers on a stir plate and added a stir bar to the chambers to ensure the circulation of water. We measured dissolved oxygen concentration in mg L^{-1} for one hour using a PreSens OXY-4 SMA (G3) Multi-Channel Oxygen Meter. We also measured the dissolved oxygen concentration in a chamber with no mussel present to account for any background change in oxygen.

To test *whether exposure to microplastics alters feeding rates in ribbed mussels*, we allowed the mussels to rest in seawater filtered to 0.7 μm for ~23 hours following the respiration measurements. Then we placed them individually in beakers filled with 500 ml of seawater filtered to 0.7 μm. Next, we fed them algal culture using the same dry weight proportion as described above. We used an aeration device to ensure the circulation of algal culture throughout the seawater. After five minutes to allow for circulation, we took 10 ml samples from each beaker to measure the initial cell concentration. These samples were immediately fixed with 1 ml of 37% formaldehyde and stored in complete darkness. After a one-hour feeding period, we took another set of 10 ml samples from each beaker to measure the final cell concentration. These samples were also fixed with 1 ml of 37% formaldehyde and stored in complete darkness. Then we measured the number of cells in these 10 ml samples using a Beckman Coulter Z1 Particle Counter. Since initial cell counts varied, we divided the initial number of cells in the entire 500 ml beaker by the change in cells over the one-hour feeding period to determine the clearance time–how long it would take each mussel to clear all of the cells in its respective beaker.

Results

Bioaccumulation of microplastics in mussels across a gradient of urbanization

Across all of the sub-estuaries within Waquoit Bay, we found a total of 594 microplastics in our 22 mussel samples. Every mussel we analyzed contained microplastics. The number of microplastics per mussel ranged from 3 to 129. We found that microplastics in a mussel increased exponentially with the level of urbanization in the watershed (Figure 3). We found a similar relationship between the number of microplastic fragments and watershed urbanization

(Figure 4). More specifically, microplastic pellets–a certain kind of fragment–also increased exponentially with the level of watershed urbanization (Figure 5). 61% of all microplastics found in our samples were pellets. Contrastingly, microplastic fibers were found at consistent levels across different levels of watershed urbanization (Figure 6).

Filtering and bioaccumulation of microplastics by mussels

In the microplastic assays, ribbed mussels filtered a significant (paired t-test, $p < 0.0001$) amount of microplastics from the water (Figure 7). Mussels reduced initial microplastic concentrations in the beakers by 62% during the three-hour exposure. The control beaker–with no mussel–only saw a 20% reduction in microplastic concentration. Since the beakers with mussels had a more dramatic decrease in concentration than the control, we could confirm the decrease was due to mussels filtering microplastics rather than microplastics adhering to the beaker walls, sinking, or floating to the water surface.

The mussels from the assays accumulated microplastics in their biomass during the three-hour exposure period. The number of microplastics ranged from 37 to 114 across the four mussels exposed to microplastics that we analyzed. The average number of items per mussel was 59.

Effect of microplastic exposure on mussel respiration and clearance time

Exposure to microplastics had no clear impact on the respiration rates of ribbed mussels (t-test, $p = 0.3056$, Figure 8). Mussels from both treatments appeared to respire at about the same rates following the experiment.

Ribbed mussels that were exposed to microplastics had a longer clearance time than mussels that were not exposed. While not significant (ANCOVA, $p = 0.2188$), the difference in clearance time between mussels from each treatment was approximately six minutes if the mussels had the same soft tissue wet weight (Figure 9).

Discussion

Urbanization of coastal areas is not going away, estuary-adjacent lands are projected to increase in population and development throughout the 21st century (Neumann et al. 2015). This pattern in tandem with increasing rates of plastic production globally (Figure 1) poses a huge threat to coastal ecosystems. Rapid coastal urbanization has the potential to increase microplastic inputs to estuaries globally. Microplastic bioaccumulation in mussels and other filter-feeding bivalves will become increasingly pressing as more land is developed in estuarine watersheds like those on Cape Cod. Other studies have also shown increased input of microplastics to coastal ecosystems with the level of watershed urbanization (Y. Huang et al. 2020, Lloret et al. 2021).

Our study revealed that the number of microplastic fragments and fibers in mussels have varying relationships with the level of watershed urbanization. This indicates that fragments and fibers have different modes of introduction and transport throughout Waquoit Bay. While fragments appear to be introduced at a local level more in-line with the level of urbanization, fibers are prevalent across all levels of urbanization. This suggests fibers can be transported long distances. Previous studies of this estuary have come to similar conclusions (Lloret et al. 2021). Further, fibers have been found in remote areas such as national parks or wilderness areas in the United States (Brahney et al. 2020). Fibers have even been shown to become airborne and travel in the wind (Liu et al. 2019). With increases in global plastic production (Figure 1), there will likely be localized increases in microplastic fragments to estuaries and filter feeders where urbanization is prevalent. However, microplastic fibers pose the risk of infiltrating a greater range of ecosystems regardless of urbanization in the area.

Interestingly, microplastic pellets alone increased exponentially with the level of watershed urbanization. Microplastic pellets–also known as primary microplastics–originate from personal care products such as cosmetics and face scrubs. They are also used in the process of air-blasting which cleanses heavy machinery, boat hulls, etc. from old rust and paint (Cole et al. 2011). Since pellets made up such a large proportion of the microplastics found within Waquoit Bay mussels, further research could explore what sources are contributing to this specific form of microplastic contamination.

We found that ribbed mussels filtered a significant amount of microplastics from a highly concentrated microplastic exposure. In just three hours, the mussels were able to accumulate many particles within their biomass. While this occurred in the lab, our urbanization analysis shows that mussels accumulate microplastics in estuaries like Waquoit Bay as well–sometimes without a high level of human development nearby. Mussels are not isolated in these estuarine ecosystems. Other organisms prey on these filter feeders to create a large coastal food web. Further research could explore if the microplastics accumulating in ribbed mussels may be climbing the trophic pyramid into predators like the blue crab (Zhu and Gosnell 2021). Additionally, while humans don't eat the ribbed mussel, humans do eat other marine filter feeders. If organisms like oysters, quahogs, and soft-clams accumulate microplastics in the same fashion as the ribbed mussel, there is potential for

microplastics to be entering human diets. As plastic production increases globally and more microplastics enter aquatic food webs through the diets of filter feeders, the impact of microplastics on those organisms and the large food webs they support will become increasingly pressing.

Respiration rates were shown to be consistent whether or not mussels were exposed to microplastics. Since respiration is one indicator of stress, this suggests mussels are perhaps not stressed by the presence of high concentrations of microplastics in the surrounding water. This has interesting implications; as microplastic concentrations in seawater increase bioaccumulation in mussels and other filter feeders would become a major threat, since these organisms do not seem to react and protect themselves against the presence of these particles in the water. However, our number of samples was limited to four per treatment due to time constraints. Other studies have shown that respiration rates can be impacted by exposure to microplastics (X. Huang et al. 2022). With more replicates, a trend showing a change in respiration with microplastic exposure may emerge.

While not significant, clearance time did increase for mussels exposed to microplastics. This could be due to microplastics causing mussels to feed at lower rates due to related stressors. Other studies have shown similar decreases in feeding rates when marine organisms are exposed to high concentrations of microplastics (Wright et al. 2013). Similar to our respiration measurements, our number of samples was limited to four per treatment due to time constraints. With more replicates, a stronger relationship between clearance time and microplastic exposure may emerge–furthering the need for more research into why microplastics reduce the ribbed mussel's feeding rates and what impact that reduction has on the organisms' overall health.

Other studies have explored how microplastics impact the overall energy budget of mussels (X. Huang et al. 2022). Respiration and clearance rates are two very important components of an organism's energy budget. Understanding the energy budgets of these filter feeders allows for a broader understanding of how microplastics impact the health of these creatures. Further research could explore assimilation efficiency and excretion rates in addition to respiration and clearance rates so this energy budget could be calculated. As urbanization and plastic production increase in Waquoit Bay and globally, understanding how microplastics impact the health of organisms near the base of the food web will be essential to learning how these particles affect entire ecosystems.

Figures

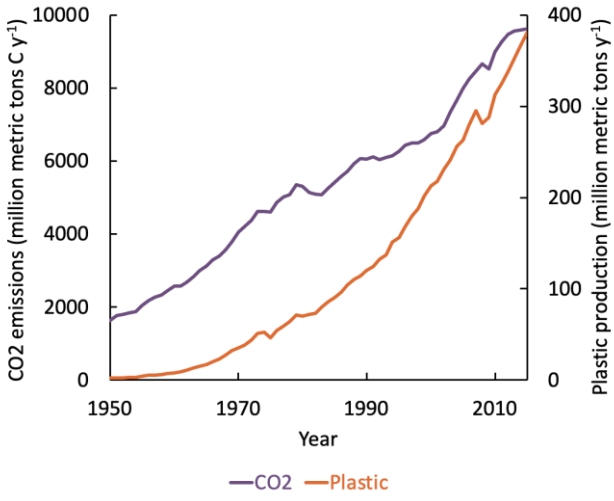

Figure 1. Global rate of plastic production is increasing faster than the rate of CO_2 emission. Data from Geyer, Jambeck, and Law (2017) and Gilfillan and Marland (2021).

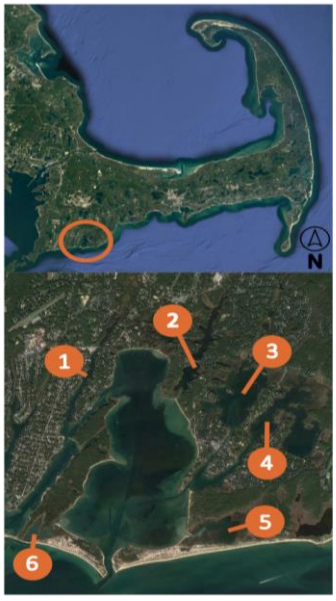

Figure 2. Location of Waquoit Bay on Cape Cod and sites that we visited in Waquoit Bay: 1 - Childs River, 2 - Quashnet River, 3 - Hamblin Pond, 4 - Jehu Pond, 5 - Sage Lot Pond, 6 - Timms Pond

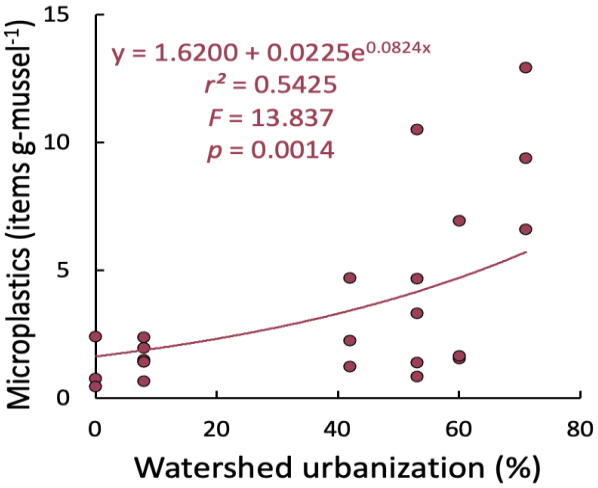

Figure 3. Ribbed mussel bioaccumulation of microplastics increases with level of watershed urbanization.
We standardized microplastic items by the soft tissue wet weight in g of each mussel. We determined significance by log transforming the data and performing a linear regression.

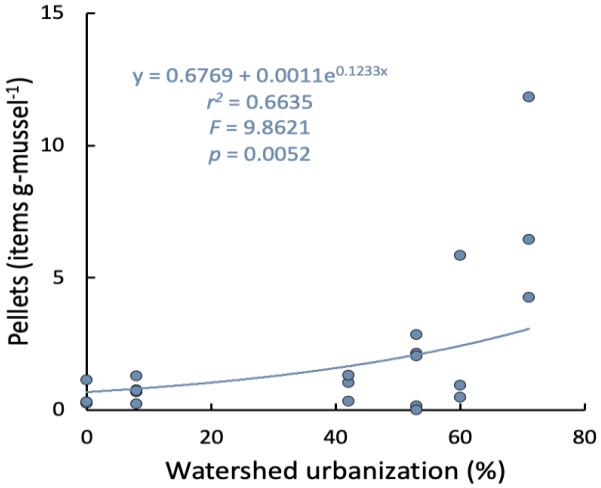

Figure 5. Ribbed mussel bioaccumulation of microplastic pellets increases with level of watershed urbanization.
We standardized microplastic pellets by the soft tissue wet weight in g of each mussel. We determined significance by log transforming the data and performing a linear regression.

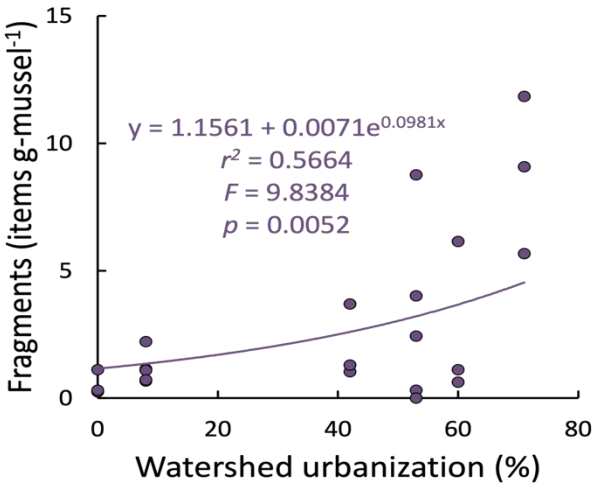

Figure 4. Ribbed mussel bioaccumulation of microplastic fragments increases with level of watershed urbanization.
We standardized microplastic fragments by the soft tissue wet weight in g of each mussel. We determined significance by log transforming the data and performing a linear regression.

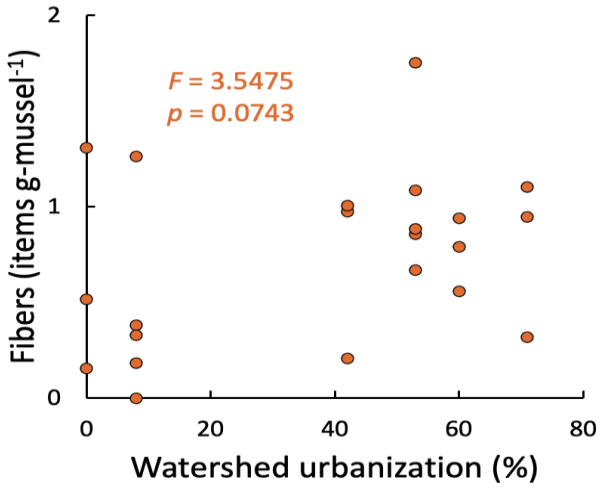

Figure 6. Ribbed mussel bioaccumulation of microplastic fibers consistent across different levels of watershed urbanization.
We standardized microplastic fibers by the soft tissue wet weight in g of each mussel. We determined significance by log transforming the data and performing a linear regression.

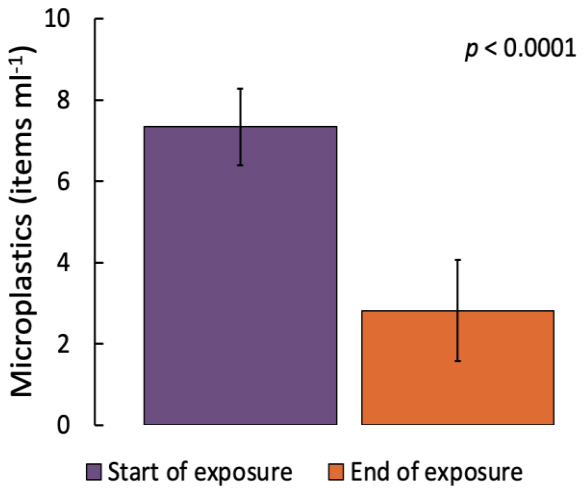

Figure 7. Average microplastic concentration in the water at the beginning and end of the microplastic assays. The bars represent standard error. We determined significance using a paired t-test.

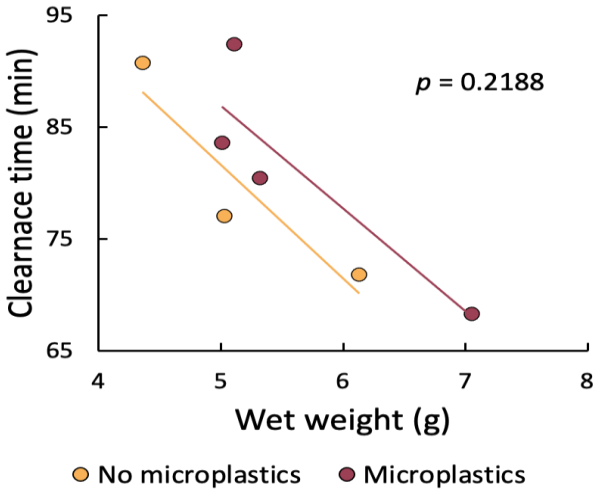

Figure 9. Clearance time given a mussel's soft tissue wet weight and treatment. We determined significance using ANCOVA.

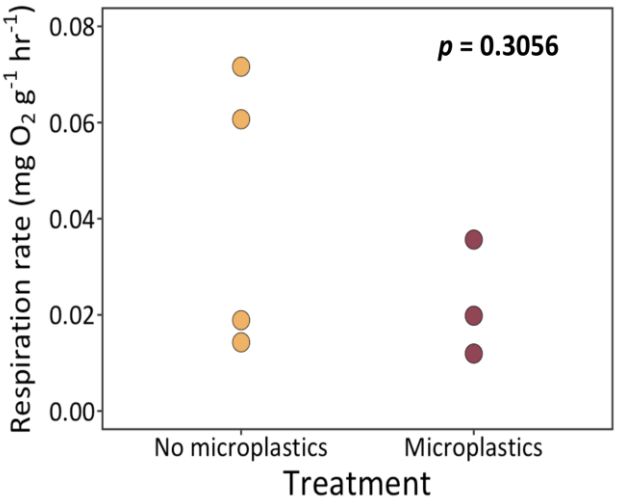

Figure 8. Respiration rates of mussels given treatment. We standardized rates by soft tissue wet weight of mussels (g). We determined significance using a t-test.

Tables

Site	Watershed urbanization (% of area)
Childs River	71
Quashnet River	53
Hamblin Pond	60
Jehu Pond	42
Sage Lot Pond	8
Timms Pond	0

Table 1. Level of watershed urbanization (%) across the six sample sites (Data from Lloret et al., 2021).

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Dispensary Cannabidiol (CBD): Nothing to Worry About!

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Despite U.S. Food and Drug Administration approval of cannabidiol (CBD) liquid (Epidiolex®), patients with epilepsy still supplement prescription treatments with dispensary CBD. This study aimed to evaluate the therapeutic effectiveness of dispensary CBD. We retrospectively collected dosage information, CBD serum levels, efficacy, and adverse effects from patient charts (children, adolescents, adults) (n=18). All 18 patients showed no clinical benefit from dispensary CBD, as detectable serum levels never reached a therapeutic range (6 patients had barely detectable levels that were below laboratory reporting thresholds). Minute levels of tetrahydrocannabinol (THC) were found in 3 patients, and moderate levels were found in 1 patient. Dispensary CBD failed to reach effective therapeutic levels in these patients. The presence of THC demonstrates the current lack of regulation of dispensary CBD. Anecdotal reports of clinical effectiveness should be considered an effect of prescription antiseizure medications and not dispensary CBD.

Introduction

The burden of epilepsy has been reduced by a vast array of new antiseizure medications, with more potent and selective treatments constantly in production or trials; nevertheless, generalized seizures continue to produce complicated cases for patients trying to maintain their quality of life, and for physicians attempting to refine treatment regimens. Focal seizures are likewise similarly complex and comprise the most common form of epilepsy worldwide (World Health Organization). One possible treatment modality is cannabidiol (CBD), which is composed of 1 of 2 active ingredients found in the *Cannabis sativa* and *Cannabis indica* plants and has been utilized as a treatment for various diseases. CBD acts on multiple targets and increases adenosine uptake into neurons, which acts as an inhibitory signal, and decreases the intracellular calcium release that precedes excitatory neuronal activity (Gray and Whalley 2020). These unique modulations make it distinct from other antiseizure medications that primarily act on sodium and potassium currents. Likewise, its active metabolite—7-hydroxy-cannabidiol, produced from CYP2C19 metabolism of CBD—has been shown to have a mild anticonvulsant effect and can be affected by other antiseizure medications that inhibit CYP2C19 (Lucas et al. 2018).

In 2018, the U.S. Food and Drug Administration (FDA) provided initial approval for Epidiolex®, the prescription formulation of cannabidiol, indicated for seizures associated with Lennox-Gastaut Syndrome and Dravet syndrome for patients aged 2 years and older. An indication for Tuberous Sclerosis Complex was added by the FDA in July 2020 (Jazz Pharmaceuticals). Prescription CBD is a mostly purified plant-based CBD product, has proven efficacy as an antiseizure medication, and lacks the psychoactive properties of tetrahydrocannabinol (THC) (Abu-Sawwa et al. 2020, Devinsky et al. 2020, Ryan 2020). The label for the prescription formulation of CBD now indicates approval for use in treating seizures associated with Dravet Syndrome, Lennox-Gastaut Syndrome, and Tuberous Sclerosis Complex (Jazz Pharmaceuticals). Prior study has demonstrated its effectiveness in reducing seizures while also providing behavioral and sleep benefits (Zhornitsky 2012). Following prescription CBD's approval, patients have continued to use other cannabis products, which were studied to investigate their medicinal content. However, dispensary CBD products are neither FDA approved nor regulated, which results in uneven quality and unpredictable levels of CBD (and possibly THC) across products

(Gurley et al. 2020, Miller et al. 2022, Spindle et al. 2022) Ultimately, using these products could lead to unpredictable treatment responses.

Even before the FDA approved the prescription formulation of CBD, many epilepsy patients obtained internet-based dispensary CBD products to supplement their prescribed treatment regimen, and dispensary supplementation has occurred even with patients taking prescription CBD. We, therefore, sought to document and evaluate the pharmacology of internet dispensary CBD in a real-world clinical setting. We performed a retrospective chart review of patients from the epilepsy clinic at our institution with seizures and reported taking dispensary products purchased from various internet services to examine serum levels of CBD in patients and evaluate the pharmacokinetic profile of dispensary products.

The purpose of this study is to demonstrate the pharmacokinetics of internet and store-bought dispensary CBD. We hypothesize that subtherapeutic levels of CBD will be found in patient serum levels with a concomitant lack of seizure suppression efficacy. We hope that awareness of assayed CBD serum levels (and the ability to perform these easily within the clinical setting) in patients on these non-prescription CBD products will better help inform care.

Methods

Institutional review board approval and approval to waive consent were obtained from The University of Tennessee Health Science Center (22-08763-XP). We performed a retrospective chart review of all patients followed at the Comprehensive Pediatric Epilepsy Program clinic at Le Bonheur Children's Hospital in Memphis, TN who were reportedly taking a non-prescription internet or store-obtained cannabidiol product (i.e., dispensary cannabidiol). We reviewed records for clinic patients from April 2015 to May 2022, which included patients from several age ranges: children (2–11 years of age), adolescents (12–17 years), and adults (≥18 years). Adult members present in the study were long-term patients who began epilepsy treatment in early childhood and continued seeking care at Le Bonheur Children's Hospital. Patients using a combination of brand (i.e., Epidiolex®) and dispensary products were excluded from the study to ensure that only the dispensary product was responsible for serum levels.

Patients were not provided with dispensary cannabidiol but instead added it to their regimen without prior consultation with the treating physician. When discovered, patients were informed about the questionable efficacy and risks involved with using a non-pharmaceutically regulated substance.

Data collected include patient demographics (age, sex, weight), epilepsy type and etiology, dispensary cannabidiol brand name, daily dosage, serum level, number of prior antiseizure medications, any reported clinical effects, duration on the product, and reasons for discontinuation. At follow-up appointments, the treating physician ordered random levels of the cannabidiol product, and serum concentration was obtained through reports from NMS Labs (Horsham, PA) and Quest Diagnostics (New York City, NY), who currently use a reporting limit of 1 ng/mL. This limit was based on much higher doses of cannabidiol but is still applicable given the high variability of CBD levels. Both labs use High-Performance Liquid Chromatography and Tandem Mass Spectrometry to determine serum levels (Franco et al. 2022, Malaca et al. 2021). We set the starting therapeutic range for serum CBD at the 100–150 ng/mL range, as determined by previous studies (Cohen et al. 2022, Szaflarski et al. 2019, Uttl et al. 2021). Patients reported self-initiating treatment on dosages based on anecdotal evidence from dispensers, current users, and recommendations provided on the internet dispensary websites. Because of uneven dosages of dispensary CBD across patients, we reported only the stable dose that patients were on at the time serum levels were obtained.

Results

A total of 18 patients added dispensary CBD to their prescribed antiseizure regimen, consisting of 8 males and 10 females. The overall age range was 2–32 years—50% (n=9) were between the ages of 2–11 years (children), 33.3% (n=6) were 13–15 years (adolescents), and 16.7% (n=3) were 18 years or older (adults). All patients had a diagnosis of generalized seizures except for two, who were diagnosed with focal seizures with secondary bilateral tonic-clonic activity. We found that 89% (n=16) of patients had a seizure etiology with a genetic abnormality. Nine patients had significant cognitive delay associated with their seizure type (Table 1).

No new side effects were found in this study, nor were any side effects reported by patients. A total of 13 patients eventually discontinued dispensary cannabidiol in favor of pursuing prescription cannabidiol (n=7), after epilepsy surgery (n=1), or due to a perceived lack of efficacy (n=5).

Because each patient used a different product, there was little-to-no consistency in dosage and serum levels. Six patients had serum levels of CBD below the laboratory reporting level of 1 ng/ml (Table 1). Two patients modified their dosages of

dispensary CBD but still maintained extremely low levels. Patients used cannabidiol from 12 different dispensaries: 6 from Charlotte's Web, 2 from Haleigh's Hope, 1 from Buffalo River, 1 from Phoenix Tears, 1 from Ancient Nutrition, 1 from Care by Design, 1 from PlusCBD, 1 from Palmetto Harmony, 1 from Tenne CBD, 1 from Pure Cannaceuticals, 1 from TreatWell CA, and 1 from Texas Original. Of the 12 brands represented in the study, Charlotte's Web hemp oil was the most readily obtained and showed higher CBD levels in 2 patients (21 ng/mL and 31 ng/mL); however, even these were well below the therapeutic range (100–150 ng/mL). Three patients were taking enzyme-inducing medications at the time of initiation, and their average cannabidiol serum level was 1.52 ng/mL, barely above the detection limit. The remainder had an average cannabidiol serum level of 11.14 ng/mL. Children taking dispensary CBD had an average serum concentration of 9.4 ng/mL with adolescents and adults having 7.4 ng/mL and 14.2 ng/mL, respectively.

Discussion

This study demonstrated the inability of non-prescription cannabidiol to achieve minimally therapeutic serum levels, which is consistent with the low levels of cannabidiol contained in the various products. These subtherapeutic levels explain the lack of side effects as well as the perception of no significant improvement in seizure control. This information is especially pertinent to families who assume their family member with Dravet Syndrome, Lennox-Gastaut Syndrome, or Tuberous Sclerosis Complex may benefit from dispensary CBD; likewise, it is helpful for medical professionals to dissuade caregivers from purchasing these products. Other studies of dispensary CBD for seizures and epilepsy have noted a lack of data regarding safety, effectiveness, and dosing (O'Connell et al. 2017, Amann et al. 2022, White 2019). Not only were subtherapeutic and inconsistent levels of CBD found, but also the presence of THC, which were attributed to the lack of regulation of dispensary products (Amann et al. 2022, White 2019).

For those whose seizures would benefit from CBD, this study supports the usage of the prescription formulation cannabidiol over CBD obtained from the internet or local sources.

Given the large presence that dispensaries such as Charlotte's Web and Haleigh's Hope have online and the patina of legitimacy online sources give to dispensary products, the lack of detectable or therapeutic CBD in dispensary products is vitally important for clinicians, patients, and families to understand. All of our patients taking CBD from these and other sources showed serum levels that were undetectable or well below the therapeutic range, which only begins between 100 to 150 ng/mL (Cohen et al. 2022, Szaflarski et al. 2019, Uttl et al. 2021); therefore, this minimal concentration suggests that there is no benefit to adding these products to a treatment regimen. The lack of side effects in our patients additionally supports this claim because adverse effects would only be seen in concentrations around or above the therapeutic range. Sleepiness was reported in 1 patient, but their CBD level was below the reporting limit, and they had recently initiated an antiepileptic drug that was known to cause somnolence. Interestingly, some patients showed extremely low levels of THC, which was expected because the product they chose reported a low concentration of THC, but 1 patient with Texas Original showed a much higher concentration of THC than expected. This finding suggests that Texas Original may contain more THC than reported on its bottle label and website and highlights the minimal supervision of the dispensary CBD industry.

Limitations of this study include its small sample size and reliance on patient reports for dosage and any clinical or adverse effects. The true effect of these dispensary products may not be ideally represented in this study; therefore, larger studies should expand upon the relationship between serum level and seizure control. Additionally, a deeper investigation into the content of each product will be beneficial in identifying the risks involved and encouraging regulation of such dispensary products. Finally, there are many more dispensary products on the market than those reported here. We only had access to the products that our patients were taking. Obtaining serum levels of every dispensary CBD product is helpful to guide the clinical decision-making, as we suspect higher dosages of some products may produce a therapeutic CBD serum level, which would be important to know when converting to prescription cannabidiol.

Conclusion

This study supports the clinical practice of obtaining serum levels of cannabidiol and THC in patients treated with internet-based dispensary products. We found that all 18 of our patients had no therapeutic level and would potentially benefit from switching to prescription cannabidiol. Even those with a recorded serum level above the reporting limit of 1 ng/mL never achieved therapeutic levels of 100–150 ng/mL. The presence of THC above advertised levels in 1 patient also demonstrates the current lack of regulation of dispensary CBD. Knowing serum

levels in patients taking dispensary cannabidiol may help in determining the starting dose for prescription cannabidiol when physicians discuss switching to the prescription formulation. Given that serum levels obtained with internet-based dispensary cannabidiol are likely to be nontherapeutic, it is very unlikely that dispensary cannabidiol contributes any to seizure control. Anecdotal reports of clinical effectiveness should be considered an effect of prescription antiseizure medications and not dispensary CBD.

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Declaration of Conflicting Interests

The Authors declare that there are no conflicts of interest related to the manuscript

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Epigenetic Drugs Could Function as a Therapeutic Method of Treating Metabolic Syndrome

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In this review of epigenomic contributions to metabolic disease, I propose environmental factors impacting the epigenome can result in altered phenotypes typical of metabolic syndrome in model eukaryotes that can be inherited transgenerationally, suggesting that treatment of metabolic diseases with epigenetic therapeutic agents could possibly have beneficial impacts on patients with direct or indirect alterations to their epigenome that promotes metabolic disease phenotypes. In this paper, I cover how the alteration of DNA methylation, histone acetylation, and histone methylation patterns can result in metabolic disease in both F0 and downstream generations. Additionally, I propose these epigenetic markers be targeted therapeutically to treat or relieve symptoms of metabolic disease.

Introduction

According to the National Institutes of Health (herein referred to as NIH), approximately 33 percent of American adults have metabolic syndrome, also known as insulin resistance syndrome. This disease is clinically defined as a collection of physiological symptoms like high blood pressure, high blood sugar levels, and low high-density lipoprotein (HDL) cholesterol levels that increase one's propensity for more severe diseases like diabetes, stroke, and coronary heart disease (NIH 2022). These diseases are known to be among the deadliest diseases in developed countries like the United States (Murphy et al. 2021). These metabolic-related diseases can be linked to altered epigenomes that impact both parent and offspring (King and Skinner 2020). As such, epigenetic regulation of metabolic genes is essential to the understanding of these diseases.

The field of epigenetics is defined as the study of non-genetic factors changing gene expression without affecting the DNA sequence (Al-Hasani et al. 2019). Therefore, epigenetics work by upregulating or downregulating gene expression, creating altered phenotypes without any edits to the genome. Chemical markers that cause epigenetic alterations, defined as the epigenome, typically consist of DNA methylation, histone modifications, or other methods of chromatin remodeling (Al-Hasani et al. 2019). DNA methylation is typically associated with gene repression, while histone acetylation is associated with gene expression (Al-Hasani et al. 2019). Histone methylation can be associated with either increased or decreased expression, depending on the location (Li et al. 2007). Chromatin remodeling via methylation, acetylation, phosphorylation, ubiquitination, and other molecular additions to histones works to relax or constrict chromatin structure (Li et al 2007). Tightly wound chromatin, called heterochromatin, prevents convenient transcription of the DNA and represses gene expression, while a more relaxed state, called euchromatin, allows for easier transcriptional access and an increased level of expression (Al-Hasani et al. 2019). Regulation of these epigenetic markers is mediated by the enzymes responsible for these epigenetic additions, such as methyltransferases, deacetylases, or kinases (Al-Hasani et al. 2019). Thus, changes in the epigenome occur due to altered activity of these regulatory enzymes. The production of epigenetic markers can be caused by environmental stressors and are propagated within the organism via changes in the germline epigenome (Ben Maamar et al. 2021).

Studies have shown the rapid increase in obesity prevalence in humans cannot be explained via purely genetic mechanisms (Ling and Rönn 2019). Thus, it can be assumed that there is some environmental component to obesity that explains the rapid growth of obese populations within the last century. As Ling and Rönn noted in their 2019 paper, obesity has increased rapidly over the past 50 to 100 years. This suggests that environmental stressors could be constantly impacting the epigenome of individuals at every generation, prompting similar changes across populations and generations. However, research has also signaled that altered epigenomes in germ cells could propagate these diseased phenotypes to offspring without any subsequent exposure to the environmental triggers that influenced their parent's germline epigenome (Wen et al. 2020). Thus, it seems that the field of epigenetics shows promise as an area of study in the investigation of how metabolic diseases manifest. The inheritance of epigenetic markers will be crucial to understanding the propagation of metabolic disease phenotypes from generation to generation. Epigenetic transgenerational inheritance, defined as the passing down of phenotypes via epigenetic mechanisms in the absence of the environmental

stressors that originally caused the phenotype, serves as the mechanism by which environmental stressors can cause disease-producing phenotypes across generations (Hanson and Skinner 2016).

The targeting of epigenetic markers in disease phenotypes, whether they be inherited or acquired, is a common area of investigation for drug therapies, most famously in cancer. Methylation patterns have been studied as potential targets for therapeutic intervention in cancer. Drugs like 5-azacytidine, which inhibit DNA methyltransferases (herein referred to as DNMTs), have shown promise in treating cancer (Gomez et al. 2020). Additionally, histone deacetylases inhibitors (HDACis) have been shown to reverse the downregulation of tumor suppressor genes by interfering with histone deacetylases, which function to silence genes by removing histone acetyl tags (Gomez et al. 2020). Thus, therapeutic agents to alter epigenetic markers are common treatments for diseases with epigenetic roots.

In this review, I propose environmental factors impacting the epigenome can result in altered phenotypes typical of metabolic syndrome in model eukaryotes that can be inherited transgenerationally, suggesting that treatment of metabolic diseases with epigenetic therapeutic agents could have beneficial impacts on patients with direct or indirect alterations to their epigenome that promotes metabolic disease phenotypes. In this paper, I will cover how the alteration of DNA methylation, histone acetylation, and histone methylation patterns can result in metabolic disease in both F0 and downstream generations. Additionally, I will cover how I propose these epigenetic markers be targeted therapeutically to treat or relieve symptoms of metabolic disease.

Results

Alterations in DNA methylation patterns result in metabolic syndrome

Alterations in DNA methylation patterns on chromosomes can result in abnormal expression of genes (such as silencing essential genes or expressing normally silenced genes), resulting in an altered phenotype for the individual that results in symptoms of metabolic syndrome (Liberman et al. 2019). DNA methylation patterns are normally responsible for regulating gene expression, transposons, and alternative splicing mechanisms (Smith and Meissner 2013; Lev Maor et al. 2015). Thus, it can be assumed that any changes to methylation patterns or how they are established and maintained could result in differential gene expression. Some researchers hypothesized that changes in DNA methylation might explain symptoms of metabolic disease. A 2013 study by Xu et al. linked obesity to epigenetic mechanisms by studying methylation patterns in leukocytes of obese versus lean children. Researchers found regions in the epigenomes where mean methylation levels differed between groups, termed differentially methylated CpG sites or DMCs (Xu et al. 2013). Epigenome-wide analyses (EWAS) found these differentially methylated sites to be located near metabolic genes, suggesting that epigenetic regulation of these regions could be responsible for altered metabolic processes and abnormal phenotypic presentation (Xu et al. 2013). Thus, researchers demonstrated that DNA methylation patterns could be responsible for regulating metabolic pathways as atypical patterns correlate with phenotypic symptoms of metabolic syndrome (Xu et al. 2013).

Aberrations in DNA Methyltransferase (DNMT) activity cause the expression of obese-like phenotypes

Studies in mice have shown how altered expression of DNA methyltransferases (DNMTs) can result in phenotypic changes consistent with obesity and metabolic abnormalities (Smith et al. 2021). DNMTs function to methylate the epigenome and can generally be split into two groups: DNMT1s (responsible for maintenance of methylated tags) and DNMT3s (responsible for *de novo* methylation) (Beard et al. 1995; Okano et al. 1999). In a 2021 study, Smith et al. mutate *dnmt3a* genes in mice to observe the effects of abnormal epigenetic regulatory enzymes on the epigenome. Point mutations at a specific site in DNA that codes for the *dnmt3a* gene demonstrated how dysfunction of DNMTs resulted in the expression of phenotypes consistent with overgrowth and obesity. Smith et al. created heterozygously mutated mice of the *dnmt3a* gene via point mutation of a histone residue for an arginine residue at the 878th position in the *dnmt3a* gene (labeled Dnmt3a$^{R878H/+}$ mice) and observed differences in various physical characteristics associated with growth. Figure 1A shows heterozygously mutated mice exhibit elevated weight gains as they grow and develop, compared to control mice ($p < 0.0001$). Figure 1B demonstrates a visual representation of the weight differences between control and Dnmt3a$^{R878H/+}$ mice. Thus, panels A and B show Dnmt3a$^{R878H/+}$ mice have higher body weights than control mice (Smith et al. 2021). Figures 1C and 1D demonstrate slightly increased femur growth in Dnmt3a$^{R878H/+}$ mice, compared to WT mice ($p = 0.0049$). However, humerus length is not significant between the groups ($p = 0.1306$). Figure 1G demonstrates a significant increase in the mass of fat tissue in Dnmt3a$^{R878H/+}$ mice above the

age of 6 months, compared to WT mice of the same age group (Smith et al. 2021).

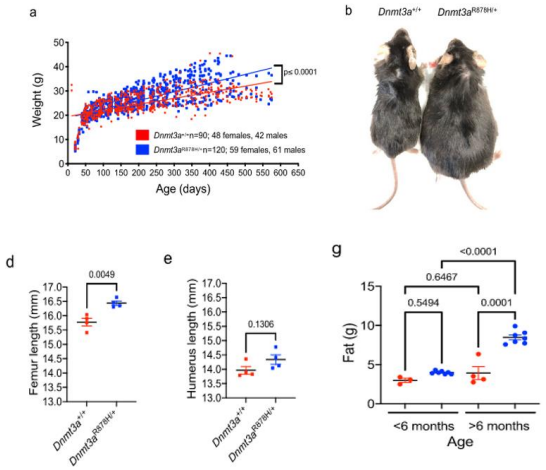

Figure 1. (Panels 1 a, b, d, e, g): Various phenotypic differences between Dnmt3a$^{R878H/+}$ (blue) mice and Dnmt3a$^{+/+}$ (red) control mice. a) Mice with DNMT3A deficiency exhibited elevated weight compared to control mice as they age, post weaning ($p < 0.0001$). b) Side by side comparisons of control versus Dnmt3a$^{R878H/+}$ mice at one year of age. d, e) Graphical comparison of femur ($p < 0.05$) and humerus ($p > 0.05$) lengths in Dnmt3a$^{R878H/+}$ mice and Dnmt3a$^{+/+}$ control mice. g) Mass of fat tissue in Dnmt3a$^{R878H/+}$ mice and Dnmt3a$^{+/+}$ control mice before and after 6 months of age. No significance existed between groups at ages less than 6 months, but elevated mass of fat tissue was present for Dnmt3a$^{R878H/+}$ mice after 6 months of age, $p = 0.0001$ (Smith et al. 2021)

The slight significance of the femur data and the lack of significance of the humerus data indicate that mutations in the *dnmt3a* gene do not massively impact bone growth. However, overall weight and amount of fat tissue remain affected by the mutation. Smith et al. also identified that mice with the *dnmt3a* mutation had hypomethylated regions, having only 50% of the average WT methylation pattern (Smith et al. 2021). Since these mice are heterozygous for this mutation and exhibit half of the gene's normal function, it can be assumed that there exists dose-dependent expression of this gene, based on the functionality of each allele. As a result of this study, it is shown that lower expression of functioning DNMT3A proteins, responsible for *de novo* methylation, is correlated with overgrowth and obese-like phenotypes. Therefore, the establishment of normal methylation patterns can be associated with healthy individuals, while abnormal methylation patterns can be associated with individuals suffering from obesity and by extension, metabolic syndrome.

The study performed by Smith et al. provides evidence to suggest that the manner by which symptoms of metabolic syndrome are expressed may be through the regulation (or dysregulation) of methylation patterns in the epigenome. Thus, regulation of the epigenome seems to be connected to the expression of metabolic syndrome.

Environmental pollutants are correlated both with disease and epigenetic markers, suggesting that changes in the epigenome are the mechanism by which environmental chemicals cause disease.

Endocrine-disrupting compounds (EDCs) are chemical compounds that inhibit hormone signaling and can have a variety of impacts on the body. Most notably, they are implicated in the expression of diseases like cancer and a variety of other diseases (Tiffon 2018). As there exists a connection between EDCs, epigenetics, and these diseases, the mechanism by which EDCs cause expression of diseased phenotypes is proposed to be epigenetic in nature (Cano et al. 2021). Thus, the epigenetic mechanism by which environmental triggers are related to disease provides an interesting opportunity to further link metabolic syndrome to epigenetics. In a 2020 study, Wen et al. investigated how exposure to DEHP, a known EDC, would impact gene expression of mice descended from the exposed generation. One of their genes of interest was *tet2*, a Ten-eleven translocation (TET) demethylase, which functions to remove methylation markers from the epigenome (Liberman et al. 2019). Wen et al. investigated *tet2* gene expression in F1 - F3 generations of mice whose F0 generation was exposed to varying levels of DEHP during gestation of F1 offspring. Females from F1 offspring were mated with control male mice to produce F2, and the same procedure was followed to produce F3 offspring (Wen et al. 2020). Relative mRNA expression levels of *tet2* were tested in mice at postnatal day 8 (PND 8, data seen in panel A) and postnatal day 60 (PND 60, data seen in panel B). The results showed significant alterations in *tet2* expression for all generations at some concentrations and stages. F1 generation showed no significant difference at PND8 and elevated expression levels when exposed to 200 µg, 500 mg, and 750 mg of DEHP at PND 60. F2 generation showed significantly increased expression at 500 mg and 750 mg conditions for both time periods. F3 generation showed significantly elevated levels at PND 8 in 200 µg and 750 mg conditions but significantly decreased expression levels in 200 µg and 500 mg conditions for PND 60 (Wen et al. 2020).

Data from Wen et. al. shows that as a result of ancestral DEHP exposure, *tet2* mRNA is differentially expressed in F1 – F3 mice at different stages and DEHP concentrations, compared to vehicle controls. This suggests that there will be an altered amount of TET2 protein in these individuals. As TET2 functions to demethylate methyl markers in the epigenome, an abnormal amount of these proteins would result in an atypical epigenome, which could result in altered gene expression and altered phenotypic expression. While they do not directly investigate any phenotypic consequences of this altered expression of *tet2*, Wen et al. note that altered expression of *tet2* could explain how DEHP, known to cause health problems, functions as a toxin and negatively impacts those exposed to it (Wen et al. 2020). Thus, I propose that alterations of the epigenome via TET demethylases (or other epigenetic regulatory enzymes) serve as the mechanism by which EDCs cause diseases like metabolic syndrome or cancer. It can be hypothesized that altered *tet2* gene expression would result in altered TET2 production, which would function to abnormally demethylate areas of the genome. This could alter the expression of genes responsible for metabolic processes, resulting in altered metabolic phenotypes. Thus, changes to methylation patterns due to environmental stressors can be used to explain the emergence of metabolic abnormalities and diseased phenotypes.

DNA methylation patterns explain the connection between prenatal stress and adulthood expression of metabolic syndrome

Research suggests that the establishment of DNA methylation patterns after subjection to prenatal stress could be responsible for altered metabolic processes that could result in metabolic syndrome or more severe diseases in humans (Tobi et al. 2018). Previous research suggests a relationship between a poor prenatal environment and adulthood disease propensity (Barker 1990). Tobi et al. provide evidence that DNA methylation patterns are the link between these two correlated events. DNA methylation patterns were observed in individuals subjected to famine conditions *in utero* and compared to DNA methylation patterns of siblings of these individuals who had not endured *in utero* famine conditions (Tobi et al. 2018). The data showed that methylation patterns at a variety of metabolically related genes explained approximately 80% of the correlation between prenatal stress and altered triglyceride levels and showed a connection between methylation patterns and body mass index (BMI). Thus, they concluded that the link between prenatal stress and metabolic disease propensity can be explained epigenetically, specifically through DNA methylation patterns (Tobi et al. 2018). Therefore, evidence suggests alterations of DNA methylation patterns to be a cause of metabolic syndrome in humans.

Alterations to histone epigenetic markers can also confer changes in metabolic regulatory processes, resulting in symptoms of metabolic disease

Histone modifications can also result in altered phenotypes that result in a higher chance of metabolic dysfunction (Xu et al 2021). Histones, as the main proteins responsible for chromatin organization, can be impacted by chemical compounds that bind to the proteins, such as methylation, acetylation, phosphorylation, etc. (Liberman et al. 2019). Regulation of these histone proteins is crucial to gene expression as they are responsible for silencing or promoting expression of genes, depending on how tightly or loosely wound the DNA is around the histones (Liberman et al. 2019). Thus, this is another area of epigenetic interest regarding the relationship between epigenetic mechanisms and metabolic diseases.

Changes in epigenetic histone acetylation patterns via nutritional stress result in altered expression of metabolic genes

Changes in histone epigenetic markers have been linked to metabolic disease phenotypes in primary human vascular cells (Pirola et al. 2011). Pirola et al. investigated the role of glycemic control in the management of diabetes. They cited prior research that found strict regulation of glucose levels to be essential for diabetes management (Holman et al. 2008). They also cited evidence that the effects of high glucose exposure can be delayed and long-lasting, an effect they named “hyperglycemic memory” (Pirola et al. 2011). Other research connecting diabetes to histone H3 tail modification led them to investigate the connection between histone modifications, glucose exposure, and expression of metabolic disease symptoms. They investigated the relationship between histone acetylation and gene expression and the relationship between mRNA expression of some metabolic genes and glucose exposure. They established that increased gene expression was associated with an increased number of acetylation markers, which suggests histone acetylation promotes the upregulation of genes.

Data collected by Pirola et. al. shows that gene expression is correlated with an increased number of

acetylation tags, represented as $\log_2$ number of tags. On average, genes with higher levels of expression have more acetyl groups bound to H3 tails. This means acetylation of histone proteins could cause upregulation of genes in the region of that histone. In their paper, Pirola et al. graph relative mRNA expression of genes involved in metabolic processes in low glucose and high glucose conditions. The data show that for most genes, a high glucose condition promotes higher mRNA expression than a low glucose condition. Thus, it can be concluded that high glucose environments result in higher levels of metabolic gene expression. It is correlated that this process is mediated by acetylation markers when combining the data presented above. Therefore, Pirola et al. lay out a model of how symptoms of metabolic syndrome can be acquired. First, exposure to nutritional stress results in alterations to the epigenome via increases in acetylation marker levels. Second, the change in acetylation markers causes elevated expression of genes involved in metabolism (Pirola et al. 2013). An increase in expression of these genes is a shift away from homeostatic balance, meaning metabolic processes will occur abnormally, resulting in metabolic dysfunction and thus, metabolic syndrome. Therefore, the data presented by Pirola et al. suggests a connection between environmental stressors, histone acetylation patterns, and the altered expression of metabolic genes that could cause metabolic dysfunction.

Alterations in histone methylation patterns in diet-induced obese mice suggest a mechanistic connection between epigenetics and metabolic syndrome.

As previously mentioned, environmental stressors have connections to both epigenetics and disease. One of the most studied stressors in this context is diet. Evidence in mice models shows that mice subjected to a high-fat diet, labeled as diet-induced obese (DIO), demonstrate altered patterns of histone methylation in liver and primary hepatocyte cells, suggesting environmental stressors (i.e., diet) regulate the epigenome and affect the expression of metabolic processes (Nie et al. 2017). Nie et al. induced obesity in experimental mice via a high-fat diet, then tested methylation and acetylation markers in liver and primary hepatocyte tissue. The results of their study show altered levels of histone methylation and acetylation at six specific locations. They also performed Western blotting on H3K36me2 in liver and primary hepatocyte tissue and found increased levels of H3K36me2 in both tissue types for DIO mice, compared to control levels (Nie et al. 2017).

A

Ratio of Change (DIO and Chow fed)	
Sites	Average Ratio
H3K4me2	0.39 +/- 0.11 **
H3K4me1	0.61 +/- 0.21 *
H3K36me1	0.58 +/- 0.33 *
H3K36me2	1.73 +/- 0.44
H2AK5ac	0.57 +/- 0.07 **
H2AK9ac	0.57 +/- 0.15 *

B

Figure 2. The differences in methylation and acetylation markers in control (chow) and DIO mice. A) The table shows the six sites of methylation and acetylation in which DIO mice significantly differed from the chow condition. B) Representation of Western blot of H3K36me2 levels between the two groups in liver and primary hepatocyte tissue shows increased H3K36me2 in DIO mice in both tissue types, compared to chow-fed mice.

Nie et al. show through their research that there is a significant difference in epigenetic marker levels between mice with obese phenotypes and those with an average metabolic phenotype. Since the only experimental difference between the two groups was diet, it can be concluded that nutritional stress influences both histone methylation patterns and phenotypic expression of obesity. Therefore, combined with the data from Pirola et al., Nie et al. show that alterations in histone epigenetic patterns could serve as a possible explanation for the mechanism by which nutritional stress (or some other environmental stressor) might cause altered metabolite levels and disrupted metabolic processes. The alterations to metabolic homeostasis that potentially occurs via histone methylation or acetylation pattern modifications could then result in various symptoms of metabolic syndrome.

Epigenetic markers that confer phenotypes of metabolic syndrome can be passed down between generations.

A newer model of phenotypic inheritance, called transgenerational epigenetic inheritance, suggests that epigenetic information can be passed down to subsequent generations via the germline without continued exposure to the cause of the original

epigenetic change (King and Skinner 2020). The inheritance of epigenetic markers from generation to generation can function as an explanation for how the prevalence of metabolic disease has increased over the years without changes to the genetic pool. Thus, the study of the transmittance of epigenetic markers from generation to generation can also be used to explain the development of metabolic syndrome. This process occurs via epigenomic alterations in the germline cells of the F0 generation (Jirtle and Skinner 2007). As germline cells from the F0 generation produce the gametes that produce the F1 generation, study of the F2 and F3 generations is required to determine that phenotypes are transgenerationally inherited via epigenetic markers as these tissues are not directly exposed to the original environmental stressor (Jirtle and Skinner 2007).

Epigenetic signals that promote metabolic syndrome phenotypes can be exhibited in subsequent generations in Drosophila *models*

In their 2013 study, Buescher et al. investigate transgenerational epigenetic inheritance of abnormal metabolic phenotypes in *Drosophila*. They raised F0 female flies in a high-sugar (HS) condition and studied the body composition and sugar levels of F1 larval males. These flies were compared to control F1 flies whose parents were fed a low-sugar (LS) diet. Both groups were compared while feeding on a low-sugar diet. Results showed elevated glucose and trehalose levels and decreased glycogen and cholesterol in flies with mothers exposed to HS diet, while triacylglycerol levels were insignificant between the two groups (Buescher et al. 2013). These results show that nutritional stress in one generation can alter metabolite levels in the subsequent generation. As I have previously shown nutritional stress results in changes to the epigenome, I propose that these results show F1 individuals inherited the mother's altered epigenome, explaining their altered metabolite levels. However, these data alone do not suggest the inheritance to be transgenerational since these F1 individuals were directly exposed to nutritional stress as they originated, in part, from oocytes already present in the mother at time of nutritional stress. So, Buescher et al. performed similar analyses on F2 flies to see if these flies could inherit an altered epigenome from their grandparents.

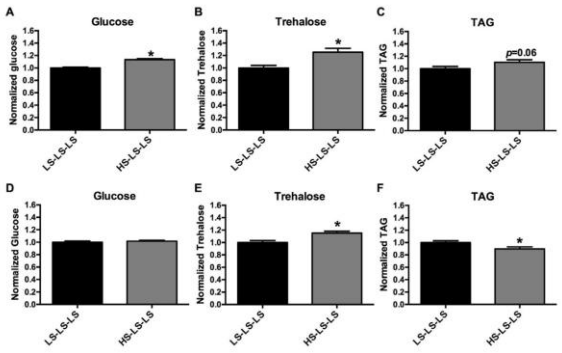

Figure 3. The body compositions of F2 Drosophila were analyzed, comparing ancestral F0 diet. Control flies (black) had a familial dietary pattern of LS-LS-LS, while the experimental group (gray) had a familial dietary pattern of HS-LS-LS. Panels A - C compared males, while panels D-F compared females. b, e) Data shows significant increases in trehalose for HS-LS-LS flies in both sexes ($p < 0.05$). a, c) HS-LS-LS males show a significant increase in glucose levels ($p < 0.05$) and barely insignificant results for triacylglycerol (TAG) ($p = 0.06$). d, f) HS-LS-LS female flies have no significant difference in glucose levels, compared to the control group. HS-LS-LS female flies have significantly decreased TAG levels, compared to the control.

They analyzed the levels of sugars and lipids of two groups of F2 male and female individuals with HS F0 ancestors or LS F0 ancestors. The results, shown above in Figure 3, demonstrate that HS-LS-LS F2 male mice showed a significant increase in glucose and trehalose levels, compared to LS-LS-LS F2 male mice. HS-LS-LS F2 female mice showed a significant increase in trehalose levels and a significant decrease in triacylglycerol levels. Glucose levels in males and triacylglycerol levels in females were insignificant (Buescher et al. 2013). These data show altered metabolite levels in both sexes of F2 flies whose grandmother ate a HS diet, meaning that the diet of the grandmother impacted the level at which metabolites were produced in F2 flies. Just as it was proposed that F1 flies inherited an altered epigenome from F0 flies, these data also suggest the inheritance of altered metabolic processes in F2 flies occurs through epigenetic mechanisms. As environmental stressors are already correlated with epigenomic changes, the work of Buescher et al. supports the hypothesis that phenotypic changes after nutritional stress are due to epigenetic changes and that inheritance of that altered epigenome explains why these traits appear in subsequent generations that were never exposed to the stressor.

Inheritance of epigenetic markers that cause obesity is present in mice models

Mice serve as a popular model for studying the links between epigenetics and metabolic syndrome. Evidence suggests that both maternal and paternal epigenetic markers can be passed on to the subsequent generation (and occasionally even further) and promote a diseased phenotype in the offspring (Fullston et al. 2013; Morgan et al. 1999). Fullston et al. demonstrate how paternal environmental factors can influence the metabolic processes of both F1 and F2 generations. They designed an experiment in which a high-fat diet-induced pre-obesity in F0 male mice without changing glucose homeostasis and tested F1 and F2 generations (all fed on control diet) for obesity and insulin resistance. The data showed a significant increase in body weight of F1 males ($p = 0.02$) and F1 females ($p = 0.005$) as they enter maturity (Fullston et al. 2013). Results also showed that paternal diet did cause obesity in adult female F1 offspring. Fullston et al. also showed dysfunction in glucose tolerance and insulin sensitivity at various stages for both F1 male and female mice (Fullston et al. 2013) Results in the F2 generation were much murkier, but some evidence shows inheritance of metabolic dysfunction in certain subsets of the F2 group (Fullston et al. 2013). Due to the non-Mendelian nature of the inheritance pattern and some sex-specific results, researchers concluded alterations in phenotypes must be epigenetic in nature, as opposed to fully genetic (Fullston et al. 2013). Thus, evidence suggests a transgenerational epigenetic inheritance of metabolic syndrome and its phenotypes. These conclusions could serve to potentially explain why obesity rates and incidences of metabolic syndrome have risen in developed countries over the past 50 years (Murphy et al. 2021).

Drugs that target epigenetic markers could be a mechanism to treat metabolic syndrome

Epigenetic drugs are known methods of treating cancer and can serve as a model for how therapeutic treatment of metabolic syndrome could work.

In some cancers, investigation of methylation patterns of the tissues can often indicate which cells are cancerous. For example, studies have shown that the loss of methylation markers on repetitive sequences is a common indicator of cancer as it results in the expression of genes intended to be constitutively silenced. In other studies, hypermethylation of tumor suppressor genes can also indicate cancer (Baylin and Jones 2016). In cancers with epigenetic elements, treatments often target these altered epigenetic markers to regulate an out-of-control epigenome. Often, these drugs are DNMT inhibitors (DNMTis) or HDAC inhibitors (HDACis) and function to prevent alterations to the epigenome of cancerous tissues (Gomez et al. 2020). As previously established, epigenetic mechanisms are linked to metabolic syndrome through a variety of marker types. So, epigenetic markers that trigger metabolic syndrome could be treated similarly. Thus, I propose that drugs that function as epigenetic agents in cancer could be co-opted for use in treating metabolic disease.

DNMT inhibitors (DNMTis) and HDAC inhibitors (HDACis) could function to treat metabolic abnormalities that result from errors in DNMT function.

DNMTis function by phosphorylating cytosine nucleoside agents in an ATP-dependent manner to form mono-phosphorylated cytosine nucleotides (Stresemann and Lyko 2008). Sites in the DNA sequence where cytosine nucleotides typically exist are filled instead with mono-phosphorylated nucleotides (Hu et al. 2021). Then, DNMTs find the azacytosine-guanine dinucleotide and form a covalent bond with the cytosine ring to induce the methylation reaction (Santi et al. 1984). Finally, covalently trapped DNMTs are degraded (Santi et al. 1984). DNMTis typically target either DNMT1, such as 5-azacytidine or 5-2'-deoxycytidine, or DNMT1 and DNMT3A, such as decitabine (Stresemann and Lyko 2008). Figure 6, below, shows the steps by which DNMT proteins are degraded by DNMTi drugs. By blocking DNMT1s, DNMTis can prevent the maintenance of methylation patterns associated with metabolic syndrome. Thus, the altered expression of metabolic genes will no longer occur. These types of drugs would work best in individuals with metabolic syndrome to prevent it from progressing to more severe phenotypic expression or more serious diseases. Conversely, by blocking DNMT3A, DNMTis can prevent the generation of altered methylation patterns to inhibit the expression of metabolic syndrome symptoms in the first place. This avenue would be best used on individuals who have not yet shown signs of metabolic syndrome but have been exposed to certain environmental factors that might predispose them to these symptoms.

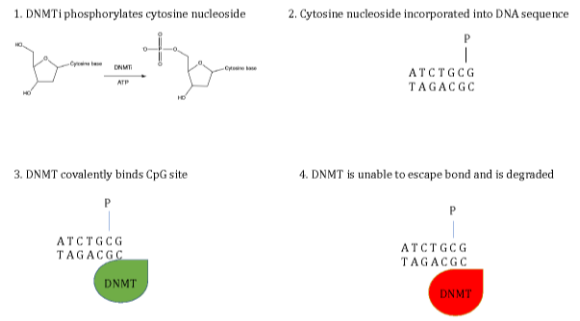

Figure 4. A visual depiction of how DNMTs are inhibited by DNMTi pharmacological agents. Green DNMT represents its active form, while red DNMT represents its degraded form.

Additionally, HDACis can be used to stabilize the epigenome by chelating a zinc enzyme cofactor, which blocks the catalytic activity of HDACs (Falahi et al. 2014). Figure 7, shown below, demonstrates the process by which HDACs become inactivated via HDACis. Without the zinc enzyme co-factor, HDACs cannot function to acetylate histones and thus, cannot cause the generation of altered epigenomes that result in overactive genes and possibly symptoms of metabolic syndrome. These types of drugs would function best in individuals with environmental predispositions to metabolic syndrome to ensure that they do not develop an altered epigenome.

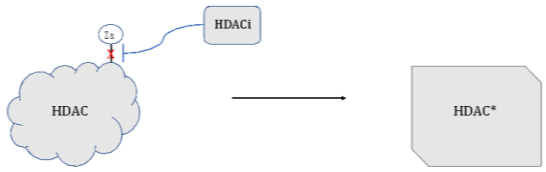

Figure 5. A visual depiction of the mechanism of HDAC inactivation via HDACis by which the zinc co-enzyme is chelated.

As they function to conserve the existing epigenome, these drugs, which are currently used mostly in cancer treatments, could be used to prevent the accumulation of epigenetic alterations that result in metabolic syndrome and attempt to stall the effects of already progressed metabolic syndrome. Therefore, I propose that these types of drugs be used to treat both individuals exposed to environmental triggers that can result in metabolic epigenetic changes and descendants of these individuals. However, future research must consider the consequences of these drugs on healthy tissues, as they are currently used to target cancerous tissue. Therefore, future research must consider the ethical and biological plausibility of this proposal as well as delve into animal model research to support this potential mechanism of treatment before we can consider this a true possibility for treating metabolic syndrome in humans.

Conclusion

In this review, I summarize the main mechanisms of epigenetic change as it pertains to the emergence of metabolic syndrome phenotypes. I discuss how changes to DNA methylation patterns and histone modifications can both be triggers for the appearance of metabolic syndrome phenotypes that have no purely genetic explanation. Additionally, I demonstrate how these epigenetic markers can originate in one individual and propagate throughout generations, resulting in the same or similar phenotypic abnormalities for generations. This possibly explains the significant increase in the percentage of humans with metabolic syndrome or other more advanced conditions. Finally, I propose a pharmacological mechanism by which these conditions might be treated at the source by using epigenetic cancer drugs as an analog for comparison. Therefore, in this paper, I have established that there is an epigenetic reason for the development and inheritance of metabolic syndrome and that pharmacological treatment of metabolic syndrome and its related issues could include the use of epigenetic drugs similar to those used in the treatment of cancer.

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Impacts of Land Use on Water Quality and Arthropod Diversity

Mia Harris, Hanna Stuart, & Jewelle Stone

Freshwater systems contain complex interactions between surrounding land usage, nutrient concentrations, and species richness. Anthropogenic influences, such as urbanization and agriculture, result in variable nutrient loads within freshwater systems and directly impact the diversity of surrounding arthropod species. This study investigated how land use affects nutrient concentrations and arthropod diversity in three lakes in the Mid-South. It was hypothesized that surrounding land use impacts the nutrient concentration and arthropod community of a freshwater lake. Water samples were collected and arthropod diversity was observed at three lakes across the Mid-South with surrounding agricultural, suburban, and forested land usages. While this study found no differences between arthropod diversity at the three lakes, the study did indicate a difference between nutrient concentrations at the three locations. The findings from this study highlight the impacts of different anthropogenic activities near freshwater systems and can inform future agricultural and urban developments near freshwater systems.

Introduction

Freshwater ecosystems have been and still are integral parts of human life (Beeton, 2002). These systems provide humans with the ability to connect communities, develop industrial applications, and participate in recreational activities (Beeton, 2002). Additionally, freshwater ecosystems are critical sanctuaries for biodiversity, supporting 10% of all organismal diversity on Earth (Stendera et al., 2012). Although these systems are frequently used and heavily relied upon, freshwater ecosystems only represent 5% of the total water present on Earth (Herdendorf, 1990). The rarity of freshwater relative to saline water makes the ecological services of freshwater bodies valuable and environmental conservation critical to the management of freshwater resources (Aylward et al., 2005).

Scientific depictions of freshwater ecosystems and their interactions with their surrounding environment have often been oversimplified (Cole et al., 2007). Recent studies indicate that freshwater ecosystems are heavily involved in nutrient cycling and distribution, participating in complex interactions with other water bodies and the surrounding land (Cole et al., 2007). These systems also have the potential to interact with excess nutrients from agricultural activities and pollutants from industrial practices (Vörösmarty et al., 2010). Furthermore, freshwater ecosystems receive resources from both point and nonpoint sources, making the management of these systems difficult and complex (Holt, 2000).

The productivity, elemental cycles, and species diversity of freshwater ecosystems can have varying sensitivities to changes in nutrient concentrations depending on location and geographical history (Turner, 1989). However, the risks of nutrient loading into a freshwater system typically correlate with surrounding land use (Nielsen et al., 2012). Urban watersheds make freshwater bodies vulnerable to anthropogenic influences, which can lead to variable loads in nitrogen and phosphorus (Tasdighi et al., 2017). Variable nutrient concentrations impact food webs and invertebrate species richness (Weijters et al., 2009). Additionally, eutrophication has been found to cause species replacement, favor specific taxa, and lead to sharp declines in species diversity (Gutierrez et al., 2020; Cook et al., 2018).

Freshwater systems support a rich diversity of arthropods, with an estimated twelve thousand species of aquatic insects in North America (Richardson, 2008). As herbivores and carnivores, arthropods maintain the function of ecosystems by consuming autotrophs, heterotrophs, and particulate organic material (Coll et al., 2002). The interactions between freshwater ecosystems and the surrounding area can expose arthropods to foreign substances, potentially impacting the diversity of arthropod populations and the productivity of freshwater ecosystems (Cole et al., 2007; Traas et al., 2004). The impact of nutrient shortages and surpluses may shift the ecological roles of arthropods, lowering the efficiency of energy transfer amongst different trophic levels within a food web (Dickman et al., 2008).

Nutrient concentrations play a central role in the maintenance of the different ecological niches that a freshwater body can provide. External factors, such as land usage, may dictate the prominence of different nutrients in freshwater lakes. Therefore, land used for agricultural activities may impact freshwater lakes differently than land used for industrial practices, and there may be specific ecological communities associated with different land usage practices. If there is an excessive abundance of one or more nutrients due to anthropogenic activities,

artificial selection may occur, promote a small number of arthropod species, and lower biodiversity. Artificial selection refers to an unnatural set of environmental conditions created by anthropogenic activities that influence how local arthropod communities evolve. With lower arthropod diversity, freshwater lake food webs and services are limited, which lowers the health of the local ecological community.

Biodiversity is critical in the maintenance and health of a freshwater ecosystem. To preserve these systems, management policies must address land use with a focused approach towards nutrient concentrations and arthropod diversity. The objective of this project was to investigate how land use affects nutrient concentrations and arthropod diversity in three lakes in the Mid-South. The findings of this study can be used to identify methods to limit the impacts of anthropogenic activities on freshwater ecosystems. The first hypothesis was that land use impacts the arthropod community of a freshwater lake, which generated the prediction that lakes located near agricultural or urbanized areas will have lower arthropod diversity than in lakes located in forested areas. The second hypothesis was that land use impacts nutrient concentration of a freshwater lake, which generated the prediction that lakes located near agricultural or urbanized areas will have higher nutrient concentrations than lakes located in forested areas.

Methods

Study Sites

The first lake sampled was Poplar Tree Lake (PTL) in Meeman-Shelby Forest State Park. PTL is a manmade lake that is used recreationally, and the surrounding regions are heavily forested. During sampling, the weather was sunny with a light breeze, and the temperature was 55°F. The second lake sampled was Memphis Lake (MEL), a manmade lake in Midtown Memphis. The land use of the MEL is suburban and recreational. The weather at this site was also sunny with light breeze, and there was a temperature of 59°F. The following week, Wapanocca Lake (WAL) was sampled in Turrell, AR, and its land use is primarily rural and agricultural. The weather at this collection site was cloudy, cold, and windy. At WAL, the temperature was 47°F.

Arthropods

To assess the number of arthropods at each study site, an arthropod collection was randomly sampled at each lake. Arthropods were observed and handled in the water, the benthic zone, and the surrounding terrestrial regions for fifteen minutes. Two stopwatches were used to record time for arthropod observations. To collect samples, the hand collecting and beating techniques were used to move and shake arthropods from vegetation (Figs. 1 and 2). Beating techniques consisted of an individual moving vegetation with a wooden rod to detach arthropods from their original locations. Additionally, a sweep net was used to sample the benthic region of the lakes, and these samples were placed into white sorting trays to maximize morphospecies observations. Arthropod specimens were observed and temporarily stored in small sample vials. Arthropods were counted and classified by general morphospecies before being released.

Figure 1. An example of the beating technique.

Figure 2. Examples of the hand collecting technique.

Nutrients

To determine nutrient concentrations for each lake, a water sample was collected from each location in large bottle. Water samples were randomly taken from the edge of each lake. From each initial water sample, twenty-four 30 mL replicates were derived and poured into 60 mL polypropylene bottles (Figure 3). For each lake, four 30 mL replicates were dedicated to total nitrogen (TN) analyses and four 30 mL replicates were dedicated to total phosphorus (TP) analyses. The replicate samples were autoclaved before analyses were performed. The second derivative method was used to measure TN. Colorimetric analysis was used to measure TP.

For the second derivative method, TN standards of 0 µg/L, 200 µg/L, 400 µg/L, 600 µg/L, 800 µg/L, 1000 µg/L, and 2000 µg/L were used. Each standard was poured into a 1-centimeter quartz cuvette, and TN was analyzed using a spectrophotometer. Each sample was scanned at 200 nanometers, and absorbance was measured at 5 nanometer intervals. This process was repeated for the PTL, MEL, and WAL sample replicates. Between each sample, the quartz cuvette was thoroughly rinsed with Milli-Q water.

For the colorimetric analysis, TP standards of 0 µg/L, 5 µg/L, 10 µg/L, 25 µg/L, 50 µg/L, 100 µg/L, and 200 µg/L were used. To create the composite reagent, pipettes were used to combine 15 mL of ammonium molybdate, 37 mL of 15% sulfuric acid, 15 mL of ascorbic acid, and 7.5 mL of potassium antimony tartrate into a beaker. Afterwards, 3 mL of the composite reagent were pipetted into each of the standards and sample replicates. Samples were left to react with the composite reagent for 10 minutes before the TP was analyzed using a spectrophotometer. The standards were poured into a cylindrical polarimeter cell, and the absorbance was read on a 10-centimeter pathlength at 885 nanometers. This analysis was repeated for each of the replicates for the three lakes. Between each sample, the cylindrical polarimeter cell was thoroughly rinsed with Milli-Q water.

Figure 3. Researchers organizing samples and performing nutrient assays.

Statistics

Variance analyses were used to examine and compare the relationships between land use, arthropod diversity, and nutrient concentrations. TN and TP across lakes were analyzed for significant differences using a one-way analysis of variance (ANOVA) test. The same test was used to analyze

differences between specific nutrient concentrations amongst lake pairings. The number of morphospecies identified for each lake were analyzed for differences using a chi-squared test. Differences between the number of individuals counted in each of the study lakes were not analyzed. The presence of an outlier (WAL) would have produced a false significant result in statistical tests; therefore, the number of individuals found at each lake was not included in the statistical analyses. All statistical results have a level of confidence of 95%.

Results

There was no difference amongst the number of morphospecies found for each study lake (Fig. 4, p = *0.06*). PTL showed the greatest arthropod diversity with 18 identified morphospecies (8 aquatic, 10 terrestrial), followed by 10 morphospecies in MEL (6 aquatic, 4 terrestrial) and 10 morphospecies in WAL (1 aquatic, 9 terrestrial). The number of individuals counted across the study lakes exhibited great variation. The fewest number of individuals was counted at MEL**,** where 34 arthropods were found. Slightly more individuals were counted at PTL, where 49 arthropods were identified. WAL was an outlier in the individual counts data as 297 individuals were counted. However, an estimated 250 of those individuals (about 84%) were ants from an anthill that was recently disturbed. No statistical tests were performed on individual count data from the study lakes due to this outlier.

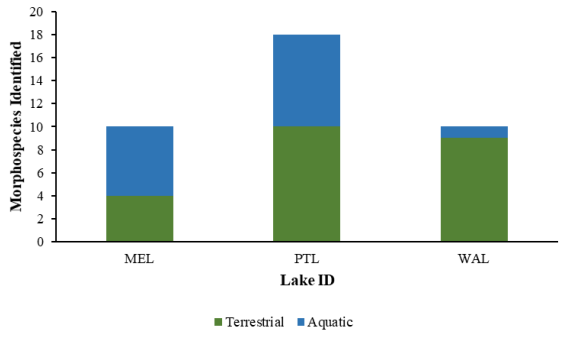

Figure 4. **Morphospecies by habitat found.** Identified morphospecies in MEL, PTL, and WAL. The number of morphospecies identified across all study lakes was not different (chi-squared test, *p = 0.06*).

There was a difference in TN amongst the study lakes (Fig. 5, *p = 0.03*). Mean TN was determined for each lake: MEL (564.02 ± 47.41 µg/L), PTL (1268.37 ± 298.34 µg/L), and WAL (560.55 ± 90.19 µg/L). There were no differences in TN for PTL versus MEL (p = 0.06), PTL versus WAL (p = 0.06), and MEL versus WAL (p = 0.97).

There was a difference in TP amongst the study lakes (Fig. 5, *p = <0.01*). Mean TP was determined for each lake: MEL (272.29 ± 4.21 µg/L), PTL (50.65 ± 2.9 µg/L), and WAL (298.76 ± 5.59 µg/L). There were differences in TP for PTL versus MEL (p <0.01), PTL versus WAL (p <00.01), and MEL versus WAL (p <0.01).

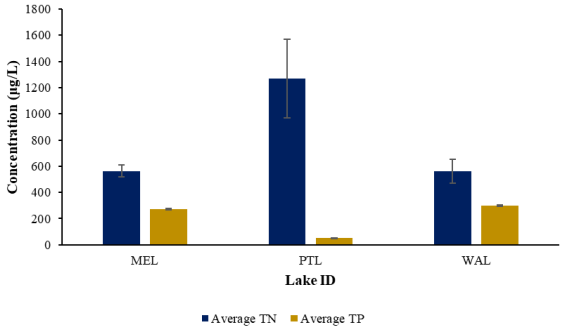

Figure 5. Average TN and TP concentrations. Mean TN and TP in MEL, PTL, and WAL. TN and TP across samples from all study lakes were different (one-way ANOVA, *p = 0.03;* one-way ANOVA, *p = <0.01*).

Discussion

Arthropod Diversity

The statistical analyses did not support the first hypothesis that different land use impacts arthropod diversity (Fig. 4). Moreover, no difference was found between the observed number of terrestrial and aquatic arthropods at PTL, MEL, and WAL (Fig. 4). Though these findings do not indicate that there is an interaction between different land uses, there were two factors in the study that may have influenced the experimental conclusions.

The first factor that may have influenced the data was the different weather conditions for each day of the field sampling. On the first day of the field study, arthropod samples were collected at PTL and MEL under warm, sunny conditions with a slight breeze. In contrast, arthropod samples were collected at WAL under cold, cloudy, and windy conditions. This difference in climatic conditions may have impacted the location and behavior of the arthropods, making some species and populations more accessible than others.

The second factor that may have influenced the data was the sampling site at WAL, which contained an active anthill with a large colony of individuals.

The presence of this colony impacted the number of individuals that were counted at that location, which may influence how WAL is represented quantitatively. Compared to PTL and MEL, WAL had a much larger and biased individual count (Fig. 4).

In contrast to this smaller-scale study, more extensive studies focusing on arthropod diversity in relation to land use have indicated that species richness has an inverse relationship with landscape manipulation and agricultural practices (Hendrickx et al., 2007). When land use intensity is high, species richness is low (Attwood et al., 2008). Additionally, the intensification of anthropogenic land use practices may cause species and taxonomic loss that may decrease the functional distinctness and the number of ecological niches available to an arthropod community (Birkhofer et al., 2015). These studies suggest that there is an interaction between land use and arthropod diversity.

Nutrient Concentrations

Statistical analyses supported the second hypothesis that different land uses affect lake nutrient concentrations (Fig. 5). When compared broadly, TN did not significantly differ between PTL, MEL, and WAL. However, when TN was compared in specific lake pairings, there was not a significant difference in TN amongst any of the pairings (Fig. 5). In the broad comparison across all three lakes, the significant difference between TN was likely a result of the larger variations between the three lakes. However, this variation was not detected in pairing analyses, as TN was similar across PTL, MEL, and WAL.

There was also a significant difference in TP when the three lakes were widely compared through a statistical analysis. Moreover, when the lakes were compared in pairs, TP significantly differed in each of the lake pairings (Fig. 5). These findings suggest forested, urban, and agricultural land uses affect lake nutrient dynamics differently. TP was higher in urban and agricultural watersheds, which suggests that anthropogenic activities significantly impact lake nutrient concentrations.

Several limitations to the nutrient concentration analyses may have impacted these findings. Foremost, nutrient concentration analyses may have been limited by lake water sampling techniques. Research indicates that sampling should be extensive in terms of quantity, depth, and seasonality (U.S. Geological Survey, 2018). Three study sites were visited at the beginning of the spring months when the lakes were all beginning to mix from winter stratification. When water samples were randomly collected, a medium-sized sampling container was filled from the edge of each lake. While this sampling technique provided accurate nutrient concentrations at specific sampling locations, these nutrient concentrations might not have reflected the nutrients throughout the entirety of the water column in each lake.

These analyses may have also been impacted by anthropogenic efforts to control lake aesthetics and eutrophication. While PTL and WAL do not have visible nutrient management systems, MEL has a fountain that continuously circulates water and dissolved oxygen throughout the water column. The presence of dissolved oxygen creates an oxidized environment, which strongly influences redox conditions within freshwater systems. The presence of oxygen at the lower depths of the lake also prevents the release of nutrients stored in the sediment, such as ferric iron and phosphorus (Wang et al., 2008). Consequently, the TP and the potential for eutrophication decrease substantially. Urban land usage may increase TP within MEL, but the cycling fountain may have reduced the available phosphorus in the water column.

Furthermore, PTL, MEL, and WAL each have different anthropogenic origins. While PTL and WAL are both recreational reservoirs, MEL was constructed for aesthetic purposes. Though the lakes that were sampled in this study were not formed naturally, both TN and TP do not significantly differ between natural and manmade lakes (U.S. Environmental Protection Agency, 2009). Therefore, the three sample lakes accurately represented the influence of surrounding land usage on TN and TP.

The brief duration of this experiment may have limited the generalizability of these findings. Lakes and reservoirs are highly variable ecosystems that are impacted by a wide array of factors, including seasonal variations, discharge, and temperature (U.S. Geological Survey, 2018). While discrete sampling cannot represent large ecosystem fluctuations, long-term sampling and analyses can account for high amounts of variation. Other studies have also indicated the need for longitudinal studies to specifically assess nutrient concentrations within reservoirs (Yurista et al., 2004). Moreover, long-term studies have the ability to accurately represent the dynamic relationship between watershed land usage and nutrient concentrations (Tasdighi et al., 2017). This experimental design included single arthropod and nutrient samples at each lake and reservoir, which produced discrete results for ecosystems that are highly variable. Therefore, these findings may not portray accurate quantifications of year-round arthropod diversity and nutrient concentrations for these study sites.

Conclusion

These findings suggest that there is a relationship between land use and lake nutrient concentration. Although this study does not propose a relationship between land use and arthropod diversity, future studies should examine these relationships more thoroughly. The design and limitations of this study may have overlooked potential interactions between anthropogenic activities and lake dynamics. Therefore, future investigations should aim to include more sampling techniques, sites, and replicates to provide a more comprehensive understanding of the relationships between land use, nutrient concentrations, and arthropod diversity.

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Neuroinflammation Negatively Impacts Neuronal Signal Transmission

Erica Mosby

Neuroinflammation is characterized as an inflammatory response in the brain or spinal cord. Neurodegenerative diseases, like Alzheimer's disease and Multiple Sclerosis, are associated with a detrimentally high neuroinflammatory response and cognitive impairment. The neuroinflammatory response is mediated by the production of cytokines, mast cells, reactive oxygen species, and secondary messengers. The aforementioned substances have apoptotic properties, which in abundance over a repeated period, damages neurons and thus weaken signal transmission. Therefore, neuroinflammation negatively affects synaptic transmission because neuroinflammation releases cytokines, overactive cerebral mast cells, and ROS.

Introduction

An inflammatory response in the brain or spinal cord is described as neuroinflammation. It is caused by damage to or pathogen invasion into the central nervous system (CNS). The hallmarks of neuroinflammation include the overproduction of cytokines, mast cells, reactive oxygen species (ROS), and secondary messengers (DiSabato et al 2016). These hallmarks are not inherently deleterious, but when they continually amass in a localized region of the CNS over an extended period, cytokines, mast cells, and ROS become detrimental to neurons, hindering their efficiency and efficacy (DiSabato et al 2016). The neuronal damage begins with mast cells. As a primary defense mechanism against pathogens and tissue damage, mast cells in the brain release the chemical mediators, nitric oxide and ROS (Lyman et al 2014). These mediators regulate the neuroinflammatory response by inducing apoptosis in the surrounding cells and activating microglia. However, when produced in excess, nitric oxide and ROS react with molecules, like water, to produce toxins (e.g., hydrogen peroxide) that poison the microenvironment and kill neurons (Beckhauser et al 2016). In response to this toxicity, mast cells mediate the release of cytokines from microglia in order to regulate the amounting neuroinflammatory response (Sandhu et al 2021). The activation of microglia is not always helpful because the cytokines that they release also induce apoptosis and inflict additional tissue damage. These components of neuroinflammation become trapped in a positive feedback loop, leading to further damage of the neuron, myelin, and synapses (Fig. 1).

The immediate and chronic consequences of neuroinflammation result in myelin reduction and axonal degeneration (DiSabato et al 2016). Along with the increase in apoptosis, degeneration may be a result of increased synaptic pruning. Neuroinflammatory conditions dysregulated synaptic pruning by promoting excessive pruning and therefore alterations in synaptic activity (Mottahedin et al 2017).

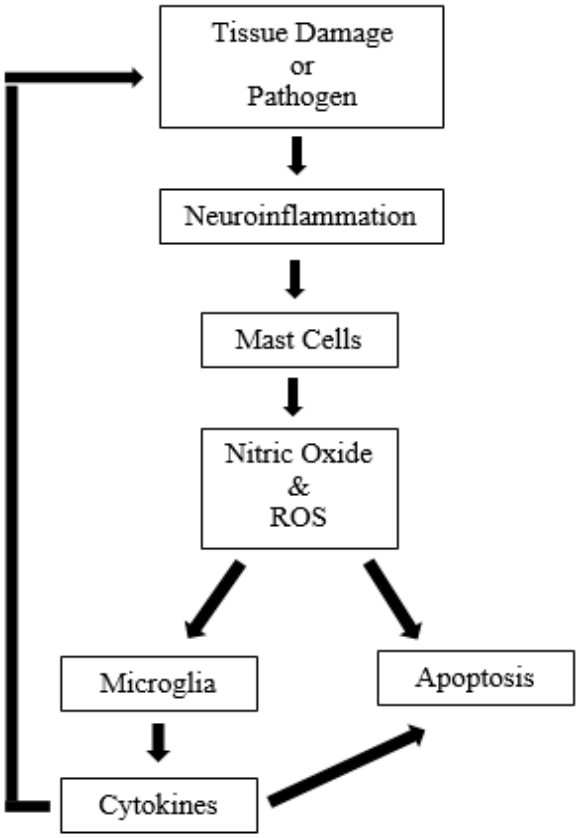

Figure 1 | Positive feedback loop of the neuroinflammatory response. The neuroinflammatory response is initiated by a pathogen or site of tissue damage in the brain or spinal cord. The immediate response to adverse stimuli is mast cell production. Mast cells produce nitric oxide and ROS that prompt apoptosis to kill pathogens and damaged cells. However, when released in abundance, nitric oxide and ROS react with other molecules in the microenvironment to produce harmful substances. Microglia are then activated by their chemoreceptors that respond to changes in pH. When the environmental change is noted, microglia release cytokines to regulate the neuroinflammatory response. However, because the

cytokines also induce apoptosis, they cause more tissue damage. As a result of tissue damage, the neuroinflammatory response starts again. The neurons and myelin are now trapped in a positive feedback cycle of the brain responding to damage and inflicting damage. This is how neuroinflammation becomes problematic, leading to impaired neuronal signal transmission, and thus neurodegenerative diseases.

Degeneration of the neuron and myelin leads to impaired synaptic transmission and decreased action potential propagation, respectively. Therefore, synaptic dysfunction is known to be associated with neuroinflammation (Lyman et al 2014). With respect to neurodegenerative diseases, Alzheimer's disease and multiple sclerosis are promoted by neuroinflammation (DiSabato et al 2016). They are also associated with impaired cognition—likely as a result of synaptic dysfunction and reduced propagation efficiency caused by neuroinflammation. This pathology implies that therapeutics for neurodegenerative diseases should target mechanisms involving the upregulation of mast cells, cytokines, nitric oxide, and ROS in order to improve cognitive function for patients struggling with Alzheimer's disease and multiple sclerosis. Therefore, the goal of this review is to identify and explain the neuroinflammatory mechanisms that lead to reduced signal transmission.

Accumulation of Mast Cells

Because mast cells are white blood cells, they are the primary defense mechanism against attacks on the brain and spinal cord. Thus, they have various receptors that respond to changes in the environment. For the neuroinflammatory response, specifically, mast cells respond by synthesizing and secreting pro-inflammatory mediators that induce neuronal death (Kempuraj et al 2017). These chemical mediators include ROS, cytokines, and growth factors (Li et al 2017). All of these mediators are needed because they appease damage and kill pathogens. However, the problem arises when damage to the CNS is persistent, so mast cells are then overproduced. Consequently, the accumulation of mast cells leads to the overproduction of cytokines and ROS, both of which lead to cell death. Therefore, mast cells indirectly limit synaptic signal transmission by decreasing the number of neurons in the brain via chemical mediators.

An example of the cognitive deficits caused by neuronal death from an amassment of mast cells is patients who have undergone neurosurgery. Neurosurgery takes months to recover from, not only because it is a majorly invasive surgery, but also because it initiates the positive feedback loop of the neuroinflammatory response. After this form of surgery, many older patients are prone to having neuroinflammation-related post-operative cognitive dysfunction (POCD) (Terrando et al 2011). POCD leads to various cognitive impairments in speech, learning, memory, and spatial orientation that last for a brief period after neurosurgery (Terrando et al 2011). These impairments are caused by the overproduction of mast cells, which secrete cytokines that damage synapses (Terrando et al 2011). POCD symptoms were replicated in albino Rattus norvegicus rats to provide insight into the role of mast cells in POCD. Susu Zhang, Hongquan Dong, Xiang Zhang, Nana Li and Jie Sun et al. implanted cannulas into the right lateral ventricle of rats using stereotaxic surgery in order to ascertain the effects of neurosurgery in humans. Half of the rats who had surgery were given a mast cell degranulation inhibitor to stop secretion in mast cells. The other half of the rats who had surgery were given a vehicle drug. The data showed that the rats who were given the control drug experienced reduced cognitive function—as measured by freezing behavior and number of mistakes made while completing a previously learned maze (Zhang et al 2016). They concluded that the cognitive deficits in POCD are caused by the accumulation of mast cells in the active neuroinflammatory response. The mast cells secrete cytokines which damage synapses and perpetuate weakened neuronal signaling.

Reactive Oxygen Species

On the other hand, if mast cells are overproduced, then their additional cell mediators, like ROS, will be produced in surplus too. These species also limit synaptic transmission because when in excess, they poison the neuronal microenvironment by reacting with nearby molecules. Common ROS are diatomic oxygen, superoxide, hydrogen peroxide, and hydroxyl ions. To provide one illustration of many for how toxicity accumulates in the microenvironment, hydrogen peroxide, which is toxic to cells in high amounts, will be used as an example. The mechanism of action for ROS is the activation of various cellular responses (Chen et al 2010). The cellular response triggered by hydrogen peroxide is increased activity in calcium channels (Beckhauser et al 2016). When excess hydrogen peroxide is released by mast cells during neuroinflammation, it overstimulates the calcium channels on the synapse. Therefore, the synaptic clefts receive an influx of calcium. Calcium then binds to an intermediate protein called calmodulin.

Calmodulin's role is to report the changes in calcium levels to other enzymes. One of these enzymes is NADPH oxidase in the electron transport chain. Because oxygen is the last electron acceptor in the electron transport chain, when NAPDH oxidase activated, it turns diatomic oxygen into superoxide (Fig.2). When superoxide reacts with water, it produces hydrogen peroxide, thus reactivating the calcium channels and further poisoning the neuronal microenvironment. The surplus of calcium leads to excitotoxicity along glutamatergic pathways (Chen et al 2010). Cell death along this pathway leads to dysregulated and decreased long-term potentiation (LTP) (Chen et al 2010). The overproduction of ROS activates cellular responses that lead to neuronal death and diminish signal transmission.

Alternate Perspective on ROS

Despite the deleterious effects of ROS on neuronal signaling, they serve as modulators for memory formation in dendritic spines (Massaad et al 2011). Memory formation requires the induction of LTP using AMPA receptors, NMDA receptors, and activation of the extracellular signal-related kinases (ERK) pathway (Kishida et al 2005). Activation of the ERK pathway is dependent upon activation of the NMDA receptors. Research shows that when mice were given general antioxidants, activation of the NMDA receptor-dependent ERK pathway was inhibited (Kishida et al 2005). This inhibition implies that ROS are needed in order to activate the NMDA receptors and thus form memories (Kishida et al 2005). In addition to ROS, nitric oxide contributes to the maintenance and induction of LTP directly by increasing the amount of time that calmodulin remains in the postsynaptic cleft (Massaad et al 2011). Thus, calmodulin can further stimulate NADPH oxidase to produce more ROS that will continually modulate memory formation. Alternative perspectives on ROS demonstrate that ROS are necessary for memory formation. These researchers explain that ROS only become harmful to neuronal signal transmission when they are overproduced by responses, like neuroinflammation. ROS indirectly limit learning and memory via neuroinflammation.

Overproduction of Cerebral Cytokines

Lastly, increased cerebral cytokine production decreases LTP. The interleukin-1 beta (IL-1ß) cytokine is one of the primary cytokines that microglia produce in response to stress (Ramesh et al 2013). The production of IL-1ß has an onset as early as 1 hour post-stressor (Uceyler et al 2007) and can accumulate for up to 35 days (Ruohonen et al 2005). IL-1ß in low amounts is shown to be necessary for LTP to occur, but it is detrimental to the neuron in large amounts (Lynch et al 2015). The concentration of IL-1ß cytokines determines the impact of LTP in the hippocampus (Lynch et al 2015). The impact of LTP is determined because as the concentration of

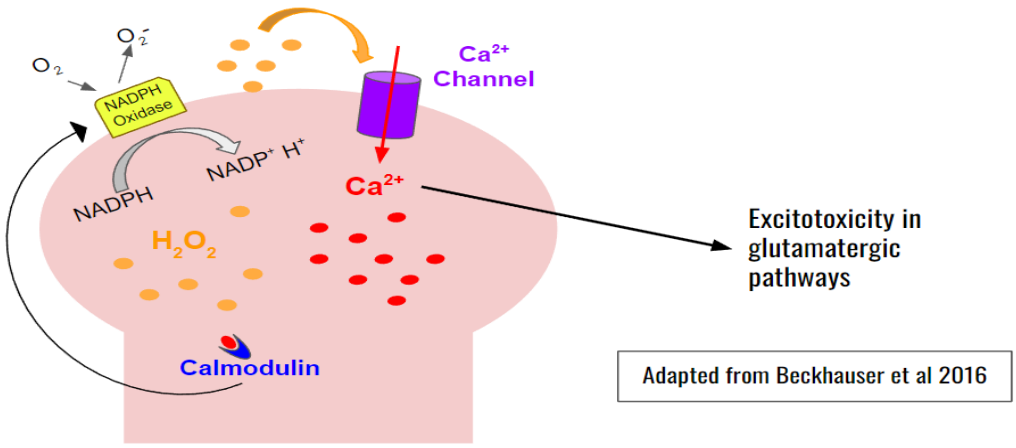

Figure 2: Hydrogen peroxide poisons neurons by overstimulating calcium channels. Hydrogen peroxide stimulates the calcium channel, leading to an influx of calcium. Calcium binds to calmodulin. Calmodulin then activates NADPH oxidase, which produces superoxide. Superoxide combines with water to produce more hydrogen peroxide. The overproduction of hydrogen peroxide from neuroinflammation leads to cell death along excitatory, glutamatergic pathways because calcium is poisonous in high concentrations.

IL-1ß cytokines bound to the interleukin-1 receptor (IL-1R) on the postsynaptic cleft increases, the concentration of AMPA receptors on the postsynaptic cleft decreases (Prieto et al 2019). Therefore, research supports the claim that there is a negative correlation between cytokine concentration and sustainability of LTP (Lynch et al 2015). As shown in figure 3, the mechanism by which IL-1ß suppresses LTP is by inhibiting phosphorylation of the receptor tyrosine kinase (RTK) on the neuron in order to reduce the amount of AMPA receptors present (Azad et al 2020; Prieto et al 2019) (Fig.3). When cytokines are placed into an *in vitro* neuronal environment, neurexin-1β proteins, which are used to bridge the pre- and postsynaptic clefts, and AMPA receptors are diminished (Prieto et al 2019). For LTP to occur, a synaptic connection has to be strengthened via frequent activation, and there needs to be numerous AMPA receptors. Phosphorylation of the phosphatidylinositol-3-kinase (PI3K) Akt pathway is necessary to bolster AMPA receptors on the postsynaptic cleft, and thus foster LTP.

LTP is also needed for fear conditioning. Elevated IL-1ß cytokine levels in the hippocampus during neuroinflammation lead to decreased fear conditioning (Hein et al 2010). Induced conditioning was shown by decreased freezing behavior in rats injected with IL-1ß solution when they were conditioned to startle at a loud noise in a particular context (Hein et al 2010). Another type of cytokine is interleukin-12 (IL-12), which is known as a pro-inflammatory cytokine (Derecki et al 2010). Common of all cytokines, IL-12 has also been shown to perpetuate the neuroinflammatory response by fostering neuronal tissue damage (Derecki et al 2010). Lastly, in accordance with IL-1ß, IL-12 is also associated with decreased LTP. The overproduction of IL-12 leads to decreased levels of brain-derived neurotrophic factor, which is a crucial molecule that is needed for learning and memory (Derecki et al 2010). Cytokines weaken neuronal signal transmission by damaging neurons and their synapses.

Conclusion and Future Research

Neuroinflammation is the mechanism by which the CNS responds to dangers in its environment. This response can quickly become detrimental to neurons when the inflammatory response persists and cycles. Unfortunately, the positive feedback loop of neuroinflammation is a common denominator across neurodegenerative diseases, like multiple sclerosis and Alzheimer's disease. Neuroinflammation and neurodegenerative diseases are both associated with cognitive deficits due to limited neuronal signaling, so the two variables are hypothesized to be in a causal relationship with one another. Based on this review, the conclusion that neuroinflammation limits neuronal signal transmission because it mediates the release of overactive cerebral mast cells, ROS, and cerebral cytokines is supported. The overproduction of mast cells indirectly leads to neuronal damage and death via secretion of ROS and cytokines. ROS react with nearby molecules to produce toxic chemical substances that kill cells, but other times, ROS are useful for memory formation. Finally, increased concentration of interleukin cytokines decreases LTP,

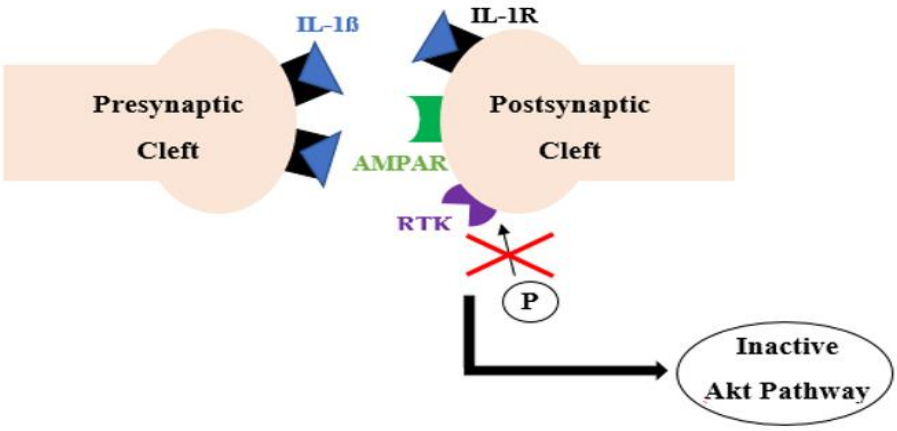

Figure 3 | IL-1ß inhibits Akt pathway leading to decreased AMPA receptors and LTP. When IL-1ß binds to IL-1R on the postsynaptic cleft, it inhibits phosphorylation of RTK. RTK is one of the receptors that activates the Akt signaling pathway by binding a phosphoryl group (Azad et al 2020). When the phosphoryl group cannot bind, the Akt pathway is inhibited. As a result, this limits the production of AMPA receptors on the postsynaptic cleft. IL-1R stands for interleukin-1 receptor. AMPAR stands for AMPA receptor. RTK stands for receptor tyrosine kinase. P represents a phosphorus group.

learning, and memory. All of these factors negatively impact neuronal signal transmission.

This review provides mechanisms that can be used as treatment targets for neurodegenerative diseases that are prompted by neuroinflammation. For example, the drug rilonacept is a competitive, cytokine inhibitor that blocks the interleukin-1 receptor (Ji et al 2018). This drug has been shown to decrease pain caused by neuroinflammation in patients with arthritis by inhibiting the positive feedback cycle (Ji et al 2018). In addition, this study is also limited because it does not provide insight into how to preemptively regulate the neuroinflammatory response before it becomes a cycle. Thus, future research should focus its attention on the primary stages of the response. Therefore, more research is needed on mast cell inhibition since mast cells initiate and then mediate the neuroinflammatory response.

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Okapis (*Okapia johnstoni*) Do Not Exhibit Variable Spatial Patterns Around People and Conspecifics

Hanna Stuart, Ashlee Caruana, Sarah Boyle

*The okapi (*Okapia johnstoni*) is an understudied, Endangered species found within the forests of the Democratic Republic of Congo. Since their discovery in the early 1900s, okapi populations have rapidly declined from a range of environmental, political, and biological factors. Though wild populations are solitary, captive okapis frequently encounter humans and conspecifics, and individuals have exhibited variable behavioral and spatial patterns. Our study investigated which variables influenced how two captive male okapis, Miraq and Riley, used their enclosure spaces at the Memphis Zoo. We hypothesized that okapis' locations are influenced by their proximity to conspecifics and the public. Using scan sampling at two-minute intervals, we recorded the behavioral and spatial patterns of the two okapis in their separate enclosures. Our study shows that Miraq and Riley exhibited similar spatial patterns within their enclosures, suggesting that these individuals have not restricted their ranges based on the presence of conspecifics or people. By understanding how variables influence okapis' enclosure usage, zoos can mitigate the variables that decrease individuals' ranges within their enclosures and increase animal welfare.*

Introduction

The okapi (*Okapia johnstoni*) is the closest, extant relative of the giraffe (*Giraffa camelopardalis*) (Hassanin et al., 2011). Okapis were discovered in the early 1900s and are native to the forests of the Democratic Republic of Congo (DRC) (Johnston, 1900; Stanton et al., 2014). The okapi is classified as Endangered due to its rapidly declining populations, which are estimated to contain a few thousand remaining individuals (Kümpel et al., 2015a; Mallon et al., 2015). Within the DRC's Okapi Wildlife Reserve, okapi populations have decreased due to environmental, political, and biological factors (Kümpel et al., 2015b; Medrode and Cowlishhaw, 2006; Schwarzenberger et al., 1999; Wilkie et al., 1998).

During times of conflict and economic instability, protected species in the DRC have been aggressively hunted and overexploited (Medrode and Cowlishhaw, 2006; Wilkie et al., 1998). Okapis have become increasingly prevalent in the DRC's bushmeat trade and have been hunted for their skin and meat (Nixon and Lusenge, 2008; Vliet et al., 2012; Wilkie et al., 1998). Habitat loss through deforestation has also directly impacted okapi populations, reducing okapis' natural ranges, nutritional resources, and shelter from predation (Kümpel et al., 2015b; Kümpel et al., 2018). Despite in-situ and ex-situ conservation attempts, okapis have been unable to replenish their diminishing numbers with fourteen-month gestation periods and viviparous births (Mallon et al., 2015; Schwarzenberger et al., 1999). Though okapi populations have steadily declined since their discovery, okapis remain understudied in wild and captive settings (Kümpel et al., 2018).

In the wild, okapis are solitary individuals, and when observed with other individuals, groups typically consist of a female with her calves or a female in proximity to a male (DeRosa et al., 2004). However, captive okapis are arranged into enclosures based on sex and relatedness, and individuals exhibit variable behavioral and spatial patterns when encountering people and conspecifics (DeRosa et al., 2004; Troxell-Smith and Miller, 2016; Troxell-Smith et al., 2017). Enclosure architecture, enrichment, and proximity to people can also influence the behavioral repertoires and spatial patterns of captive okapis (Troxell-Smith et al., 2017).

To better understand how captive okapis respond to their environments, we examined which variables influenced how okapis used their enclosure space. We hypothesized that okapis' locations are influenced by their proximity to a) conspecifics and b) the public. We predicted that okapis would spend less time near another okapi and near the public compared to other spaces in the enclosure, relative to the available space in each location. Behavioral studies of captive okapis can inform zoos how individuals respond to different factors in their environment, especially conditions that okapis do not encounter in their natural habitat. By better understanding the variables associated with okapis' spatial patterns and behaviors, zoos can then increase the welfare of captive okapis while mitigating the declining populations of wild okapis (Kümpel et al., 2015b).

Methods

Two male okapis, Miraq and Riley, were studied in the Zambezi River Hippo Camp at the Memphis Zoo during the Fall of 2022 (Fig. 1). The okapis inhabited adjacent enclosures separated by a wooden fence. In the left yard, Miraq was housed with two white storks (*Ciconia ciconia*), and in the right yard, Riley was housed with dik-diks (*Madoqua kirkii*) and guineafowl (*Numida meleagris*). On the public trail, zoo visitors could view the okapis through wire fencing.

The okapis' enclosures were mapped using Google Earth Pro satellite imagery from March 2018. Using ArcGIS Pro, a numbered grid was overlaid onto the enclosure map using the WGS 1984 coordinate system. A boundary was defined around the public fence and the okapis' shared fence to indicate proximity to people and another okapi (Fig. 2).

At two-minute intervals, researchers used scan sampling to record the behavior and grid location of the okapis (Martin and Bateson, 2007). One researcher recorded data for Miraq while the other researcher simultaneously recorded data for Riley, alternating the subjects of data collection each week. Data were collected for two consecutive hours each week for four weeks, resulting in a total of sixteen hours of data between both okapis.

We calculated the percentage of scans that each okapi spent near a conspecific, near the public, and in other locations. On the grid map, we classified the percentages into five classes using Jenks Natural Breaks. We divided the okapis' scans by the number of accessible grids in each location to standardize the data. We conducted chi-square tests for Miraq and Riley to compare the number of scans each okapi spent near a conspecific, near the public, and in other spaces, relative to the available space in each location. We conducted further chi-square tests to compare the okapis' scans predicted by available space in the three defined locations.

Figure 1. Miraq (left) and Riley (right) at the Memphis Zoo.

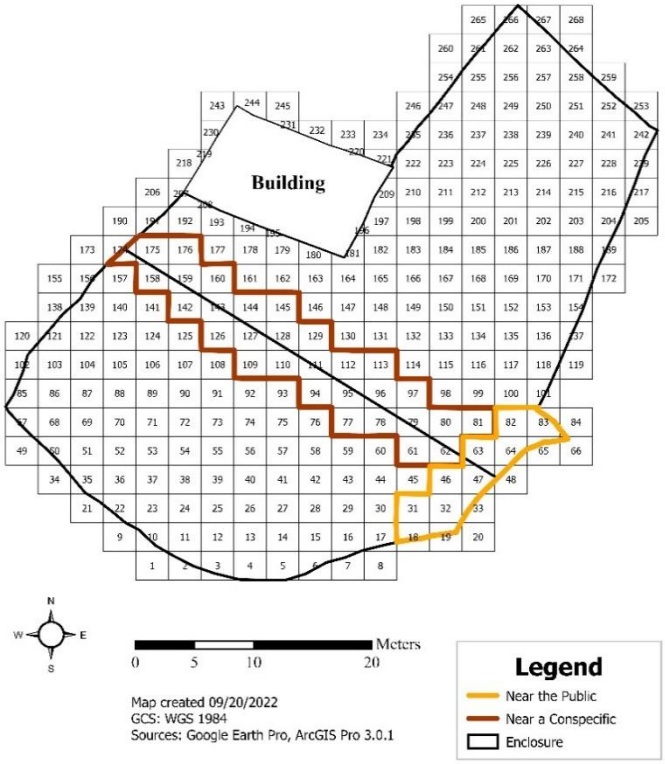

Figure 2. The gridded enclosure map used to collect spatial data.

Results

Neither Miraq ($X^2_2 = 0.52$, $p = 0.77$; Fig. 3) nor Riley ($X^2_2 = 1.12$, $p = 0.57$; Fig. 3) spent different amounts of time near a conspecific, near the public, and in other areas of the enclosure, relative to the available space in each location (Fig. 4). Miraq and Riley did not spend different amounts of time, relative to the available space, near a conspecific ($X^2_1 = 0.19$, $p = 0.66$; Fig. 5), near the public ($X^2_1 = 0.00$, $p = 0.95$; Fig. 5), and in other locations ($X^2_1 = 0.03$, $p = 0.87$; Fig. 5).

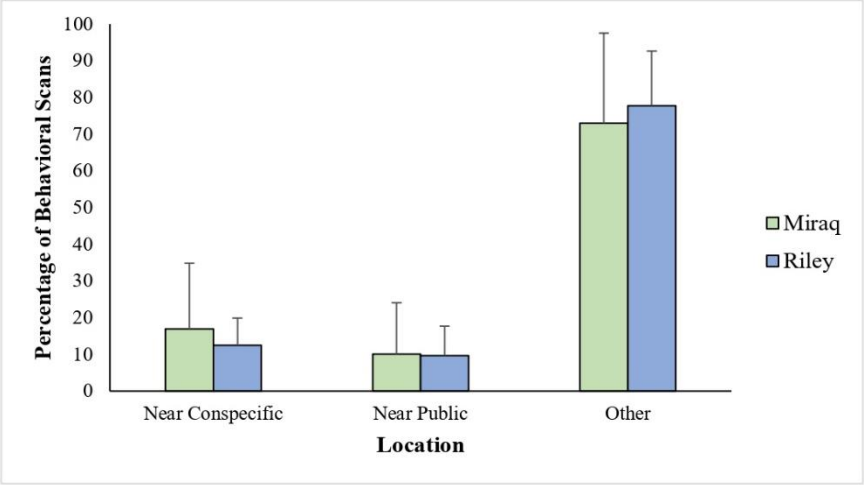

Figure 3. The percentage of behavioral scans near another okapi, near the public, and in other locations for Miraq and Riley (percent + standard error).

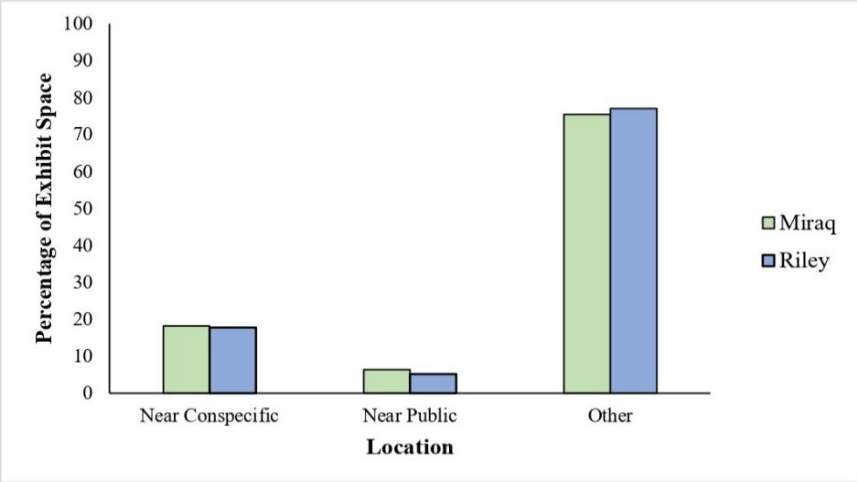

Figure 4. The percentage of space near another okapi, near the public, and in other locations for both Miraq and Riley's enclosures.

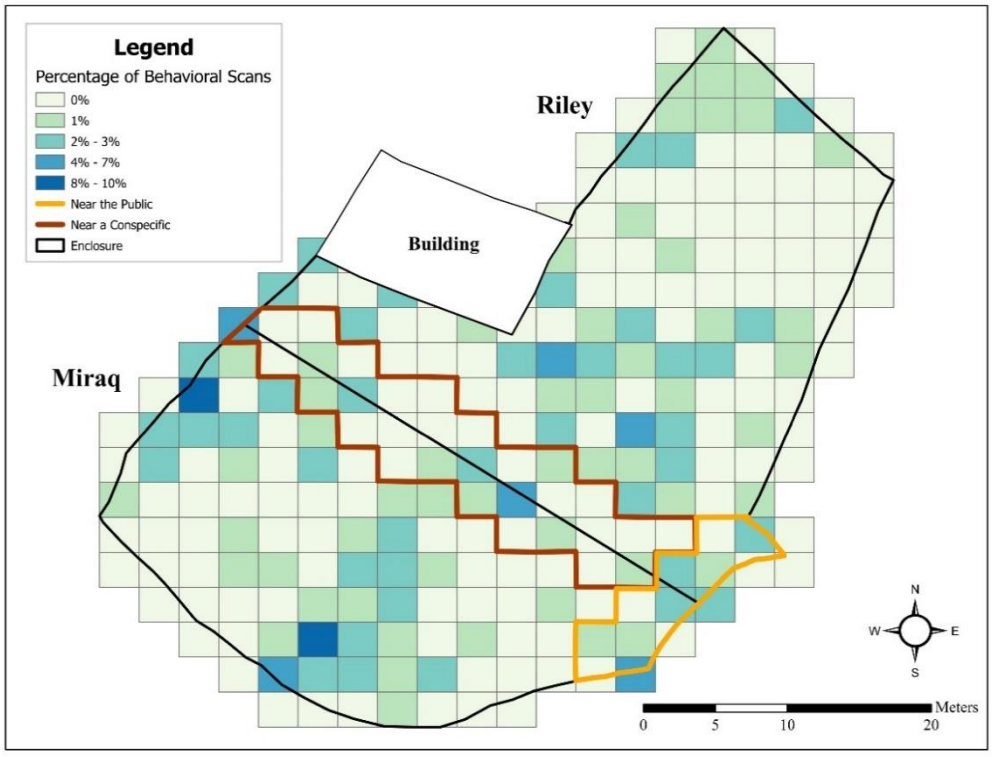

Figure 5. The percentage of behavioral scans recorded in the gridded enclosure map. Miraq was housed in the west yard, and Riley was housed in the east yard. Grids outlined in brown represent areas near another okapi, and grids outlined in yellow represent areas near the public trail.

Discussion

Our study examined if okapis exhibit variable spatial patterns near conspecifics and people. Our analyses indicate that the okapis at the Memphis Zoo exhibited no differences between the time spent near another okapi, near the public trail, and in other enclosure locations, relative to the total space available in each location. Miraq and Riley each spent similar amounts of time in the three

locations. While our findings do not suggest a relationship between okapis' spatial patterns and proximity to people, a similar study found that okapis forage less in areas with higher human activity (Troxell-Smith et al., 2017).

Our findings suggest that captive okapis' spatial patterns differ from wild okapis (DeRosa et al., 2004). While Miraq and Riley did not exhibit different spatial patterns around conspecifics or people, wild okapis are solitary and rarely encounter other okapis and people (DeRosa et al., 2004). Captive okapis are restricted in their space away from people and other okapis, while wild okapis have larger, heterogeneous habitats (Stanton et al., 2014). Factors such as the size and homogeneity of enclosures can impact how captive okapis use their space (Troxell-Smith et al., 2017).

At the Memphis Zoo, zookeepers regularly rotate the placement and delivery of food. Due to this inconsistency, we did not examine how the location of food sources impacts okapis' spatial patterns. However, studies have found that widely distributed food sources can impact okapis' enclosure usage (Troxell-Smith et al., 2017). Future studies should consider how factors such as food location and proximity to other species impact the spatial patterns of okapis. Additional studies could investigate animal enclosure usage while considering different times of the day and zookeepers' husbandry routines. Possible relationships between individuals' spatial patterns and behaviors should also be considered in future analyses.

The solidarity and small populations of okapis have contributed to their underrepresentation in scientific literature (Kümpel et al., 2018). Our study shows that two captive okapis exhibit similar usages of areas within their enclosures, suggesting that these individuals have not restricted their ranges based on the presence of conspecifics or people. By understanding how variables influence okapis' enclosure usage, zoos can mitigate the variables that decrease individuals' ranges within their enclosures and increase animal welfare.

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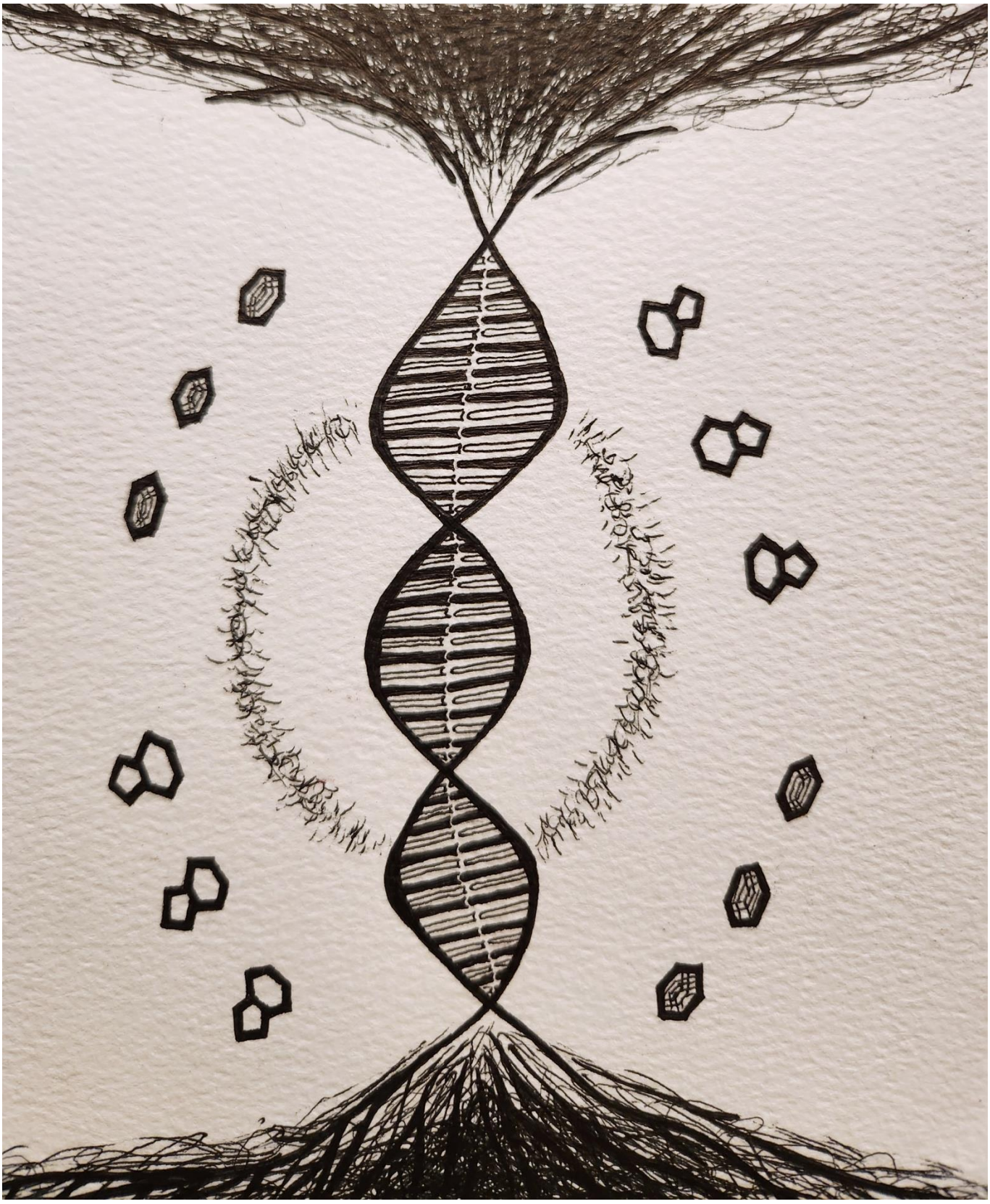

A Review of the Influences of Sex Chromosomes on the Lifespans of Male and Female Humans

Zoe Rodrigues

Across the word, human females tend to live longer than males. Though numerous factors affect lifespan, research has indicated that there may be a sex-chromosome-related reason for this consistent difference in lifespan. Currently, there are three main theories explaining how sex chromosomes may influence lifespan: the unguarded X hypothesis, the toxic Y hypothesis, and the loss of Y (LOY) chromosome hypothesis. The unguarded X hypothesis explains how the possession of a second X chromosome may protect females from body-wide expression of potentially harmful X-linked alleles. Conversely, males express all harmful X-linked alleles since their singular X chromosome would be expressed in all their cells. The toxic Y hypothesis theorizes that the Y chromosome may express satellite DNA sequences, which have been found to contribute to fatal disease development. The loss of the Y chromosome considers that age-related loss of Y chromosomes in male cells may hinder immunosurveillance and thereby contribute to a lower average male lifespan. Though all of these explanations likely contribute to the lifespan discrepancy between males and females, evidence suggests that the possession of a Y chromosome may diminish male lifespan to a more significant extent than the possession of two X chromosomes may benefit female lifespan, thus suggesting that the Y chromosome is primarily responsible for the difference in male and female lifespans.

Introduction

Across the world's numerous cultures and lifestyles, females, on average, consistently outlive males (Bronnum-Hansen & Juel, 2001; Lindahl-Jacobsen et al., 2013; United Nations, 2022). In 2019, the global female life expectancy at birth, which is the average of recorded female lifespans, was approximately 5.4 years higher than the global life expectancy of males at birth, with females having an average life expectancy of 73.8 years and males having an average life expectancy of 68.4 years (United Nations, 2022). Though this pattern has been observed at every location and period capable of being analyzed for human life expectancy (Human Mortality Database, 2022; United Nations, 2022), the exact biological reason for this phenomenon has yet to be elucidated. Understanding the biological explanation for the lifespan differences between the sexes in humans could expand our understanding of aging and longevity, which thus could provide information on how to increase human lifespan.

Such lifespan discrepancies between the sexes are not an isolated observation. Across the tree of life, the homogametic sex, or the sex with two of the same sex chromosomes, consistently outlives the heterogametic sex, or the sex with two different sex chromosomes (Xirocostas et al., 2020), which thereby indicates that sex chromosomes may somehow influence longevity. In humans, females are the homogametic sex, in that they have two X chromosomes, and males are the heterogametic sex, possessing an X chromosome and a Y chromosome (reviewed in Bachtrog et al., 2014). Many species utilize this same X/Y sex determination system, but others, such as birds, which instead use a Z/W sex chromosome system, use a different sex determination system (reviewed in Bachtrog et al., 2014). In birds, the females are the heterogametic sex and possess a Z and W sex chromosome, while males are the homogametic sex and have two Z chromosomes (reviewed in Bachtrog et al., 2014). Regardless of the sex determination system used by a species, the homogametic sex tends to outlive the heterogametic sex (Xirocostas et al., 2020). Thus, the finding that the homogametic sex lives longer than the heterogametic sex is not unique to the human species or the X/Y sex determination system, ultimately suggesting that there is an intrinsic biological reason for these longevity differences and that this reason may be associated with the possession of heteromorphic and homomorphic sex chromosomes, independent of sex. Significantly this finding conveys that it is not just females that live longer across species, but rather the sex with two identical sex chromosomes.

While lifespan differences between the sexes are likely impacted by external factors such as environment and socialization, evidence shows that sex chromosomes may influence lifespan. In a study of human male and female survival during epidemics and famines, it was found that females, even in infancy, exhibit higher survival than males (Zarulli et al., 2018). Additionally, as previously mentioned, in ZZ/ZW species and XX/XY species, the homogametic sex outlives the heterogametic sex, regardless if it corresponds with the male or female sex (Xirocostas et al., 2020). These observations suggest the presence of an inherent biological cause for longevity differences, independent of

environment and social effects because the infants shared the same environment, differences in socialization at infancy are very small, and the lifespan advantage of the homogametic sex does not appear to depend on an individual's identity as male or female.

Based on observations in other organisms, the consistent discrepancy between the lifespans of the heterogametic and homogametic sex in humans, which are males and females respectively, stems from the advantages granted by the possession of two X chromosomes and the complications that accompany the possession of a Y chromosome. In this review, three major models of sex chromosome influence on longevity are discussed: the unguarded X hypothesis, the loss of the Y chromosome, and the toxic Y effect. Here, I propose that, while both the possession of two X chromosomes and the possession of an X and a Y chromosome do impact the lifespans of females and males respectively, the largest reason for this discrepancy in longevity comes from the deleterious effects, such as an increased risk for disease development, that derive from the Y chromosome rather than the benefits of having two X chromosomes.

Sex Chromosomes Influence Lifespan

Factors unrelated to sex chromosomes, such as sex-specific behavior and gonad-associated hormones, can influence the lifespans of the sexes (Holmboe et al., 2015; reviewed in Wingard, 1984). Therefore, not all lifespan differences between the sexes solely stem from chromosome-related reasons. In some species, male-to-male aggression can lead to severe injuries and potential death (reviewed in Pandolfi et al., 2021) and, although such behavior is not as common in humans, similar sex-specific behavior could potentially decrease human males' lifespan. For example, in humans, males often engage in more reckless behaviors, such as speeding, that could lead to early death and thus a decreased lifespan (Arnett, 1996; George et al., 2006). However, while behavior differences between the sexes may impact lifespan, this discrepancy between the homogametic and heterogametic sex's lifespan persists in mice, regardless of gonad type, which suggests that differences in sex chromosomes likely contribute to lifespan differences, independent of phenotypic sex and gonadal influence (Davis et al., 2019).

Two X chromosomes are associated with higher survival in either sex

To independently elucidate the impacts of phenotypic sex and sex chromosomes on lifespan, a study used four core genotypes in mice so that the development of testes, and thus the assignment of a mouse as male, did not depend on the Y chromosome (Davis et al., 2019). As a result, they were able to produce mice with ovaries, or phenotypically female mice, with two X chromosomes as well as phenotypically female mice with one X chromosome and one Y chromosome. They also produced mice with testes, or phenotypically male mice, with two X chromosomes as well as males with one X chromosome and one Y chromosome. They monitored mouse survival and compared survival between mice with differing phenotypic sexes and sex chromosomes. This separation of sex from sex chromosomes allowed them to determine if observed discrepancies in survival derived from differences in sex chromosomes or differences in gonads (Davis et al., 2019).

When the authors compared the survival of XX mice with ovaries and XX mice with testes, the ovary-bearing mice exhibited higher survival than those with testes (Davis et al., 2019). However, there was no notable difference in survival between XY mice with testes and XY mice with ovaries (Davis et al., 2019). Therefore, since there was only a significant survival difference between mice with testes and mice with ovaries when both groups had XX sex chromosomes but not when both groups had XY sex chromosomes, the results suggest that the effects of female gonads and XX sex chromosomes may interact to bolster survival of XX females relative to their XX male counterparts, thereby conveying the presence of both an effect of XX sex chromosomes on lifespan as well as a gonadal effect, possibly through the influence of hormones produced by gonads (Davis et al., 2019). These observations also show that the effects of phenotypic sex possibly interact with the effects of XX sex chromosomes and contribute to survival differences, since the possession of testes, or the assignment of a mouse as phenotypically male, could prompt male-male aggression or competition for resources. Such violence and competition are more common in males and occur consistently, regardless of environment, which therefore could increase mortality relative to females, who more often exhibit violence only to defend offspring (reviewed in Pandolfi et al., 2021).

Knowing that XX sex chromosomes were associated with increased survival in mice with ovaries, the authors wanted to know if this advantage occurred consistently across a lifetime, specifically from 12 to 30 months of age (Davis et al., 2019). For mice with ovaries, those with XX chromosomes exhibited higher survival than those with XY

chromosomes in the latter half of the study period, towards old age (Davis et al., 2019). Interestingly, for mice with testes, those with XX chromosomes had higher survival than those with XY chromosomes in the earlier half of the study period, towards middle age (Davis et al., 2019). Because there was an increase in survival later in life, having two X chromosomes and ovaries was associated with increased lifespan, but having XX chromosomes and testes did not affect lifespan since the survival increase occurred solely during middle age and failed to increase survival at old age, which is how longevity is defined (Davis et al., 2019). These findings support that sex chromosomes could contribute to the consistent discrepancy between the lifespans of males and females in species, including humans, since XX individuals with ovaries exhibited extended lifespans compared to XY individuals with ovaries. These two groups were the same phenotypic sex, which suggests that sex chromosomes, independent of gonad differences, were able to influence survival.

The finding that the XX genotype demonstrated increased survival relative to those with the XY genotype solely in the middle years of a male mouse's life was somewhat unexpected, but it still demonstrates that the possession of two X chromosomes is correlated with increased survival, regardless of phenotypic sex, since it demonstrates that, like female XX mice, male XX mice also had increased survival relative to their XY counterparts at some point in their lifespan. It is possible that this unexpected finding in male XX mice could be explained by sex-specific behavior, like male-male aggression, or other influences related to phenotypic sex that may have led to early mortality and prevented XX sex chromosomes from increasing survival during old age, and thus increasing lifespan, in males like they appear to do in females. Therefore, this suggests that some gonadal influence or sex-specific behavioral influence likely affected the longevity of these mice and that the effects of these factors simultaneously interacted with sex chromosome influences. However, further research is required to determine if these sex-chromosome-associated differences in survival are due to the presence of the two X chromosomes increasing survival and potential longevity or the presence of one Y chromosome increasing mortality and thus decreasing lifespan.

Impact of the X Chromosome on Lifespan

The fact that females have two X chromosomes, or are homogametic, while males have one X chromosome and one Y chromosome, or are heterogametic (reviewed in Bachtrog et al., 2014), suggests that a difference in X chromosome number could contribute to lifespan differences. Although females inherit two X chromosomes, one from each parent, each cell only expresses one X chromosome due to X chromosome inactivation (XCI), whereas, in males, their single X chromosome is always expressed (reviewed in Marais et al., 2018). XCI occurs only in females and causes one X chromosome to be silenced in each cell so that each cell only expresses one X chromosome (reviewed in Panning, 2008). However, XCI occurs randomly so that, within a single female, some cells express the paternal X chromosome, while others express the maternal X chromosome (reviewed in Panning, 2008).

The unguarded X hypothesis describes how random XCI could offer an advantage to females, because, even if one of their X chromosomes carries a deleterious allele, it would not be expressed consistently throughout their bodies, since some cells will have silenced that chromosome and therefore not express that allele (reviewed in Marais et al., 2018). As a result, compared to males, who do not experience XCI, females would have fewer cells expressing harmful phenotypes stemming from the deleterious alleles of either parental X chromosome, since neither X chromosome is ubiquitously expressed throughout an individual. Conversely, because they only have one X chromosome, males are considered to have an unguarded X chromosome that is activated in all cells (reviewed in Marais et al., 2018). Males are thus more likely to express and be more severely affected by deleterious alleles that reside on their X chromosome since these alleles are always expressed throughout their bodies (reviewed in Marais et al., 2018). Indeed, this supposition has been supported by the observation that men exhibit X-linked diseases like Duchenne muscular dystrophy at greater severities than females (reviewed in Sun et al., 2022). Notably, there are more than 500 X-linked diseases that impact males to a greater extent than females (reviewed in Sun et al., 2022).

XCI skewing is associated with poor health and decreased lifespans

The longevity associated with having two X chromosomes seems to rely on "even" XCI (Gentilini et al., 2012), in which half of a female's cells silence the paternal X chromosome, or the X chromosome inherited from her father, and the other half silence the maternal X chromosome, or the X chromosome inherited from her mother. A divergence from a 50% distribution of cells expressing either X chromosome is considered XCI skewing (reviewed in Sun et al.,

2022). Higher XCI skewing is associated with quicker aging, poorer health, and decreased lifespan (Gentilini et al., 2012).

The correlation between XCI skewing and decreased longevity suggests the possible presence of the unguarded X hypothesis, which proposes that females' extended lifespans stem from XCI decreasing the degree of X-chromosome-associated deleterious allele expression (reviewed in Marais et al., 2018). Therefore, XCI skewing would cause one X chromosome to be expressed more frequently than if both X chromosomes were expressed at a 50:50 ratio, which would increase the number of cells expressing harmful mutations or alleles that could diminish health and thus decrease longevity. For example, in a prior study, a female carrier of severe hemophilia A and moyamoya syndrome (SHAM) exhibited more severe symptoms, including hemophilia, relative to her carrier mother who exhibited no such symptoms; this difference was found to be partly caused by skewed XCI, which caused the daughter to express the disease in a greater number of cells than her mother (Janczar et al., 2016). Therefore, an increase in XCI skewing in females could lead to decreased health and decreased lifespan relative to females with less XCI skewing, which ultimately supports the ideas underlying the unguarded X hypothesis by suggesting that XX sex chromosomes could potentially confer a biological advantage to females by diminishing the extent of X-chromosome associated deleterious allele expression in females relative to males.

Notably, in a study of a southern Italian population, there were distinctly more female centenarians—people older than 100 years old— than male centenarians, with a ratio of 2.2 female centenarians for every male centenarian (Passarino et al., 2002). Similar patterns were observed in other populations, though ratios varied (Deiana et al., 1999; Ribeiro et al., 2016). To analyze the influence of XX sex chromosomes on this distinct bias in longevity, a study measured the degree of XCI skewing (DS) for four age groups: centenarians, the offspring of centenarians, the offspring of non long-lived parents, and young women (Gentilini et al., 2012). Due to the age of the centenarians, it was not possible to obtain a control group that would allow the authors to determine the genetic reasons behind the centenarians' longevity and relative health (Gentilini et al., 2012). However, the children of centenarians previously exhibited higher survival and healthier aging, with fewer health problems, than the children of non long-lived parents (Terry et al., 2004), thus suggesting that the offspring of the centenarians could be used to study how XCI influenced longevity and healthy aging. Therefore, they compared the DS of centenarians' offspring with the DS of the non long-lived parents' offspring, since they were all in their late 60s to early 70s and thus comparable in age (Gentilini et al., 2012). They also analyzed the DS of young women that were considerably younger than the other groups, at an average age of 31.2 years old, to investigate the relationship between age and XCI skewing (Gentilini et al., 2012).

The study found that female centenarians exhibited significantly higher DS than both their offspring and young women, but the DS of female centenarians did not significantly differ from the DS of offspring of both non long-lived parents (Gentilini et al., 2012). However, they also found that centenarian females tended to have a lower amount of XCI skewing than would be expected given their age since XCI was also found to increase with age. Because XCI increased with age, centenarians should have had significantly higher XCI than the offspring of non long-lived parents, which were younger than them, but they instead exhibited similar XCI skewing, with no significant difference in DS between the two groups. Although both groups were similar in age, the group containing the children of non long-lived parents exhibited significantly higher XCI skewing than the group consisting of the offspring of centenarians. Significantly, the centenarians' offspring displayed a DS similar to the DS of young females, even though, on average, they differed in age by roughly 30 years (Gentilini et al., 2012).

This study ultimately allowed the authors to conclude that lower degrees of XCI skewing possibly contribute to healthy aging and longevity, as DS was found to normally increase with age, but the long-lived centenarians had a DS similar to the offspring of non long-lived parents who were approximately 30 years younger than them, and the centenarian offspring exhibited a similar DS to young women, who were roughly 30 years their junior (Gentilini et al., 2012). Also, the fact that both centenarians and their offspring displayed unexpectedly low DS given their ages suggests that there may be a genetic component to healthy aging since skewed XCI was previously connected to health problems such as the onset of hemophilia in carrier females (Janczar et al., 2016). Additionally, the fact that the offspring of centenarians had XCI skewing like that of young females appears to support the unguarded X hypothesis, as the hypothesis posits that the inactivation of each X chromosome in equal amounts of cells diminishes the expression of deleterious alleles from both X chromosomes, ultimately assuaging their potentially harmful effects. This prediction was reflected in the results, since the offspring of centenarians displayed few health

problems compared to their peers—the children of non long-lived parents—and displayed relatively low degrees of XCI skewing (Gentilini et al., 2012), which suggests that lower DS was associated with relatively good health and that this lack of skewing led to neither X chromosome being expressed more than the other, ultimately lessening the expression and effects of any harmful mutations or alleles on either X chromosome. However, further research is needed to definitively determine if XCI directly contributes to lifespan differences between males and females.

Evidence against the unguarded X hypothesis

Since heterogametic individuals are more likely to express deleterious genetic content due to a lack of alternate alleles on a second X chromosome (reviewed in Marais et al., 2018), it would therefore be expected that the heterogametic sex—males in humans—are more likely to express survival-diminishing mutations. However, in a previous study, authors utilized population genetic models of the unguarded X hypothesis which ultimately demonstrated that the effects predicted by the unguarded X hypothesis do not create enough lifespan discrepancies to sufficiently explain the large difference in mortality between the sexes (Connallon et al., 2022).

By using a simple population model in combination with parameter estimates of effects and mutation rates, the authors determined that the impact of the unguarded X chromosome in the heterogametic sex accounted for only a 1-2% higher lifespan in female mammals compared to male mammals (Connallon et al., 2022). Considering that previous studies of longevity have observed a roughly 20% increase in female lifespan compared to male lifespan (Lemaître et al., 2020; Xirocostas et al., 2020), the results of this mathematical study suggest that, alone, the unguarded X chromosome does not sufficiently explain this lifespan difference. This leaves 18-19% of the differences between male and female lifespans unexplained. Ultimately, this large difference between the calculated lifespan differences for male and female mammals and the measured lifespan differences of mammals suggests that, if the unguarded X hypothesis contributes to lifespan differences between the sexes, at least one additional phenomenon must also influence lifespan.

This inability of the unguarded X hypothesis to sufficiently explain sex-specific longevity differences has also been found in experiments of the model organism, *Drosophila melanogaster*, a species of fly (Brengdahl et al., 2018). Since the unguarded X hypothesis suggests that males exhibit decreased lifespans because they always express the genes on their single X chromosome even if they are deleterious (reviewed in Marais et al., 2018), it is predicted that inbreeding of the homogametic sex should decrease the sex's longevity, since inbreeding would cause the homogametic sex to inherit two identical sex chromosomes, thus mimicking the heterogametic sex by always expressing the same X-chromosome alleles in every cell.

To test this prediction, researchers inbred female flies so that each female fly had two genetically identical X chromosomes, which meant they expressed their X chromosomes and any X-linked recessive deleterious alleles to the same extent as their male heterogametic counterparts, essentially expressing a single allele of each gene of the X chromosome (Brengdahl et al., 2018). Per the unguarded X hypothesis, this inbreeding was expected to decrease female lifespan compared to outbred females that had two different X chromosomes. Notably, they found no significant difference in longevity between females that were inbred for their X chromosomes and females that were outbred for their X chromosomes (Brengdahl et al., 2018). This finding contradicts the predictions made by the unguarded X hypothesis, thus indicating that males' unguarded X chromosomes may have little influence on longevity differences between the sexes. Specifically, because inbred females with two genetically identical X chromosomes and outbred females with two different X chromosomes did not differ in terms of lifespan, this suggests that a lack of X-chromosome-related genetic variation—like that of males who only have one X chromosome—did not lead to diminished health and longevity. Therefore, even if inbreeding led to the ubiquitous expression of recessive deleterious alleles such as those associated with pathogenesis, the effects of these alleles were not severe enough to create a notable decrease in lifespan in inbred females. Thus, based on this data, if the unguarded X does influence lifespan, these influences are likely mild and not great enough to create the previously observed lifespan discrepancies between sexes in numerous species, including humans.

Unexpectedly, in the same experiment, males outlived both inbred and outbred females (Brengdahl et al., 2018), countering previous observations that females exhibit longer lifespans than males in numerous species (Xirocostas et al., 2020). The authors considered this unexpected observation a result of female exposure to young males, as the presence of males is correlated with decreased female lifespan, at least partly due to harm done to females during mating (Friberg, 2005; Partridge et al., 1987). However, the authors deemed the males' higher

lifespan irrelevant to the study since the predictions made by the unguarded X hypothesis were primarily concerned with lifespan differences between inbred and outbred females (Brengdahl et al., 2018).

It is important to consider that *D. melanogaster* utilizes a different dosage compensation mechanism than humans. In *D. melanogaster*, males double the expression of their single X chromosome to match the levels of X chromosome gene expression from the two X chromosomes of the females (reviewed in Ferrari et al., 2014). This mechanism contrasts with how, in humans, females undergo XCI so that only one X chromosome is expressed in each cell (reviewed in Ferrari et al., 2014), thereby matching the amount of X chromosome expression that stems from the single X chromosome in males. Typically, in flies, for any X-linked gene, there could be two different alleles, one from each X chromosome, being expressed in all the female cells, but, in this study, inbred flies had two identical X chromosomes and therefore, for any gene on the X chromosome, they expressed only one allele, albeit at a doubled level, in all their cells. This organism-wide expression of a single allele for genes on the X chromosome mimicked the expression levels of male flies, which double the expression of the alleles on their single X chromosome, ultimately mirroring the number of X-linked alleles that are expressed in human female cells as each cell of a human female only expresses the alleles of one X chromosome. As a result, these findings could potentially be extended to humans, but we must exercise caution and remember that these sex chromosome systems are not identical. Regardless, this study demonstrated that, alone, the unguarded X hypothesis does not sufficiently account for the longevity differences between male and female flies, which, in conjunction with the statistical findings that the effects of the unguarded X hypothesis are not large enough to explain the observed mammalian lifespan differences, suggests that the unguarded X hypothesis is likely not responsible for most of the sex-specific lifespan differences in humans.

Impact of the Y Chromosome on Lifespan

While previous data provided potential evidence that the possession of two X chromosomes contributes to the lifespan differences between the sexes, the possession of a single Y chromosome could also influence these lifespan discrepancies, possibly by contributing to increased male mortality relative to females. Thus, though the unguarded X hypothesis is a possible mechanism that promotes sex-specific differences in lifespan, it is only one mechanism among many. Two main models that delineate the Y chromosome's possible impact on lifespan are the loss of Y chromosome model and the toxic Y effect (reviewed in Marais et al., 2018), both of which describe potential mechanisms by which the Y chromosome decreases male longevity by triggering pathogenesis and diminishing male health.

Mosaic loss of the Y chromosome is connected to disease risk

Mosaic loss of the Y chromosome, or LOY, in which cells lose their Y chromosomes, becomes more common in males as they age (Jacobs et al., 1961; Pierre & Hoagland, 1972). LOY results in aneuploidy, or an abnormal number of chromosomes, in affected cells because it causes them to lack a Y chromosome; however, not all cells are affected, which is why this loss is often considered "mosaic" (reviewed in Barros et al., 2020). In the peripheral blood of human males, LOY is the most common genetic variant in somatic cells, and it is associated with a heightened risk of all-cause mortality as well as mortality associated with non-hematological cancer, or non-blood cancers; therefore, men with higher degrees of Y chromosome are at greater risk of death (Forsberg et al., 2014). In addition to an increased risk of cancer and all-cause mortality, LOY is also correlated with the pathogenesis of Alzheimer disease, one of the most common neurodegenerative diseases (Dumanski et al., 2016). Even when data was controlled for confounding variables such as age, males with Alzheimer disease had higher LOY than those without (Dumanski et al., 2016). Therefore, LOY is at least associated with mortality specific to males, and its presence may indicate an increased risk of death. If LOY was the cause of this disease development and mortality, it would solely affect males, since females do not have Y chromosomes to lose, and this could explain the discrepancy between male and female lifespans.

Numerous types of sex-unspecific cancers are more prevalent in males than in females such as urinary tract cancer, esophagus squamous cell carcinoma, and stomach cancer (Cook et al., 2011; Radkiewicz et al., 2017). Notably, a study that examined sex-specific risk for 39 types of human cancers found that males had a higher risk of developing 34 of the 39 cancers relative to females (Radkiewicz et al., 2017). The higher risk of cancer in males compared to females supports that the increased risk of cancer could be partly due to LOY since only males exhibit LOY, and it is known that LOY is associated with cancer. Additionally, in most cancer types, cancer development frequency appeared to generally increase with age for both sexes, but the

occurrence of cancer in males increased more quickly with age than in females (Radkiewicz et al., 2017).

Significantly, a study of LOY in the peripheral blood of males found that, as males age, the number of cells exhibiting LOY increases (Forsberg et al., 2014). Using statistical analysis, the authors developed curves that allowed them to depict how survival probability associated with all-cause mortality and survival probability associated with cancer-related mortality changed over time and differed between males with LOY in more than 35% of nucleated blood cells and males with LOY in less than 35% of blood cells (Forsberg et al., 2014). They also delineated how survival probability related to non-hematological cancer mortality changed over time and differed between the same two groups of males exhibiting LOY in less than and greater than 35% of their blood cells (Forsberg et al., 2014). The trends observed for all three categories of mortality showed that males with a higher percentage of cells exhibiting LOY had lower survival probabilities and survived fewer years after sampling than individuals with lower percentages of blood cells with LOY (Forsberg et al., 2014). This finding expresses that LOY may impact survival. Since higher degrees of LOY were associated with both cancer and mortality, LOY may contribute to disease development and therefore increase male mortality compared to females. However, LOY could also be a side effect of a mortality-inducing factor rather than the cause of increased mortality. Further research must be done to determine the nature of the relationship between LOY and this observed increase in mortality.

The toxic Y effect decreases male lifespan

Another possible mechanism by which the Y chromosome could negatively impact male health and promote lifespan differences between males and females is the toxic Y hypothesis, which explains how the genetic contents of the Y chromosome promote decreased health through the expression of harmful sequences, such as transposable elements (TEs), that can cause mutations and genomic rearrangements (reviewed in Marais et al., 2018). Additionally, the human Y chromosome likely possesses many mutations (reviewed in Bachtrog & Charlesworth, 2001), and, when these mutations are expressed, they could negatively impact cells by impeding regular cell function. The human Y chromosome also contains numerous repeat sequences (reviewed in Bachtrog & Charlesworth, 2001). Repeat sequences are noncoding DNA sequences, and though there are numerous types with various functions, some repeat sequences, such as satellite DNA sequences, can damage the cell by inhibiting DNA damage response and tumor suppression (reviewed in Ugarković et al., 2022).

The presence of this toxic Y effect in humans could be supported by a study that found that people with XXY sex chromosomes, or Klinefelter syndrome, on average, displayed a 2-year decrease in lifespan compared to control individuals with XY sex chromosomes, while individuals with XYY sex chromosomes exhibited a 10-year decrease in lifespan compared to control individuals (Stochholm et al., 2010). In this study, there was increased all-cause mortality in these XYY individuals, which included an increased risk of mortality from cancer and respiratory diseases (Stochholm et al., 2010). These observations could be associated with LOY, the toxic Y effect, the unguarded X hypothesis, or another XX benefit, but the shorter lifespan of people with two Y chromosomes, or the XYY genotype, compared to those with one Y chromosome, or the XXY genotype, more directly denotes the possible presence of the toxic Y effect, since individuals with two Y chromosomes lived shorter lives than those with one. This association between Y chromosome number and decreased longevity could potentially demonstrate how the "toxic" alleles, mutations, or repetitive sequences that reside on the Y chromosome could decrease male health, because, if these "toxic" sequences were to be expressed and permitted to initiate DNA damage and pathogenesis, people with more Y chromosomes, and thus more harmful sequences, should have shorter lifespans and develop more diseases than those with fewer Y chromosomes. However, even if the effects of other mechanisms, such as LOY or the unguarded X hypothesis, contributed to the observations made in this study of XYY and XXY individuals, that does not necessarily negate the study's ability to provide evidence of the toxic Y effect, as these mechanisms are not exclusive, and all could simultaneously decrease male lifespan.

The toxic Y effect decreases male health through TE expression

Evidence of the toxic Y effect has not been found in humans quite yet, but the phenomenon has been observed in other species such as *Drosophila miranda*, a species of fruit fly (Nguyen & Bachtrog, 2021). Like humans, flies utilize the X/Y chromosome sex determination system. The Y chromosomes of flies and humans are both non-recombining chromosomes and therefore accumulate repeat sequences and tend to be highly heterochromatic (reviewed in Bachtrog, 2013). However, there are significant morphological differences between the sex chromosomes of flies

and those of humans since they have evolved independently (reviewed in Bachtrog, 2013). Male *D. miranda* possess recently evolved neo-Y chromosomes that are "toxic," meaning they contain TEs and repetitive sequences and also poorly silence these elements and sequences (Nguyen & Bachtrog, 2021). When expressed, TEs, which are DNA sequences that can mobilize and insert themselves into various points in the genome, can alter the genome and result in large chromosomal rearrangements, potentially disrupting overall gene function (reviewed in Saleh et al., 2019).

In a study of *D. miranda*, males exhibited higher TE expression than females, which supports the idea that the presence of the neo-Y in males, which is known to contain many TEs, may lead to differing levels of TE expression between the sexes (Nguyen & Bachtrog, 2021). Even though the heterochromatin content of some chromosomes, including the neo-Y, was previously found to be lost with age, this did not affect TE expression, which was constant across ages in both sexes (Nguyen & Bachtrog, 2021). This consistently higher TE expression in males was explained by a high number of TEs on the neo-Y and a low amount of heterochromatin on the neo-Y from a young age, which was surprising due to the many repeat sequences on the neo-Y that were expected to be silenced via heterochromatin formation (Nguyen & Bachtrog, 2021). Thus, not only were these TEs insufficiently silenced due to inadequate heterochromatin formation on the neo-Y, but the neo-Y chromosome also contained an overabundance of TEs, and these two factors ultimately combined to contribute to sex-specific differences in TE expression (Nguyen & Bachtrog, 2021). Furthermore, when *D. Miranda* survival was monitored over time, males exhibited lower survival than females as they aged and therefore males had shortened lifespans (Nguyen & Bachtrog, 2021). These results continue to align with previous findings and patterns found across numerous species (Xirocostas et al., 2020). This insufficient silencing and larger concentration of TEs in male flies, in conjunction with the observed differences in survival between male and female flies, suggests that the genetic contents of *D. miranda*'s Y chromosome decrease male lifespan, which could ultimately indicate that the presence of the toxic Y effect leads to more TE expression that could decrease health and thereby diminish male longevity.

The neo-Y chromosome structure may also impede a fly's ability to form heterochromatin and thus silence repeat sequences on the neo-Y, thereby allowing the consistent TE expression that was observed in both young and old males (Nguyen & Bachtrog, 2021). For example, the heterochromatic regions of the neo-Y of *D. miranda* are embedded with actively transcribed genes, and the transcription of these genes may inhibit heterochromatin formation (Nguyen & Bachtrog, 2021). If this transcription prevents sufficient heterochromatin formation, it could explain why TE expression was consistent with age as, if there is little heterochromatin to start with, heterochromatin loss will not drastically affect TE expression. This hindrance of heterochromatin formation shows another way that the sequences of the Y chromosome could generate a toxic Y effect that impedes male health and increases mortality. However, while the toxic Y effect is present in *D. miranda* and occurs through TE expression, further research is required to determine if humans experience the effects of the toxic Y effect.

Heterochromatin loss diminishes male health by allowing satellite DNA expression

Since they found that TE expression was not affected by heterochromatin loss, the authors of the same study of *D. miranda* tested other types of repeat sequences, such as satellite DNA sequences, to see if they were affected by this loss (Nguyen & Bachtrog, 2021). It is important to note, however, that these sequences are not abundant on the neo-Y (Mahajan et al., 2018) and cannot reflect the presence of the toxic-Y effect in *D. miranda.* To determine how heterochromatin content at satellite sequence-containing regions of DNA differed between ages and sexes, they analyzed these regions' sex-specific and age-specific enrichment of H3K9me3 (Nguyen & Bachtrog, 2021). H3K9me3 is a histone protein modification that denotes the presence of heterochromatin (reviewed in Becker et al., 2016). When comparing young and old females, there were no significant differences in heterochromatin enrichment at regions containing satellite DNA sequences. However, old males displayed significantly less heterochromatin enrichment than young males (Nguyen & Bachtrog, 2021). From this data, we can conclude that, at DNA regions bearing satellite sequences, heterochromatin is lost with age in males but not in females, which aligns with previous findings that heterochromatin loss with age is more distinct in male chromosomes. However, the finding that heterochromatin loss occurs at regions containing satellite sequences, suggests that an increase in satellite expression could occur with age, as the heterochromatin responsible for silencing these sequences is lost.

To investigate if satellite DNA expression differed between the sexes, the authors compared the satellite expression of old males and old females as well as the satellite expression of young males and

young females (Nguyen & Bachtrog, 2021). They identified little difference in the satellite expression of young males and young females, since there was an equal number of satellite sequences expressed in either sex (Nguyen & Bachtrog, 2021). However, there was a noticeable difference in satellite expression between old males and old females, with old males exhibiting higher satellite expression (Nguyen & Bachtrog, 2021). When comparing satellite expression between ages, there was higher satellite expression in old males than young males, with 21 satellite sequences exhibiting increased expression amongst older males (Nguyen & Bachtrog, 2021). There was no significant difference between the expression of old and young females (Nguyen & Bachtrog, 2021). From these observations, the authors concluded that not only do old males have low heterochromatin enrichment at satellite-sequence-containing DNA regions, but they also express satellite sequences more frequently than young males, young females, and old females (Nguyen & Bachtrog, 2021). Therefore, since heterochromatin is lost from male chromosomes with age, this heterochromatin loss may promote the expression of satellite DNA sequences and increase the potential for harmful effects such as tumor development and DNA damage (Kishikawa et al., 2016; Miyata et al., 2021). While the finding that satellite sequence expression increased with heterochromatin loss does not indicate the presence of the toxic Y effect in flies, since few satellite sequences reside on the neo-Y of *D. miranda*, human Y chromosomes do contain a considerable amount of satellite sequences (Bachtrog & Charlesworth, 2001), with many that occur in heterochromatic areas (Altemose et al., 2014). Therefore, these findings derived from flies may delineate a possible mechanism by which the toxic Y effect occurs in humans.

Mechanisms by Which the Y Chromosome May Decrease Male Lifespan

Mechanism 1: Heterochromatin loss promotes the toxic Y effect

Like the *D. miranda* neo-Y, the human Y chromosome possesses numerous repetitive DNA sequences, but, unlike the *D. miranda* neo-Y, it has an abundance of satellite DNA sequences (Altemose et al., 2014). The abundance of satellite DNA contributes to the toxic Y effect upon expression due to its associations with pathogenesis and DNA damage (reviewed in Ugarković et al., 2022). In humans, heterochromatin loss is observed in patients with Hutchinson–Gilford Progeria Syndrome (HGPS), a disease characterized by premature aging (Shumaker et al., 2006), which indicates the possibility that heterochromatin loss is correlated with aging in humans. Additionally, Q-heterochromatin, a type of heterochromatin only present in humans, is found in seven autosomes as well as the Y chromosome ("Paris Conference (1971), Supplement (1975) Standardization in Human Cytogenetics," 1975). Q-heterochromatin regions on autosomal chromosomes are less abundant in older males than in younger males (Ibraimov et al., 2014), suggesting that heterochromatin may be lost with age, just like in flies. Furthermore, if this difference in heterochromatin abundance does demonstrate autosomal heterochromatin loss, males appear to exhibit more drastic Q-heterochromatin loss with age when compared to females (Ibraimov et al., 2014). Because the Q-heterochromatin on autosomes may be lost with age, the Q-heterochromatin on the Y chromosome could also be lost with age. In flies, the loss of heterochromatin allows the derepression of satellite DNA expression across the genome (Nguyen & Bachtrog, 2021). Since Q-heterochromatin may be lost with age and the human Y chromosome contains many satellite DNA sequences, the expression of satellite DNA sequences could increase with age.

This potential upregulation of satellite DNA is significant because satellite DNA has many different functions, some of which are harmful (reviewed in Ugarković et al., 2022). For example, in mouse pancreatic precancerous tissues, pericentromeric satellite DNA transcripts prevent the Ybx1 protein from repairing DNA, leading to an increase in mutations and chromosome instability (Kishikawa et al., 2016). In humans, one satellite RNA, which is a transcript of satellite DNA, triggers cancer development by preventing the function of a binding factor called CCCTC-binding factor, thereby provoking the expression of the inflammatory gene associated with tumorigenesis (Miyata et al., 2021). It is also known that demethylated human satellite II DNA and human satellite II RNA respectively sequester PRC1 and MeCP2, two proteins responsible for the regulation of chromatin (Hall et al., 2017). This sequestering gathers these regulatory proteins away from the rest of the nucleus during cancer, thus preventing epigenome regulation and exacerbating the lack of regulation that contributes to cancer (Hall et al., 2017). This potential tumorigenesis and mutagenesis that stem from satellite DNA expression could lead to increased mortality in human males relative to females, because they appear to lose heterochromatin from their Y chromosomes with age (Ibraimov et al., 2014). Moreover, human males have numerous satellite

DNA sequences on the Y chromosome (Altemose et al., 2014), and satellite DNA expression is associated with mortality-increasing health defects (reviewed in Ugarković et al., 2022).

In humans, the loss of Q-heterochromatin on the Y chromosome with age could permit the derepression of satellite DNA sequences, which could lead to cancer development and damaged DNA with age, ultimately creating a toxic Y effect. This phenomenon has potentially occurred in other species and could explain previous observations in which male mice exhibited lower survival with age (Davis et al., 2019) as well as why the heterogametic sex tends to die earlier than the homogametic sex across the tree of life (Xirocostas et al., 2020).

Mechanism 2: LOY impedes immunosurveillance

Another possible mechanism that may work in tandem with heterochromatin-loss-associated satellite DNA expression to decrease human male lifespan is the loss of the Y chromosome (LOY). However, the exact mechanism by which LOY occurs is currently unknown, though genetic variants, structural abnormalities of the Y chromosome, and environment are factors that appear to affect LOY risk (reviewed in Guo et al., 2020). One proposed mechanism by which LOY confers diminished health is through the inhibition of immunosurveillance, which is the process of fighting abnormal cells and preventing disease development (reviewed in Forsberg et al., 2017). This hypothesis is supported by evidence that shows that blood cells exhibiting LOY display lower levels of an immunoprotein important for immunosurveillance called CD99 (Mattisson et al., 2021), which could decrease the cell's ability to carry out immunosurveillance. This decreased immunosurveillance associated with LOY could diminish a cell's ability to suppress tumorigenesis and pathogenesis, which increases disease risk in males, thus possibly increasing male mortality relative to females. Regardless, more research is required to conclusively decide if LOY decreases immunosurveillance and how it operates.

Mechanism 3: The toxic Y effect is prompted by transcription in heterochromatic regions of the Y chromosome

The fact that female infants exhibit higher survival than male infants during epidemics (Zarulli et al., 2018) suggests that Q-heterochromatin loss with age and the subsequent derepression of mutagenic repeat sequences cannot be the only explanations for how sex chromosomes influence lifespan differences since little heterochromatin loss could have occurred during their short lifetimes. In humans, some sequences, such as sequences encoding non-coding RNA, are embedded in the heterochromatic regions of the Y chromosome but are still transcribed (Jehan et al., 2007). Such transcription of heterochromatin-embedded sequences could potentially hinder heterochromatin formation meant to silence repeat sequences, as proposed in a previous study (Nguyen & Bachtrog, 2021). Considering that the human Y chromosome contains TEs known as *Alu* and LINE elements as well as repeat sequences such as satellite DNA sequences (reviewed in Bachtrog & Charlesworth, 2001), it is possible that the combination of these elements with inhibited heterochromatin formation on the Y chromosome leads to high repeat and TE expression from the Y chromosome, even when males are young. Given that satellite DNA sequences are associated with mutations and cancer (reviewed in Ugarković et al., 2022), mutations caused by LINE elements have been found to contribute to over 120 diseases (reviewed in Saleh et al., 2019), and *Alu* elements have been known to inhibit genes responsible for tumor suppression (reviewed in Slebos et al., 1998), the expression of these sequences from the Y chromosome could be detrimental to male health. Therefore, the mere possession of a Y chromosome could be a genetic liability and ultimately lead to decreased male health and longevity, regardless of age.

Conclusion

Human females have consistently out-survived males in nearly all observable periods and regions of the world (Human Mortality Database, 2022; United Nations, 2022). Though numerous environmental factors likely impact sex-specific longevity differences, studies of infant survival during famines and epidemics show that females regularly outlive males, even in similar environments and at young ages when behavior and social interactions do not significantly differ between sexes or impact survival (Zarulli et al., 2018). This ultimately suggests that there is some intrinsic biological factor that contributes to these lifespan differences.

Data suggest that the consistent difference between male and female lifespans could stem from the possession of two X chromosomes in females, which may bolster female health by assuaging the effects of disease-causing alleles associated with the X chromosome. It could also stem from the possession of a single Y chromosome in males, which potentially leads to DNA damage and pathogenesis. Ultimately, it is likely that both the possession of XX sex chromosomes and XY sex

chromosomes influence the lifespans of females and males, respectively, and therefore promote the lifespan discrepancy between the sexes. However, I proposed that any advantages stemming from having two X chromosomes have only minor effects on the lifespan differences between males and females, while the Y chromosome contributes the most to the differences between male and female lifespans.

This paper discussed three prominent models of how sex chromosomes impact the longevity difference between males and females: the unguarded X hypothesis, the loss of the Y chromosome, and the toxic Y effect. Through the analysis of multiple studies, it was found that the unguarded X hypothesis is likely to have only a small role in decreasing male lifespan relative to female lifespans and that the toxic Y effect and loss of the Y chromosome likely decrease male longevity to a more significant extent. Finally, three potential mechanisms by which the Y chromosome may decrease male lifespan were delineated. Two of the three mechanisms propose that the genetic content and nature of the Y chromosome create scenarios that enable the expression of repeat sequences and possibly TEs, which could potentially lead to DNA damage and disease development. The remaining mechanism describes how the loss of the Y chromosome may be connected to an inability to suppress tumor development and therefore eventually result in tumorigenesis and death.

Future studies should further explore the proposed relationships between lifespan and sex chromosomes as well as the proposed mechanisms by which the Y chromosome diminishes male health. Further experimental evidence of these mechanisms and relationships is required to extend many of the proposed findings and relationships from this paper to humans. Though conducting human-based studies is difficult due to ethical and sample-related constraints, further evidence for the presence of the toxic Y effect, loss of Y chromosome effects, as well as the unguarded X hypothesis in humans could be obtained by using model organisms more closely related to humans such as mice or primates.

Searching for further evidence of the relationship between sex chromosomes and lifespan in humans as well as searching for the presence of the proposed mechanisms by which the Y chromosome increases disease propensity is a vital step toward understanding the causes of discrepancies in longevity between males and females. Ultimately, elucidating the influence of sex chromosomes on longevity could provide useful insight into understanding what contributes to longevity and early mortality, ultimately enabling us to better understand human life.

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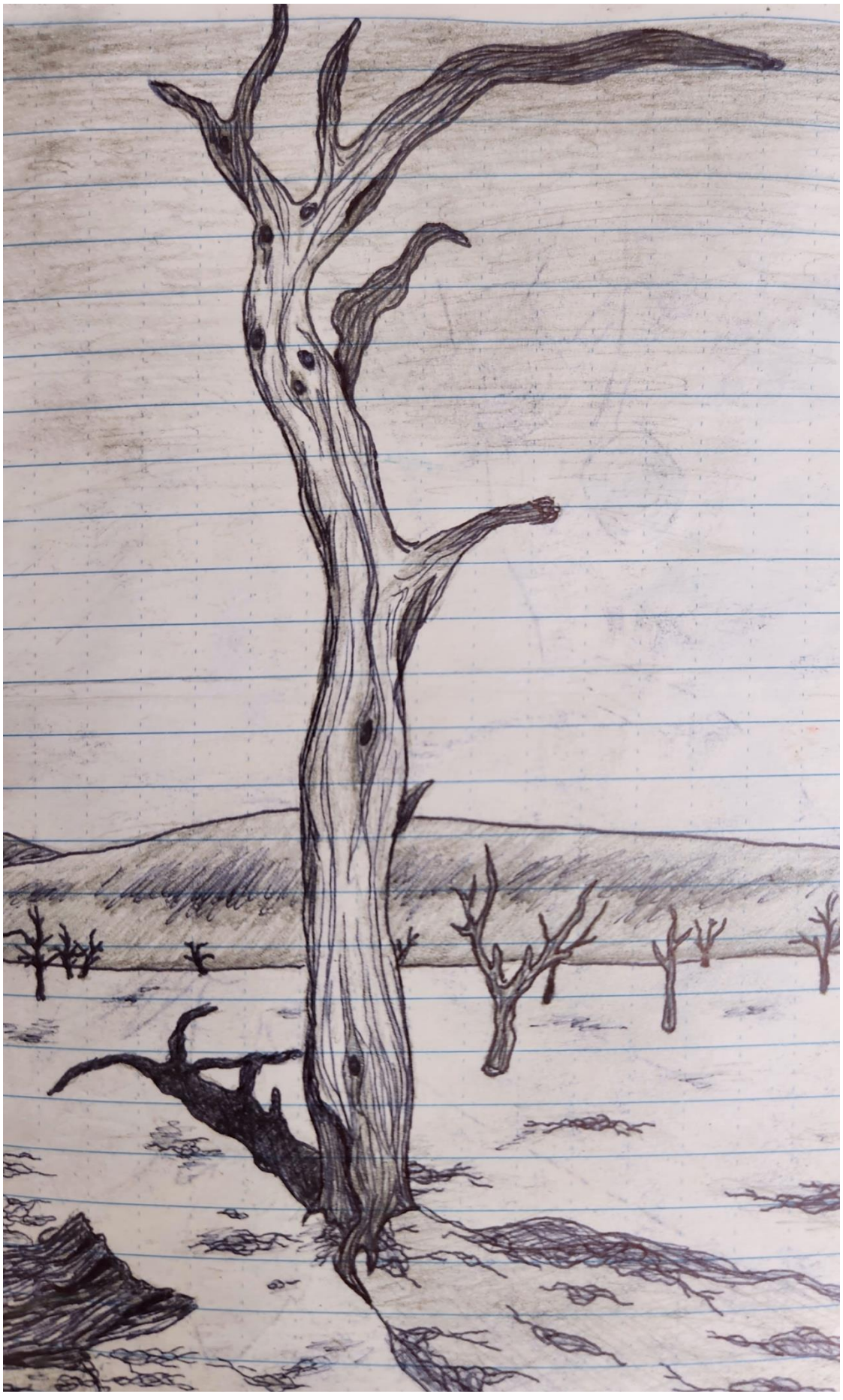

The United Nations Effect: Climate Change Research Methods and the Marginalization of the 2SLGBTQIA+ Community

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This paper reviews the impact of climate change research methods, particularly those of the United Nations, on the marginalization of sexual and gender minorities. There is no doubt that prevailing systems of oppression allow cisgender, heterosexual men to avoid the brunt of certain aspects of climate change. There is some literature on the impact of climate change on women compared to the impact of climate change on men, but there does not seem to be much literature about the impact of climate change on members of the 2SLGBTQIA+ community. Consequently, it is impossible to identify if there is a disproportionate climate change impact on the 2SLGBTQIA+ community. I will cite the lack of or limited research on this problem to highlight systems of oppression that perpetuate the marginalization of sexual and gender minorities. It is vital that research on the discriminatory nature of climate change is extended to sexual and gender minorities outside of white, cisgender, or heterosexual normativity, and that the current lack of research is recognized and addressed to ensure that impacted communities can receive necessary resources.

Climate change is not new to academic discourse. Virtually all of academia has concluded that the planet we live on is rapidly changing and becoming less capable of supporting life (NASA, 2023). What is *comparatively* new to academic discourse, however, is the idea that the severity of the impacts of climate change that are experienced by humans follows the same social stratification with which we have constructed our societies. Despite its novelty, the idea that climate change disproportionately affects those who suffer from socioeconomic inequalities is relatively undisputed.

Most of the climate change research that is available to the public is done at a national level. This gives way to significant inconsistency in climate change information from nation to nation. In their article "Climate change: Does international research fulfill global demands and necessities?" Doris Klingelhöfer, Ruth Müller, Markus Braun, Dörthe Brüggmann, and David A. Groneberg note that the wide array of approaches to climate change research and its publication can make it difficult to find reliability in climate change information, and, by extension, in approaches to mitigate climate change concerns within each country (Klingelhöfer et al., 2020). This wide and relatively unregulated variety highlights the necessity of a unifying force; one that is international by nature. Enter the United Nations, who are at the forefront of international research into climate change and other humanitarian crises (and the *intersection* of climate change and other humanitarian crises) and are arguably the foremost source on global climate change information. A United Nations paper found that climate change inequality is characterized by a cycle: an initial inequality, or the social stratification that causes a certain group to be oppressed, causes disadvantaged groups to suffer greater from the impacts of climate change, which in turn results in greater subsequent inequality. There are three main ways in which this cycle materializes: (1) an increase in exposure to the impacts of climate change, (2) an increase in susceptibility to damage that is caused by climate change, and (3) a decrease in the disadvantaged group's ability to cope with and recover from the climate-related damage suffered (Islam & Winkel, 2017). This cycle primarily affects sexual, gender, racial, and ethnic minorities, and is, by nature, intersectional.

A relevant example of this cycle is provided by Hurricane Katrina in New Orleans, United States. Economic and racial oppression caused much of New Orleans' low-lying districts populated primarily by Black people. Even though these areas suffered the worst damage, the recovery efforts proceeded at strikingly lower rates than in areas populated by wealthier, primarily white residents. With such a discouraging level of investment in these areas, many residents displaced by the hurricane did not return. Those in charge of public resources used this low rate of return as justification for their shortcomings in investment, and the aforesaid cycle emerged. Almost 100,000 Black people did not return to New Orleans after Hurricane Katrina, compared to approximately 11,500 white people. Those who were able to return, most of whom were white, had much better labor market outcomes than non-returnees, thereby perpetuating the continuation of this cycle. The experience of Black residents of New Orleans following Hurricane Katrina exemplifies that racial discrimination leads to inadequate allocation of resources to disadvantaged groups, resulting in the exacerbation of existing inequalities (Islam & Winkel, 2017).

A significant amount of valuable research has been done on the gendered nature of climate change.

It is important first to acknowledge that women of color face a disproportionate impact from the climate crisis. As Heather McTeer Toney points out in her *New York Times* article, "Black Women Are Leaders in the Climate Movement," many Black women, particularly in the southern United States, live in communities with polluted air and water, and work in industries such as housekeeping and hairdressing where they often are surrounded by toxic chemicals and have limited food options that are often impacted by pollutants such as pesticides. Rarely are black women's voices seen or heard as part of national conversations about policy solutions, the green economy, or clean energy (Toney, 2019).

The United Nations has been a major player in the development of research on the disproportionate impact of the climate crisis on women. Dr. Balgis Osman-Elasha highlights some of the major social, economic, and cultural factors that cause people to be more vulnerable to climate change, and that these factors disproportionately do not impact men (one can assume that she is referring to cisgender men). She notes that women make up the majority (70%) of the world's impoverished population and are proportionally more dependent on threatened natural resources. Women tend to have less access than men to resources such as land, credit, agricultural inputs, decision-making structures, technology, training, and extension services that would benefit their capacity to adapt to circumstances brought about by climate change (Osman-Elasha).

The consistency with which the United Nations leave sexual and gender minorities out of their research framework perpetuates the marginalization of sexual and gender minorities, or members of the Two-Spirit, Lesbian, Gay, Bisexual, Transgender, Queer, Intersex, Asexual plus (2SLGBTQIA+) community, particularly with regard to their experiences in the climate crisis. An article titled "Explainer: How gender inequality and climate change are interconnected," on the United Nations Entity for Gender Equality and the Empowerment of Women's (also known as UN Women) website, is the first reference I could find to the interconnectedness of climate change and the oppression of sexual and gender minorities within the United Nations climate change research framework. It is important to note that the article was written about a week ago at the time of my writing this, and the discussion about sexual and gender minorities was brief and at the very end of the article. The unnamed author(s) mention(s) that "it is clear that climate change risks are acute" for "LGBTIQ+ people," although they fail to provide much specificity or any research. They then go on to quote Matcha Phorn-In, a lesbian feminist defender of human-rights: "'If you are invisible in everyday life, your needs will not be thought of, let alone addressed, in a crisis situation.... Humanitarian programmes tend to be heteronormative and can reinforce the patriarchal structure of society if they do not take into account sexual and gender diversity.... In addressing structural change, we are advocating for and working towards equality of all kinds'" (2022). I think this is an incredibly powerful and pertinent quote. I also cannot help but notice the irony behind its being used in a United Nations article: sexual and gender minorities, like the quote implies, are often left out of the climate change research framework of humanitarian programs–and the United Nations itself is one of those programs.

Outside of the United Nations, there is quality research outlining the disproportionate impact of climate change on sexual and gender minorities. Two-spirit individuals face some of the same problems as cisgendered women in the climate crisis, in addition to their own unique vulnerabilities. For instance, Indigenous Americans are at a disproportionately high risk of displacement due to damage caused by climate change. So too are members of the 2SLGBTQIA+ community (Vinyeta et al., 2015). In turn, these houseless individuals would be at a higher risk to once again face the impacts of climate change, given that houseless people are by definition the most exposed to the problems caused by extreme weather (Kidd et al., 2020).

Moreover, queer and transgender youth in the United States are 120% more likely to experience houselessness than their cisgender, heterosexual counterparts, making up an estimated 40% of the country's houseless population, and queer and trans houseless individuals are often not extended adequate access to support and resources after climate related disasters (Oregon State University, 2021).

On a global scale, climate change displaces approximately 20 million people per year, and 69 nations around the world actively criminalize homosexuality. Additionally, queer and transgender refugees face systemic barriers while making asylum claims. In countries that outlaw transgender identity, a person may be forced to lie about their gender, de-transition, or suppress their sexuality to not be turned away while seeking asylum (Oregon State University, 2021). Thus, there is a clear need for research focusing on 2SLGBTQIA+ individuals. A fundamental step in the de-marginalization of sexual and gender minorities in climate change discourse would be to first acknowledge and address their struggles. The widespread exclusion, whether intentional or not, of vital information pertaining to 2SLGBTQIA+ communities from the United

Nations' climate change research framework is more than likely preventing this de-marginalization from happening.

The above cycle of (1) displacement caused by climate change, (2) houselessness/homelessness, and (3) further exposure to the impacts of climate change, perfectly mirrors the theoretical framework of Islam and Winkel's cycle in their paper for the United Nations, "Climate Change and Social Inequality." The question of whether sexual and gender minorities are disproportionately impacted by the climate crisis has been answered. Why the United Nations has yet to address this in a significant way is no small issue; it is a matter of humanitarianism; it is a matter of a marginalized and underserved community of people being neglected by those who hold the power to help confront their disproportionate suffering as a result of the climate crisis.

To achieve justice for the 2SLGBTQIA+ community, climate justice must be accomplished. It is impossible to do so without first modifying current research methods from the top down to be inclusive of the 2SLGBTQIA+ community, and as these research methods currently stand, this is simply not the case. With such inconsistency from nation to nation regarding the execution and publication of climate change research, it is essential that the United Nations, as the foremost international organization that addresses climate change, sets the precedent for inclusivity. If they do so, I am confident that other entities responsible for publishing climate change research will follow suit.

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