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UNIVERSITY OF CALIFORNIA, RIVERSIDE

UNDERGRADUATE RESEARCH JOURNAL

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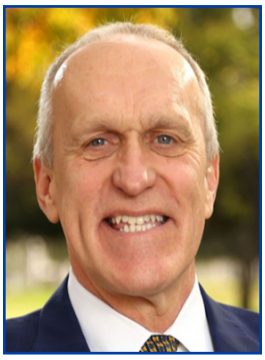


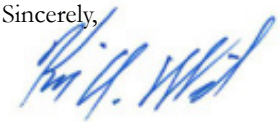
As a research university, one of UC Riverside's most important duties is the creation of knowledge. Undergraduate research is a hallmark of UCR's scholarly and educational missions and a factor in our university's impressive trajectory. With faculty-mentored research projects across a breadth of disciplines, UCR provides a wealth of opportunities for students to investigate complex questions and discover the joys of scholarly research.

As you will see in this 16th volume of the UC Riverside Undergraduate Research Journal, our students are making the most of these opportunities and accomplishing truly inspiring work. The scholarship that appears each year in this publication represents research excellence and creative endeavors of the highest order. UCR is at the forefront of discovery, with a world-class faculty, including two Nobel laureates, and state-of-the-art instructional and testing laboratories. UCR is creating a home for students, faculty, and community members to collaborate, test, and learn.

At UCR we are stewards of transformation; it is in our DNA to ensure that we create an environment and structure that fosters innovation to solve our community and the world's greatest challenges. I am impressed by the dedication and innovation displayed by the students and their mentors in pushing forward in research despite the challenges faced by the pandemic.

I congratulate all the students who contributed to this edition of the Journal, and I express my sincere gratitude to the faculty mentors and staff members that supported these students in their scholarly endeavors.

Sincerely,



Kim A. Wilcox
Chancellor

Research is a crucial component of all disciplines—from the arts to the sciences, business to engineering, philosophy to biology. Each field undertakes research in its own way, but they are united by their curiosity and desire to advance knowledge. This year's scholars conducted their research during a time of significant transition as the university returned to primarily in-person learning and research activities. The articles in these pages reflect the wide range of research and creative activities undertaken by our students—from a study of students' experiences in the US-Mexico transborder community to the biomedical applications of biodegradable salicylic acid-based compounds.

A common image of a researcher is of a figure in isolation; however, the actual practice of research is a collaborative effort. Faculty mentors encourage students to follow their curiosity, guide them in crafting important questions, and help them to develop the courage to seek answers. As a team, they advance knowledge in their disciplines and open the door to further inquiry. The Undergraduate Research Journal provides students a platform to share their research and an opportunity to continue their development as researchers.

During my time at UCR, I have watched the Undergraduate Research Journal grow from its first issue in 2006. Throughout, it has featured some of our university's best faculty-mentored undergraduate research and scholarship. The Sixteenth Edition continues to add to the university's 68-year legacy and reflects the breadth of undergraduate research at UCR. I want to thank the faculty mentors for supporting and encouraging our undergraduates, the Student Editorial Board for leading the peer-review process, the Faculty Advisory Board for its consultation and advice, and the staff of Student Engagement for their roles in producing this edition.

Sincerely,

Ken Baerenklau, Ph.D.
Associate Provost and Chief of Staff
Interim Vice Provost and Dean of Undergraduate Education

UNDERGRADUATE RESEARCH JOURNAL EDITORIAL BOARD

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It is with great pleasure that we present UC Riverside's 16th edition of the Undergraduate Research Journal. We have had the honor of collaborating with brilliant minds across campus in bringing this publication to fruition during such an unpredictable year. It is the collective efforts of everyone involved that ensures the integrity and standards of the Journal reflect the excellence that UCR represents. Congratulations to the authors for your commitment — your ability to adapt to a constantly evolving environment, one that is not conducive to traditional undergraduate research, is commendable and serves as a testament to UCR's academic and creative culture built upon the pursuit of knowledge. Your achievements found in this 16th edition will forever be a part of the Journal's legacy. Congratulations to the Student Editorial Board and Faculty Advisory Board – your diligence and dedication to the publication process have ensured the quality and success of the Journal. We are remarkably grateful to have been part of the outstanding team that made this edition possible. Sincerely,

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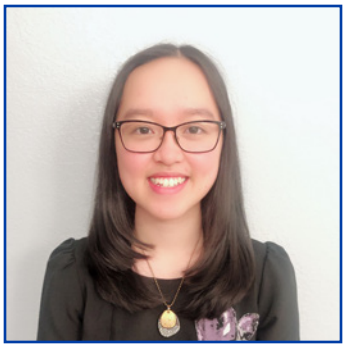

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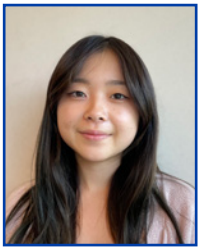

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FROM THE FACULTY ADVISOR BOARD

Since UCR's *Undergraduate Research Journal* started 16 years ago, it has published over 160 scholarly articles across many fields. These papers represent the commitment of our undergraduate students to performing independent research as part of their undergraduate experience. Because undergraduate research can often form part of a larger work with many contributors, the importance of the student's contribution can sometimes be lost. With the *Undergraduate Research Journal*, students can publish their work as first authors before the end of the academic year through a peer-review and publication process. The paper becomes a part of a student's professional experience, contributing to their record of scholarly achievement. The *Journal*'s submission and review process is run by undergraduates who form the Student Editorial Board, working with members of the Faculty Advisory Board. We owe a debt of gratitude to the students for their professionalism and dedication for the review and preparation of the articles in this issue. We are also grateful for the participation of the members of the Faculty Advisory Board in guiding the reviewers. I would like to thank Gladis Herrera-Berkowitz for her work in supporting and guiding the process every year, Lisa Des Jardins for providing Graphic Design assistance, as well as Undergraduate Education for their funding support. If you are interested in publishing your undergraduate research at UCR, consider submitting to our next issue!

Prof. Morris F. Maduro
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ABOUT THE COVER

TIDES

Taken in La Jolla, San Diego. The tides can represent a lot to different people. To some, it represents persistence - with time even indestructible rocks crumble. To others, it represents change - high some days and low some nights. I find that the tides are similar to our experience in research, with every changing day giving us something new --- until finally, a new discovery washes ashore.

Joshua Wang

Joshua Wang is a 4thyear Neuroscience major and University Honors student. For the past three years, he has been involved in prostate cancer research under the guidance of Dr. Manuela Martins-Green. In 2021/2022 he served as a Chancellor's Research Fellow and the President of the Medical Emergency Education Project at UCR. In his free time, Joshua enjoys photography and rock climbing.

Dissecting Human and Influenza Virus Interaction with qFRET Technology

Chuchu Liu, *Department of Biology*
Runrui Dang, *Ph.D. Student, Department of Bioengineering*
Jiayu Liao, *Ph.D., Department of Bioengineering*

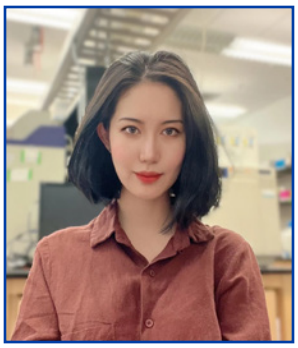
ABSTRACT

Influenza viruses cause seasonal epidemics and occasional pandemics around the world. During each flu season, IAV and IBV viruses are circulated widely in the community, with IAV being the dominant circulating virus and IBV accounting for 25% of all flu cases on average. Due to the significant threat posed by the flu virus, international organization, including the World Health Organization (WHO), have risen to prominence in limiting its global effect. Despite the vaccinations and anti-flu medications that have been developed to combat influenza, drug resistance development highlights the necessity of further studies for influenzas virus pathogenesis and new therapeutic development. Förster resonance energy transfer (FRET) is a technique for detecting protein interactions *in vitro* and *in vivo* that is widely employed in biological and biomedical research. Here we report that the IBV M1 protein has a high affinity with human SUMOylation enzymes, the conjugating enzyme UBC9 and the ligase PIAS1, and conform M1 can be SUMOylated determined with a quantitative FRET (qFRET) assay developed in our lab. Understanding the viral infection process and developing new treatment methods requires identifying and deciphering the host route of viral infection. It is critical to comprehend the viral infection process and develop new therapeutics. Blocking the host human SUMOylation pathway is particularly effective for IBV reduction. Our research provides a direct interaction of human proteins with influenza B protein, providing new insights into human-virus interactions for future therapeutics development. .

KEYWORDS: influenza virus, protein interaction affinity, SUMOylation, quantitative FRET assay

FACULTY MENTOR - Dr. Jiayu Liao

Dr. Jiayu Liao is an assistant professor in Bioengineering at UCR. Dr. Liao received his Ph.D. at UCLA in 1999 and was a founding faculty member of the Bioengineering University's department of California, Riverside. Dr. Liao's research at UCR has focused on the host-virus interaction and the creation of unique highly sensitive and quantitative FRET technology platforms for fundamental and biochemical engineering in SUMOylation and other protein-protein interactions. Dr. Liao was a senior research fellow at the Novartis Research Foundation's Genomic Institute and the Scripps Research Facility before coming to UCR.



Chuchu Liu

Chuchu Liu is a fourth-year Biology student in the Department of Natural and Agricultural Sciences. She is a volunteer in Dr. Liao's Lab and does research on high-throughput screening. She has previously worked in a Molecular Imaging Laboratory at the West China Second University Hospital of Sichuan University (WCSUH-SCU). Her work focused on targeting heart inflammatory molecules caused by diabetes with nanoprobe technology, MR imaging of myocardial infarction tract by neutrophilic granulocyte, and T-cell tracking based on magnetic hyperthermia. Chuchu's interests include science promotion, STEM education, and research. She hopes to get her Ph.D. in Bioengineering in the future.

Dissecting Human and Influenza Virus Interaction with qFRET Technology

INTRODUCTION

One of the two types of viruses causing the COVID-19 pandemic in the 21st century is influenzas, which are classified as influenza A, B, C, and D (IAV, IBV, ICV and IDV)[1]. Despite the development of vaccines and antiviral medications, the number of deaths caused by influenza remains high each year, notably high specifically during the 2009 H1N1 pandemic. In the recent flu season of 2017-2018, it is estimated that flu caused approximately 51,000 deaths and 710,000 hospitalizations, making it one of the most life-threatening infectious disease[2]. The origins of IBV were initially discovered in 1940, and later, the second lineage was found in 1983, the Yamagata- and Victoria-strains[3]. During each influenza season, these two IBV subtypes are co-circulated, contributing considerably to the influenza illness burden over the years. Although IBV's ability to cause serious disease and mortality in particular groups has increased, the science and medical communities have underestimated IBV's contributions to the disease burden, despite the fact that it is considered to produce less severe symptoms than other IAV strains. IBV, for example, has been found to cause serious sickness in children and has a substantially higher fatality rate than IAV[4]. Several studies, however, have found IBV significantly improved their ability to cause severe disease and mortality in specific groups, such as infants and young children, and that they contribute considerably to yearly sickness, accounting for 37% of the overall economic losses of influenza[5]. A recent study indicated that, during 2004 to 2013, IBV-related morbidity was substantially greater than that caused by IAV in infants[6]. For HIV patients, IBV was likewise linked to a greater rate of hospitalization than IAV. Also, during the 2010-2011 influenza season, IBV accounted for just 26% of prevalent influenza strains, yet they were responsible for 38% of pediatric mortality. These findings disprove the theory that IBV produces fewer and milder symptoms than IAV.

Protein interactions are important in all physiological processes and pathogenicity in all organisms, ranging from transcription, signal transduction, and the cell cycle to cancer and neurodegenerative illnesses[7]. A number of approaches to determine protein interaction affinities have been developed[8]. Surface plasmon resonance (SPR), nuclear magnetic resonance (NMR), calorimetry (e.g., ITC-isothermal titration calorimetry and DSC-differential scanning calorimetry), radio-labeled binding tests, ultracentrifugation, and fluorescence polarization (FP) are a few examples of these methods. In fundamental science and translational study, these technologies have substantially enhanced the understanding of protein interactions and dynamics. Protein associations and affinity variations play a key role in normal living and illness. Expensive assay tools and lengthy procedures, on the other hand, cannot always yield trustworthy and consistent findings. One of the issues of current methods for protein interaction affinity determination is that they all require large amounts of purified proteins. Large-scale proteome systems' affinity and dynamics for complicated proteins, on the other hand, are mostly unexplored. As a result, protein-protein interaction experiments are currently quite scarce.

Quantitative tricubic analysis or ratiometric fluorescence signals were previously employed in quantitative Förster resonance energy transfer (qFRET) imaging and biochemical approaches to produce Förster resonance energy transfer (FRET) signals; theoretically, these methods could be used to measure protein interaction affinities (K_D)[9]. The quantitative tricubic technique, on the other hand, necessitates the determination of both the fluorophore's molar extinction coefficient and FRET effectiveness. It is also challenging to transform the approach into a methodological framework since it demands an estimate of the FRET efficiency and several instrument-related factors during the measurement. Without omitting the direct emission of the donor and acceptor signals, FRET analysis methods employ point-to-

point subtraction to assess the FRET signal as well as the ratio of fluorescence emitted at the acceptor and donor wavelengths. When using titration ratio FRET tests to measure protein interaction affinity, the K_D values are often greater than when using SPR or ITC[10].

Because of the availability of numerous fluorescence parameter estimations and the difficulties of determining absolute FRET signal, current approaches for K_D measurement in mixtures lack precision and robustness. We recently developed a cross-wavelength correlation coefficiency method to dissect the absolute FRET signal from the direct emissions of free donor and acceptor and determine the protein-protein interaction affinity K_D, which is very consistent with the values determined with SPR or ITC, but with a significant reduction of time and process[11-13]. FRET-based K_D determination is very sensitive and reliable technique. K_D determination approach based on the qFRET has a number of benefits over other approaches. First, protein interaction measurement is carried out in solution, mimicking the physiological milieu of live cells. Other approaches, such as SPR or ITC, on the other hand, need coupling proteins on solid chip surface, all of which may disrupt protein conformation. The qFRET assays is also extremely sensitive. Because the quantity of fluorescent proteins in FRET tests may range from nM to mM, depending on the detector used, a high concentration of protein is not necessary to determine the K_D. Third, FRET-based K_D tests are non-hazardous to the environment, and protein labeling techniques are ubiquitous. Here, we report that we used the qFRET method to determine the interaction affinity of human SUMOylation E2 conjugating enzyme, Ubc9, and E3 ligase, PIAS1, with the IBV M1 protein.

MATERIAL AND METHODS

1.1 Molecular cloning of DNA constructs

The pET28b (+) constructs for CyPet-SUMO1, UBA2, AOS1, and UBC9 were cloned as described in earlier work [1]. The Ypet-M1 was created by amplifying the open reading frame of YPet using primers and a Linker sequence and ligating it into the pET28b (+) vector (Millipore Corporation, Billerica, MA). TOP10 DH5a *E. coli* bacteria were used to amplify all plasmid DNA constructs.

1.2 Protein expression and purification

The pET28b (+) constructs encoding CyPet-SUMO1, AOS1, UBA2, UBC9, YPet-Linker3-M1, CyPet-UBC9, and CyPet-PIAS1 were transformed into BL21 DE3 *E. coli* cells. LB plates with 50 g/mL kanamycin were used to plate the transformed *E. coli*. A single colony was inoculated into 5 mL LB media with a starting culture of 50 µg/mL kanamycin. Each starting culture was transferred to 1 L 2XYT media with 50 µg/mL kanamycin and cultured for 3 hours at 37 °C with 180 RPM in shaker. 0.35 mM IPTG was used to induce recombinant protein expression overnight at 20 °C with 180 RPM in shaker. The next day, the bacterial cells were collected at 4 °C for 5 minutes at 8000 xg in centrifuge. The bacterial pellet was resuspended in 30 mL binding buffer (20 mM Tris-HCl pH 7.4, 500 mM NaCl, and 5 mM imidazole). An ultrasonic liquid processor was used to lyse the cell suspension (Misonix, Farmingdale, NY). After centrifugation at 4°C for 30 minutes at 35,000 xg, the supernatant was transferred to Column with Ni^{2+}-NTA agarose beads

(QIAGEN, Valencia, CA). Two-column volumes of Wash Buffer 1 (20 mM Tris-HCl pH 7.4, 0.5% (v/v) TritonX-100, and 1.5 M NaCl), two-column volumes of Wash Buffer 2 (20 mM Tris-HCl pH 7.4, 0.5 percent TritonX-100, and 1.5 M NaCl), and one column volume of Wash Buffer 3 (20 mM Tris-HCl pH 7.4, 500 mM NaCl, and 10 mM imidazole) was used to purify before adding 700µl elution buffer to elute the protein (20 mM Tris-HCl pH 7.4, 20 mM NaCl, and 400 mM imidazole). The dialysis buffer was used to dialyze recombinant proteins overnight at 4°C (20 mM

Dissecting Human and Influenza Virus Interaction with qFRET Technology

Tris- HCl pH 7.4, 50 mM NaCl, and 1 mM DTT). Protein concentrations were assessed using the Bradford assay with known quantities of bovine serum albumin (Thermo-Fisher Scientific Inc., Rockford, IL) as standards, and protein purity was evaluated using SDS-PAGE following Coomassie G-250 staining (Bio-Rad, Hayward, CA). Fluorescence intensities recorded on a FlexStationII384 were used to calculate fluorescent-fusion protein concentrations (CyPet-SUMO1 and Ypet-Linker3-M1) (Molecular Devices, Sunnyvale, CA).

1.3 qFRET dissociation constant (K_D)

The dissociation constant K_D was determined by making the receptor concentration at a content number, 0.20μM ([R]Total, the concentration of CyPet binding protein UBC9 or PIAS1) and increasing the ligand concentration ([L]Total, the concentration of YPet binding protein M1) from 0 to 4μM. In a total volume of 60 μl, CyPet and YPet binding proteins were combined and interacted with each other. Each condition (0.2 μM CyPet binding protein UBC9 or PIAS1 interact with 0, 0.05, 0.1, 0.2, 0.3, 0.5, 1.0, 2.0, 3.0, 4.0μM YPet binding protein M1, respectively) was repeated three times. The samples were incubated for 15 minutes at 37°C. The samples were then transferred to a Greiner 384-well plate (Sigma-Aldrich). $FlexstationII_{384}$ was used to determine the fluorescence emissions.

Emission intensities were recorded at three wavelengths following excitation at 414 nm and 530 nm after excitation at 475 nm. The relationship between K_D and E_{mFRET} is shown in the **Equation 1**.[3]

$$E_{mFRET} = E_{mFRET} * \frac{[L]_{Total}-[R]_{Total}-K_D+\sqrt{([R]_{Total}+K_D-[L]_{Total})^2+4*K_D*[L]_{Total}}}{[R]_{Total}+K_D-[L]_{Total}+\sqrt{([R]_{Total}-[L]_{Total}+K_D)^2+4*K_D*[L]_{Total}}} \quad Equation1$$

1.4 qFRET in vitro SUMOylation assay

In a total volume of 60 μL, all components of the SUMOylation experiment (0.5 μM CyPet-SUMO1, 0.1 μM E1, 0.2 μM E2, 0.25 μM E3 PIAS1, and 2 μM YPet-Linker3-M1) were mixed in SUMOylation buffer (50 mM Tris-HCl pH 7.4, 1 mM DTT, and 4 mM $MgCl_2$). The sample reaction mixture was incubated in an Eppendorf tube at 37 °C after adding 6 μL of ATP (20mM stock). All sample mixes were then placed in a Greiner 384-well plate (Sigma-Aldrich). $FlexstationII_{384}$ was used to monitor fluorescence emissions (Molecular Devices, Sunnyvale, CA). Following excitation at 414 nm, emission intensities were measured at 475 and 530 nm, including 530 nm after excitation at 475 nm [3].

1.5 Em_{FRET} Analysis

The CyPet and YPet were fused to the SUMO1 and M1, respectively. Excitation and emission peak wavelengths for CyPet and YPet are 414 nm / 475 nm and 475 nm /530 nm, respectively. When the FRET pair (CyPet and YPet) is close together (between 1 and 10 nm), the donor's excitation at 414 nm causes an energy transfer from the donor to the acceptor, resulting in the donor's quenching and the acceptor's excitation. FRET can occur when YPet-M1 is SUMOylated with a CyPet-SUMO1, culminating in a 530 nm emission with a 414 nm excitation. Anything that hinders SUMOylation (such as the lack of ATP or the addition of STE-025), on the other hand, has no effect on the emission at 530 nm.

The development of the SUMO1-M1 complex was tracked using actual FRET emission (E_{mFRET}). E_{mFRET} was defined as illustrated in **Equation 2** [4]. To calculate the true FRET emission, direct emissions at 530 nm from free CyPet-SUMO1 and YPet-M1 must be calculated and subtracted from the overall emission intensity at 530 nm. The E_{mFRET} was calculated using a previously published

spectrum analysis to account for the components to the exhaust emissions at 530 nm. Real FRET emission (E_{mFRET}), CyPet direct emission, and YPet direct emission are three fractions of total fluorescent emissions at 530 nm given a 414 nm excitation (E_{mTotal}).

With a ratio coefficient of α= 0.368, the direct fluorescence contribution of the CyPet at 530 nm is proportionate to its peak emission at 475 nm (FL_{DD}) when stimulated at 414 nm. With a ratio value of β= 0.029, YPet's direct emission at 530 nm is proportionate to its emission at 530 nm given a 475 nm excitation (FL_{AA}). For each sample, the fluorescence signal was measured from 400 to 600 nm.

$$Em_{FRET} = Em_{Total} - \alpha * FL_{DD} - \beta * FL_{AA} Equation2$$

RESULTS

This research demonstrates that the human SUMOylation pathway is required for the IBV life cycle[14]. The SUMOylation pathway, a pathway required for the IBV life cycle, can impede IBV viral replication. SUMO is a member of the superfamily of ubiquitin-like polypeptides that uses a multistep enzymatic cascade to modify protein function and stability. The whole SUMOylation process involves a SUMO-activating enzyme E1 (Aos1/Uba2), a SUMO-conjugating enzyme E2 (Ubc9), and SUMO ligases E3 (PIAS1 family, RanBP2/Nup358, Pc2)[15]. It is generally assumed that E3 is not needed *in vitro* SUMOylation and plays an essential role *in vivo* SUMOylation for efficiency and specificity[16]. Thus, understanding the affinities of SUMOylation E2 and E3 for the IBV proteins is critical for further study of IBV M1 functionality during the infection process.

A straightforward way of tracing the reaction and collecting the results is to use the FRET signal to monitor the SUMOylation process. In the first study, the FRET signal was greatly significant when the CyPet-SUMO1 was

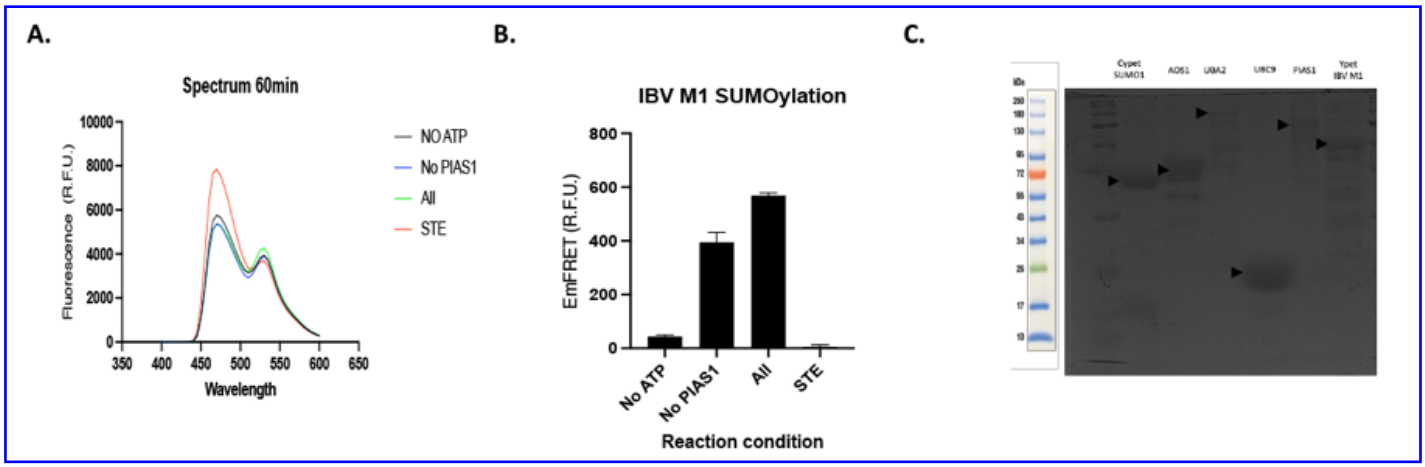


Figure 1. (A) Using the FRET assay, the FRET spectrum of the in vitro SUMOylation process of IBV M1 protein. CyPet-SUMO1, E1, E2, E3, YPet-M1, and ATP (ALL and green); CyPet-SUMO1, E1, E2, E3, YPet-M1, and ATP (no PIAS1 and blue); E1, E2, E3, YPet-M1, and no ATP (NO ATP and black); CyPet-SUMO1, E1, E2, E3, YPet-M1 (ALL plus STE and red). **(B)** IBV M1 SUMOylation quantitative FRET signal (EmFRET) from **(A)**. **(C)** An in vitro biochemical test of IBV M1 protein SUMOylation followed by a Western blot employing an anti-SUMO1 antibody. The SUMOylation processes were carried out in solution under different circumstances with and without the SUMOylation inhibitor, STE. Lane 1: CyPet-SUMO1, E1, E2, E3, YPet-M1, -ATP; Lane 2: CyPet-SUMO1, E1, E2, E3, YPet-M1, +ATP; Lane 3: CyPet-SUMO1, E1, E2, E3, YPet-M1, +ATP; Lane 4: CyPet-SUMO1, E1, E2, YPet-M1, +ATP+STE02

conjugated to the YPet-M1 due to the close closeness of the FRET donor and acceptor (**Figure 1A** ALL green). The SUMOylation inhibitor, STE025, blocks the SUMOylation process, as seen by the decrease of the FRET signal (**Figure 1A** STE and red). The SUMOylation E3 ligase PIAS1 was also included in this SUMOylation experiment. When protein levels are low, the SUMOylation E3 ligase plays a significant role in *in vivo* SUMOylation, although it is not required in the in *vitro* SUMOylation process. After adding PIAS1 to the reaction, we saw a significant increase in the FRET signal, which was consistent with previous findings (**Figure 1A** No PIAS1 in blue and ALL in green). We also examined the proteins expressed in bacterial cells, CyPet-SUMO1, ASO1, UBA2, PIAS1, and YPet-M1, in the SUMOylation reactions by SDS-PAGE gel followed with Coomassie-blue staining (**Figure 1C**). All the proteins are well expressed except the PIAS1, which expression level was low.

In this study, we used the qFRET to determine the affinities of Ubc9 and PIAS1 for the IBV M1 protein. The Ubc9 or PIAS1 was fused with FRET donor, CyPet, and IBV M1 was fused with FRET acceptor, YPet, respectively. Those three proteins were expressed in E. coli strain Bl21 (DE3). After proteins were purified through the Ni-His affinity column, the FRET donor, CyPet-Ubc9 or CyPet-PIAS1 was set in a constant concentration 0.2μM, and then the FRET acceptor, YPet-M1 was titrated in different concentrations from 0 to 4μM. The algorithm developed in our previous work can extract the absolute FRET signal, which corresponds to the interactions from the total fluorescence signal[11].

The **Figure 2** shows good sigmoidal curves of the absolute FRET signal (E_{mFRET}) of Ubc9 and PIAS1 with IBV M1. The K_D values calculated by our developed equation for Ubc9-M1 or PIAS1-M1 interactions were 0.20 μM and 0.22 μM, respectively, indicating a very high affinity of both SUMOylation enzymes E2 and E3 for the IBV M1. These K_D values show that the IBV M1 protein is a good substrate of SUMOylation enzymes.

DISCUSSION

Viruses employ host factors to infect hosts and replicate their genomes[17,18]. Depending on the roles of host factors, these components can be important or essential for

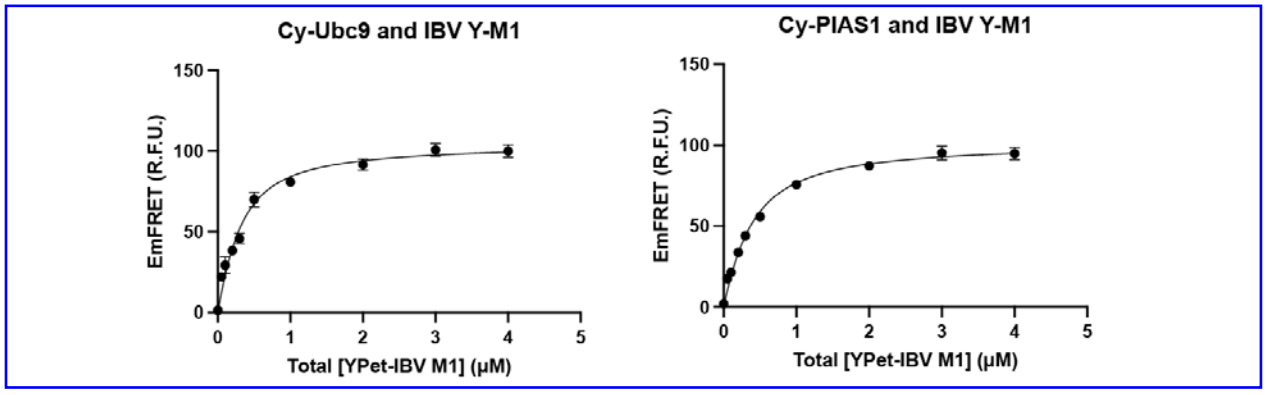


Figure 2. Ubc9, the SUMOylation E2 conjugation enzyme, and PIAS1, an E3 ligase, have a very high affinities for the IBV M1 protein, as demonstrated by a qFRET test. **(A)**. Using the qFRET assay in solution, the interaction affinity KD value of 0.2 M between Ubc9 and M1 was obtained. **(B)**. The qFRET assay was used to measure the contact affinity KD value of 0.22μM between PIAS1 and M1.

viruses. A novel antiviral method that targets host factors against infections has been proposed and developed in some efforts. SARS-CoV-2 patients are protected by antibodies that block the human receptor ACE2 and the viral S protein interaction. Here, we show that suppressing the human SUMOylation as a new approach for anti-IBV infection therapy, which is compatible with previous research and theory18. Identification and characterization of the essential host factors for viral replication offer considerable promise for revealing novel methods against IBV infection.

The IBV M1 protein is recognized by the SUMOylation E2 conjugation enzyme and E3 ligase at very high affinities, both around 0.2 μM in our qFRET assay, suggesting the potential SUMOylation of M1 under physiological conditions. Further research is required to explore the detailed functional requirement of SUMOylation for the M1 protein in viral life cycle. The IBV M1 protein is known to play essential roles in the formation of viral Ribonucleoprotein(vRNP) and the budding of the viral particle. The functional significance of M1 SUMOylation needs further investigation.

Clinical data shows that children infected with IBV are more susceptible and have higher hospitalization rates than those infected with IAV, reflecting different pathological processes and immune responses caused by IBV infection from IAV infection19. A study shows that IBV was revealed to be less sensitive to the Zanamivir and Oseltamivir than IAV in cellular assay[20]. Although there are still some differences between IBV and IAV needed to be explored, IBV has already been suggested to become a nonnegligible threaten for the anti-flu drug discovery. Therefore, novel therapies to treat influenza viruses need to be developed. Several studies have screened for host factors, factors important for IAV infection and replication. SUMOylation is a common factor identified among the genome-wide screens[21,22]. Since we confirm that the IBV M1 has a high affinity with SUMOylation E2 and E3, inhibiting SUMOylation can provide a valuable possibility for the development of a new class of inhibitors to treat influenza virus infections as well as other viruses that utilize SUMOylation. This approach illustrates the possibility of developing novel classes of anti-flu drugs.

Dissecting Human and Influenza Virus Interaction with qFRET Technology

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Has Anybody Asked How People Change Their Minds? Pre-crastination and Its Underlying Basis in Decision-Making

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ABSTRACT

Procrastination is much too familiar to us, a derogatory term taught to students as something to avoid, which, to teachers' despair, counterproductively encourages students to take up procrastination as a challenge. The opposite of procrastination, pre-crastination describes the likelihood of completing tasks early at the expense of extra effort, and may be a phenomenon as common as procrastination (Rosenbaum et al., 2014). We hypothesize that fundamentally, pre-crastination is cognitively driven, given that participants offload cognitive tasks before determining the course of action. This study took place over three experiments. Our pool of UCR undergraduate participants (N=89) made two forced yes/no responses pertaining to the same stimulus in each trial. The stimuli in the first experiment was determining chronology of number sequences while the stimuli in the subsequent two experiments was determining digit-matching. The most significant alteration was made in the third experiment, in which the second response was changed from a yes/no to a confirm/disconfirm submission. This innovative testing strategy, coined double-response in our lab, allows us to correlate response time to decision-making bases. Largely, participants exhibited a significantly longer reaction time in submitting their first response. This outcome supports our cognitive hypothesis which predicts that action-planning occurs through longer first-response times, going against the behavioral hypothesis which predicts that action is taken prematurely through shorter first-response times. Ultimately, this double-response method better helps us understand the dynamics of decision-making through pre-crastination.

KEYWORDS: pre-crastination, double-response, procrastination, decision-making, planning

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Has Anybody Asked How People Change Their Minds? Pre-crastination and Its Underlying Basis in Decision-Making

INTRODUCTION

The process of 2-step decision-making first established by Francis Donders was paramount in establishing that decisions are made in stages: early stages of decision-making are automatic while later stages are more calculated (1868; Rosenbaum et al., 2022 (in press)). Donders' groundbreaking study demonstrates that decision-making is a cognitively complex process that takes into account reaction times, and it has since been further studied using various methods. In leading experiments building off of reaction times in decision-making, studies involving choice reaction tasks are particularly significant in demonstrating that actions (i.e. behavioral response) are not always the final product of cognition; reaching trajectories can have multiple competing targets (Hillyard & Kutas, 1983; Luck et al., 1990; Hillyard, 1990; Song & Nakayama, 2009). For example, you may find yourself automatically reaching for a commonly used object due to muscle memory, but may correct yourself upon realizing you intended to reach for something else.

These trailblazing methods in integrating reaction time greatly influenced our lab's basis of choice reaction time (RT), more particularly the 2-choice RT strategy – introducing two possible stimuli under two responses (Rosenbaum et al., 2022 (in press)). Existing experimental RT has suggested that people continue to think even after a response has been made in their decision-making process, and furthermore, RT has been found to be longer after an error has been made compared to that of non-error trials (Danielmeier & Ullsperger, 2011; Rosenbaum et al., 2022 (in press)). This brand-new method of studying decision-making required two responses, which our participants were told could be non-identical. This procedure allowed participants the possibility to rethink their final answer before moving on to the next trial and was consequently a new variation of psychological experimentation based on choice RT in decision making, coined by our lab as the double-response method.

PRE-CRASTINATION

Pre-crastination, the hastening of tasks at the cost of additional effort, was the key discovery that gave rise to our invention of the double-response method for observing response times in our experiments. Our lab first observed the phenomenon of pre-crastination through a 2014 experiment, in which UCR undergraduates chose to pick up one of two buckets – one spaced closer to the starting point and the other further from the starting point – to carry to the finish line (Rosenbaum et al., 2014). In the experimental group, the bucket closest to the starting point is lighter in weight, whereas the bucket placed closest to the finish line is heavier; in the control group, the buckets are spaced in the same manner, but they remain empty and therefore are the same weight. Contrary to our lab's expectation, the results of both the experimental group and the control group show that a majority of participants choose to carry the bucket closer to the starting point rather than the bucket further from the starting point. Thus, participants consistently opt for the bucket that must be carried a farther distance.

This surprising outcome goes against Rosenbaum, Gong, and Potts's expectations in mapping the "biomechanical tradeoffs" analogizing weight and distance, which, in sum, intends to determine the point of indifference within the said tradeoff (2014). This experiment was sparked by an interest in human action planning, a segment of decision-making that posited the investigation into human course of action in correlation to the action's biomechanical costs. Essentially, this experiment was the first instance observed exemplifying pre-crastination and hence initiated our lab's research further into the phenomenon.

Noteworthy in the pioneering experiment run by Rosenbaum et al. (2014) is the restriction that it can only

test the behavioral account of pre-crastination, which only emphasizes the completion of a goal at the cost of extra effort. Cardinal subsequent studies carried out by Fournier et al. (2018; 2019) challenge this basis of pre-crastination by arguing that it is actually the start, not completion, of goals rooted in the tendency to pre-crastinate via offloading working memory. In 2018, Fournier carried out two experiments with physical tasks – one experiment requiring lower cognitive demand (bearing no memory load) and the other requiring higher cognitive demand (bearing high memory load) – in order to observe how increasing cognitive demand affected the tendency to pre-crastinate. Fournier's results show that the more cognitively demanding task increases participants' likelihood to plan a course of action, i.e. make a logical decision before taking action. This conclusion contradicts the method of pre-crastination proposed by Rosenbaum et. al. (2014) thus far, which asserts that actions are carried out prior to cognitive decision-making. Thus, a new potential premise for the phenomenon is introduced: the cognitive account.

Understanding the fundamental driving factor of pre-crastination is crucial in understanding its potential reflections in our everyday lives – whether it be answering emails hastily without caution, convicting people without forethought (as magnified within the criminal justice system, particularly regarding the disproportionately incarcerated people of color in American prisons), and in the most extreme case, beginning wars without proper deliberation (with the most current example being the 2022 war on Ukraine). All of these examples have the same limitation as Rosenbaum et al. (2014): they are all under the presumption that pre-crastination operates on a behavioral basis. As such, in order to determine if pre-crastination truly does explain fundamental decision-making in these events, our goal is to establish a conclusive experiment that also examines the potential cognitive basis behind the phenomenon by way of double-response RT methodology.

Behavioral vs. Cognitive Basis

Firstly, examining the driving factor – the behavioral account or cognitive account – of pre-crastinational decision-making is fundamental in building its premise.

The behavioral account describes the phenomenon of acting on impulse and is thus considered idleness aversion: doing something is its own reward (Hsee et al., 2010; Rosenbaum, 2022). Essentially, we define the behavioral basis as "the desire to act upon." For instance, in the present study, a participant would find pressing a button alone in itself rewarding, as they are simply looking to rid themselves of the action (Fournier et al., 2018, 2019; Rosenbaum et al., 2019). Moreover, many studies have demonstrated that there are no intrinsic rewards that result from pre-crastination. As summarized above, picking up the basket in the 2014 study is not rewarding in itself because a basket is always picked up and the same distance is walked (Fournier et al., 2018, 2019; Rosenbaum et al., 2019). As such, there is nothing intrinsically rewarding about picking up a basket earlier or later. Further studies have also shown that early actions reduce external rewards (Rayburn-Reeves et al., 2011; Zhu et al., 2018).

The cognitive basis is defined as the inclination to make a decision before planning a course of action. Having a lesser memory load may enable one to be more prepared to face future challenges, a concept which Wasserman (2018) referred to as the "fierce urgency of now," quoted from Martin Luther King, Jr (Rosenbaum et al., 2022 (in press)). Research has furthermore shown that when memory loads are increased, pre-crastination rates increase. This data is consistent with the postulation that pre-crastination is linked with working memory resources to clear items from a mental to-do list (Fournier et al., 2018; Patterson & Kahan, 2020).

Double-response is henceforth a testing strategy formulated in our experiments as an attempt to discriminate between the behavioral and cognitive accounts studied previously

in the field of decision-making. This new tool can bring more awareness and clarity to decision-making, which can subsequently enrich the understanding of this important topic as well.

Double-Response

The double-response method is the driving force for studying whether or not pre-crastination is built upon a behavioral or cognitive basis. In both accounts, the reaction time patterns are key in concluding behavioral or cognitive forces.

Double-response describes the method in which participants make a yes/no decision in response to one stimulus, and subsequently must also submit a second response echoing or not echoing their initial decision (Rosenbaum et. al. 2022 (in press)). We set these responses, R1, and R2 (the first response and the second response respectively), as the main means by which to build our data of interest. One set of data corresponding to R1 and R2 was the accuracy probabilities P1 and P2, respectively. The other set was the corresponding times, T1 and T2, with regards to R1 and R2 respectively. T1 and T2 were representative of the reaction times, with T1 defined as the time from when participants were first exposed to the stimulus to the time R1 were submitted, and T2 defined as the time period between R1 and R2 submissions. Thus, the total time per trial was defined as T1+T2 (Rosenbaum et. al. 2022 (in press)).

Participants were not given feedback regarding their accuracy or reaction times for the first two experiments. The only prior instructions participants were given were to respond accurately and directly. This procedure allows us to better gauge predictions for our two models of behavioral vs. cognitive bases.

In our prediction concerning the behavioral model, participants are expected to submit R1 rapidly, resulting in a characteristically short T1. This derives short latencies which cause participants to focus their primary decision-making in their R2, consequently producing a longer T2. Based on this strategy, our expected results would exhibit T1<T2 and P1<P2, mirroring the offloading of action.

Conversely, our prediction regarding the cognitive model would yield a characteristically longer T1 due to their reduction of cognitive load in R1. In their subsequent R2, their T2 would likely be shorter due to the decision largely being rectified in R1. This implies that primary decision-making was made before planning a course of action, thereby participants are more inclined to think the task through before submission of either response. This strategy will give rise to two potential patterns: (1) T1>T2, P1=P2 if no additional decision-making took place after R1, or (2) T1>T2, P1<P2 if decision-making was refined after R1. We omitted the hypothesis of T1=T2, as although we acknowledge that this outcome was a possibility, it was extremely unlikely and not relevant to our main hypotheses.

Our lab conducted three different experiments in order to test the double-response strategy and apply our cognitive vs. behavioral models of pre-crastination. Each experiment led to the development of the consecutive experiment, with Experiment 1 being the pioneering study utilizing the double-response method. Experiment 2 was thereafter introduced in order to reduce fallacies that may have given rise to biased results in the first experiment and was the primary procedure that Experiment 3 was built upon. Experiment 3 ultimately provided the most conclusive and substantial results for our hypothesis as it was based on the analyses of the previous two experiments.

EXPERIMENTS 1 AND 2

METHODS

In Experiment 1, UCR undergraduate participants (N=15) were asked to make yes/no judgments regarding 2-digit numerical sequences, read from left to right. Participants

were told to be as accurate and quick as possible in both R1 and R2. A participant may be given one of these two sequences: (a) 11 12 20 28 33 75 (b) 28 36 **45 35** 78 91. Sequence (a) formed a chronologically increasing pattern because each subsequent 2-digit number was larger than the number before when read left to right. However, (b) was not chronologically increasing because 35 was smaller than 45 and thus disrupted the smallest-to-largest-number pattern.

As per our double-response strategy, participants were told to submit two individual yes/no responses per trial. A "yes" was conveyed by pressing the j key and indicated that the participant observed a chronological sequence, whereas a "no" was conveyed by pressing the f key and indicated that there was not a chronological pattern. Experiment 1 and the subsequent experiments were written and run on MATLAB, a computing platform which automatically calibrated reaction times and accuracy. The program contained six blocks, with 24 trials per block. Half of the trials in each block randomly contained sequences that increased chronologically, while the other half contained a violation of the chronological pattern by one position.

In Experiment 2, UCR undergraduates (N=32) participated for academic credit. None of these participants participated in Experiment 1 and were once again told to be as accurate and as quick as possible in R1 and R2. Rather than showing two digits to be checked for monotonicity as in Experiment 1, participants were instead shown six three-digit numbers to check for repeats in the hundred's column, the tens column, or one's column (see **Table 1**). Participants were instructed to consult the first three-digit number as their reference point to scan for repeats in subsequent three-digit numbers' corresponding columns. If participants found a repeat in the hundreds, tens, or one's columns, they pressed "j" on the keyboard; otherwise, they were to press "f" on the keyboard. As in Experiment 1, participants were given a second response in each trial. They were told that they were being tested on their quickness and precision, but were not given any feedback regarding these two factors.

Digit Array	Repeat Column	Repeat Position
687 293 141 532 779 155	None	None
6**8**7 293 **6**41 532 779 155	Hundreds	3
68**7** 293 641 5**8**2 779 155	Tens	4
68**7** 293 141 532 77**7** 155	Ones	5

Table 1. Examples of digit arrays in Experiment 2

** The underlining and bolding of digits was not shown in the experiment.*

RESULTS

The total average of correct responses in Experiment 1 was calculated to be M=0.89, with P1 (M=0.88) observed as being smaller than P2 (M=0.90). Of this proportion, T1 (M=1.8) was seen to be notably higher than T2 (M=0.2); data showed that T2 values remained consistently shorter than T1 values throughout the experiment.

In Experiment 2, it was observed that P1 (M=.706) was slightly lower than P2 (M=.718). Similarly, T1 was seen to be longer than T2. Our data thus remained consistent with the results obtained from Experiment 1. We saw a correlation between participants' time and responses, where T2 was smaller when R2 matched R1 vs. when there was no match between R2 and R1, as shown in **Figure 1** below.

To further understand participants' accuracy in decision making, the function of Repeat Position was further analyzed in Experiment 2. It was found that when there

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were no repeats, the proportion correct was higher compared to when there were repeats. Given the presence of a repeat, this proportion became higher when the second number in the three-digit number had a repeat. Additionally, the proportion correct was found to be higher in Response 2 (Rosenbaum, et al., 2022 (in press)). T1 and T2 as a function of Repeat Position ultimately demonstrated that T1 was longer for trials without repeats than with repeats. T2 was found to be smaller than T1 but was not associated with the Repeat Position.

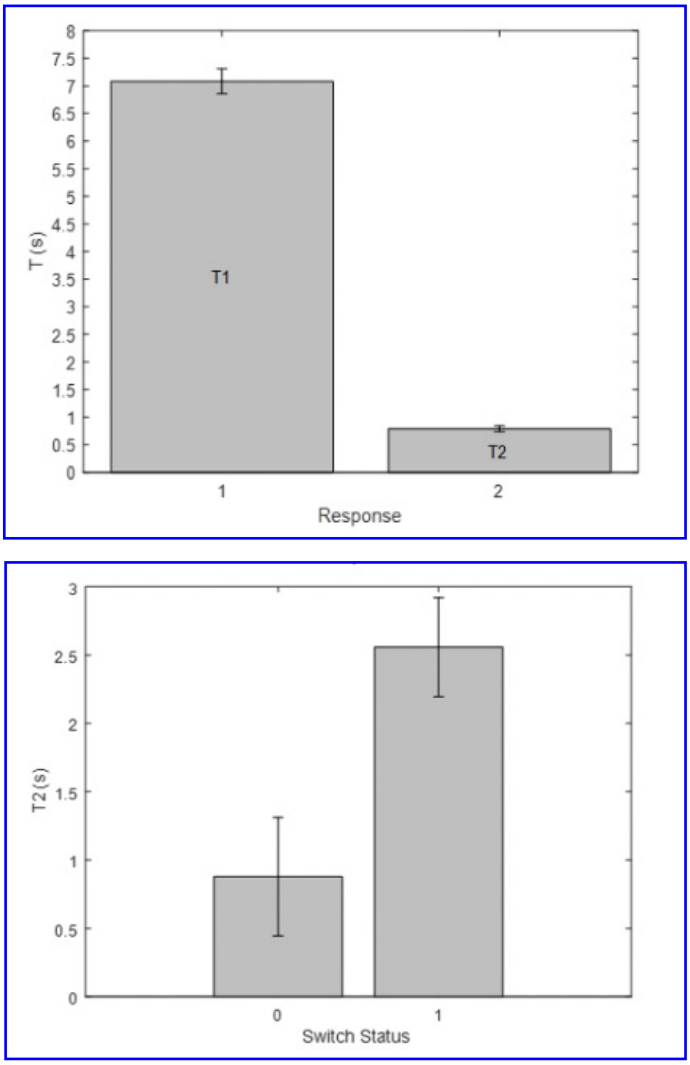

Figure 1. Experiment 2 time results. **Top image:** Mean times (±1 SE), T1 and T2, for the first and second response, respectively. **Bottom image:** T2 mean times (±1 SE) in trials without a response switch (Switch Status = 0) and with a response switch (Switch Status = 1).

DISCUSSION

Results from Experiment 1 had thus far supported the cognitive basis of pre-crastination over the behavioral basis. We observed a characteristically longer T1>T2, and a P2 very slightly, if at all, higher than P1, concluding that participants largely opted to reduce their cognitive load by completing their decision-making upfront.

In Experiment 2, methods differed slightly from Experiment 1 because it was found that a feature of the semantics affected the behavioral hypothesis, which may have made the task easier and potentially caused a fallacy. In Experiment 1, it was speculated that participants used a shortcut strategy by looking at the start and end of the sequence thereby allowing them to make a quick decision. In application, participants pressed j (yes) if the leftmost number was small and the rightmost number was large, or otherwise pressed f (no). This potential strategy may be considered a fallacy as it may have allowed participants to make quick and easy decisions, thus disrupting genuine decision-making.

In Experiment 2, the double-response method was continued, but with new, more cognitively taxing stimuli which helped remove the fallacy between numerical size and response type in Experiment 1. Instead of deciding between a 2-digit number increase, participants in Experiment 2 decided whether a 3-digit number following the first set of numbers had a repeat in the hundreds, tenths, or one's columns.

Unlike Experiment 1, in which participants may have

checked the ends of sequences to make a quick decision, Experiment 2 prompted participants to implement a more orderly method of decision-making. In both experiments, T1 was longer than T2, and P2 was slightly larger than P1. This correlation between a longer T1 and higher P2 suggested that participants were likely making a decision before taking action, supporting the cognitive hypothesis over the behavioral hypothesis. However, it was observed that participants were grouping responses, or making a single decision and submitting R1 and R2 in rapid succession, resulting in a new fallacy coined the tap-tap phenomenon. Experiment 3 was therefore created to address this potential issue and validate our findings thus far.

EXPERIMENT 3

METHODS

Response grouping (Adam et al., 2003; Miller & Ulrich, 2008; Ulrich & Miller, 2008) may have led to a term we have coined the tap-tap phenomenon, in which participants decide on one response and tap the associated key twice (for R1 and R2) in rapid succession. In an attempt to stop this occurrence in our results, we altered the semantics of R2 and emphasized that only the accuracy of R2 mattered in our participants' instructions. This change in semantics gave participants an incentive to conduct two separate motor plans instead of sticking to one response throughout a trial, therefore giving rise to slightly higher P2 and T2. In Experiment 3, participants were also given feedback on the accuracy of their final answer as a motive to answer correctly, and to ensure that they understood instructions.

To implement our adjustments, the task of R2 was changed from a yes/no judgment to a confirm/disconfirm judgment. Participants would accomplish the task by continuing to tap the j key to convey a yes (match) or the f key to convey a no (no match) for R1; however, they would thereafter press j to confirm their judgment or f to disconfirm their judgment (i.e. reverse their initial response) in R2. This inclusion of a confirm/disconfirm R2 would halt response grouping, as our program would randomly determine that pressing the j key half the time and pressing the f key the other half of the time throughout R1 and R2 would yield a correct response. This sequence made it essentially impossible to blindly group responses in order to consistently obtain a correct final answer throughout the experiment.

Seeing as the instructions for this experiment were more complex than the previous two, research assistants ensured that the participants did not begin the trials until they were coached and fully confident with the directions. As mentioned previously, participants were not given feedback on accuracy or reaction times in the first two experiments. In the third experiment, participants were given feedback regarding the accuracy of their final response in order to not only verify their understanding of the experiment, but to also check the accuracy of the results acquired from the first two experiments.

RESULTS

According to **Figure 2**, the first common response from participants was R1 and R2 being both correct (total number=4481). The second most common response was R1 and R2 both being incorrect (total number=380). The remaining two choices were chosen least commonly: incorrect and then correct (total number= 110) or correct and then incorrect (total number=68). P2 values were observed to be well predicted by P1.

Additionally, **Figure 2** demonstrates that T1 (M=9.16 s)

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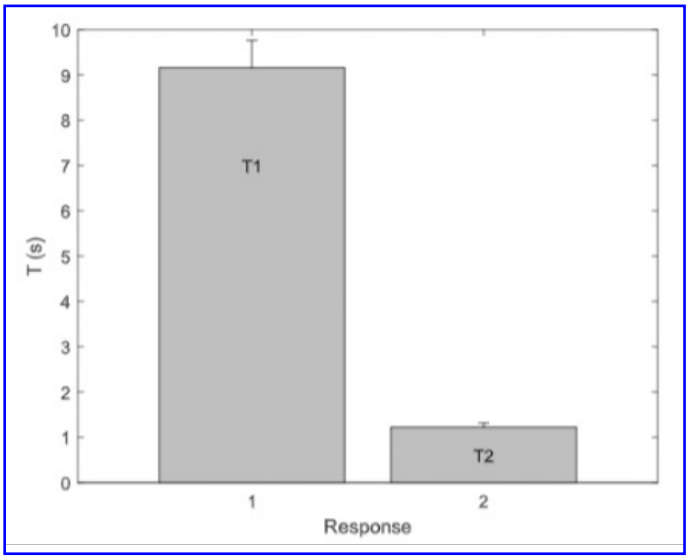


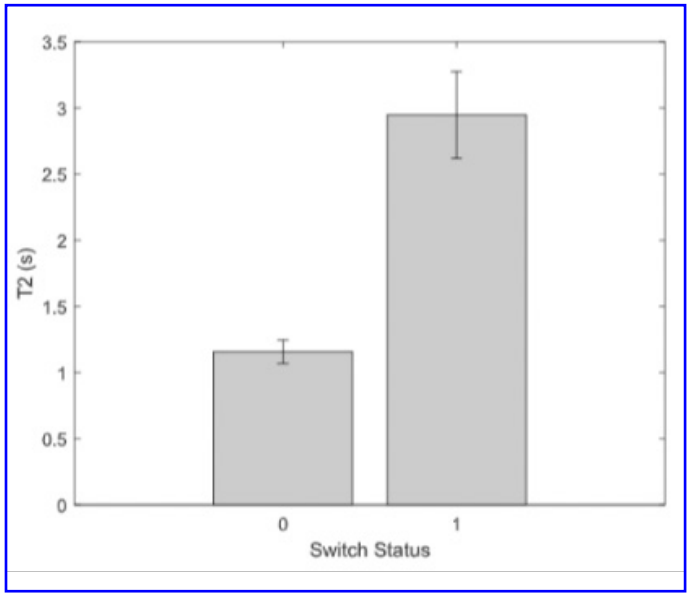
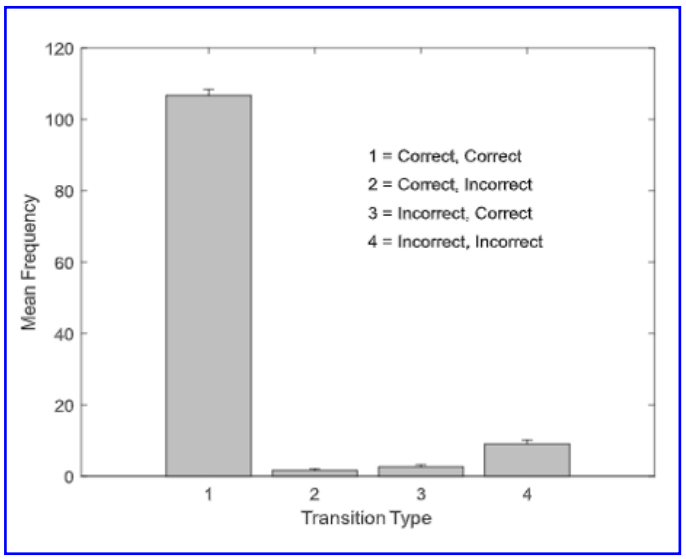


Figure 2. Experiment 3 Results. **Top image:** Mean times (±1 SE), T1 and T2, for the first and second response, respectively. **Bottom image:** T2 mean times (±1 SE) in trials without a response switch (Switch Status = 0) and with a response switch (Switch Status = 1)



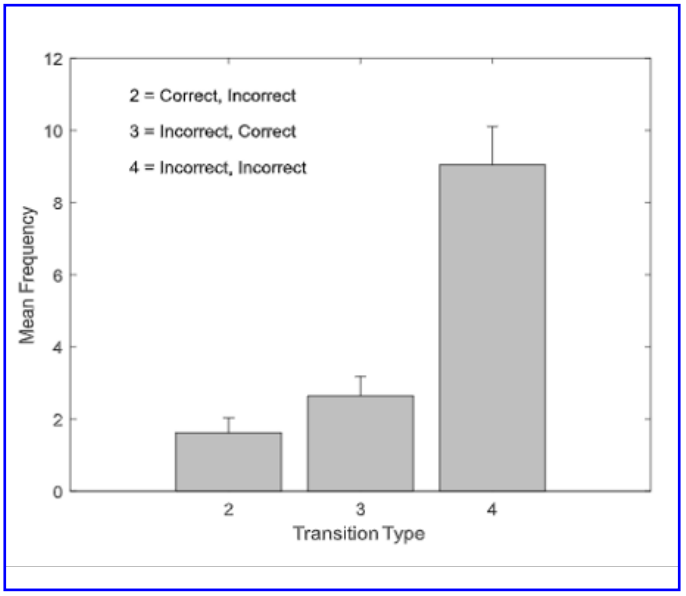


Figure 3. Experiment 3 transition types. **Top image:** Mean frequency (±1 SE) of the four transition types in Experiment 3. **Bottom image:** Blowup of frequencies from left panel.

was longer than T2 (M=1.22 s). This implies that participants were thinking about their response before responding, supporting the cognitive model. The histograms in **Figure 3** continue to point out the great difference between T2 and T1, showing T2 being characteristically shorter than T1. This trend was inconsistent in only one case, in which T2 times were found to be longer when participants switched their decision for R2 (M=1.18s), compared to the non-switch case where T2 (M=1.22). It is important to note once again that a response switch in Experiment 3 meant the participant was disconfirming their first response; however, this did not imply switching the buttons pressed.

DISCUSSION

As stated in the methods for this experiment, the potential fallacy of response grouping observed in the prior two experiments was addressed in our altered procedure. We intended to diminish response grouping by (1) changing the semantics of R2 and (2) shifting emphasis in the instructions.

Redefining R2 from a simple yes/no judgment to a confirm/disconfirm judgment caused cessation of repeat answers in R1 and R2 of each trial by encouraging participants to genuinely plan out their motor responses for both submissions. Response grouping would thus be violated for participants to receive an accurate final answer throughout the experiment.

Only underscoring the accuracy of R2 to the participants and, moreover, giving the participants feedback on the accuracy of their final answer helped to incentivize the quick behavior of R1. In other words, R1 could be given quickly at no cost to accuracy, thus leveling the playing field for both the cognitive and the behavioral hypotheses. More importantly, allowing the participants feedback on accuracy would resolve any misunderstandings and also verify the viability of previous experiments.

Experiment 3 continued to show the same results as Experiment 1 and 2, even though the task was made more challenging and the meaning of the responses changed. T1 continued to be longer than T2, demonstrating that the hypothesis made previously about their existing response grouping in Experiment 2 was wrong, given that the same results were seen in Experiment 3. This means that participants were taking their time to make a decision in all three experiments instead of making a quick and thoughtless action. Thus, these results support the cognitive model for decision-making.

GENERAL DISCUSSION

Experiment 3, the focus of our results and ultimately the concluding factor in our study, establishes that pre-crastination is built on a cognitive basis, i.e. decision-making is completed before action-planning, resulting in a delay of the first response (T1). We include experiments 1 and 2 in our article body because they necessarily built the foundation upon which our lab began to concretely build the cognitive vs. behavioral methods of early decision-making.

Through analysis and study of these two prior experiments, Experiment 3 eliminates all potential fallacies by (1) giving feedback at the end of each trial and (2) altering R2 to a confirm/disconfirm judgment as opposed to yes/no. In the end, the results of experiment 3 continue the trend of supporting the cognitive basis with P2>P1 and T1>T2, thus confirming the results of our lab's first two experiments.

This implication is significant in studies of perceptual-motor performance, as support for the cognitive basis of pre-crastination essentially enforces that pre-planning of action takes place when presented with a mentally demanding task. For instance, if one were to bear a heavy memory load (i.e.

have a lot on their mind) while walking through a crosswalk of a busy intersection, it is important to understand what would be prioritized in order to safely make it across: thinking or walking? As per our results, we hypothesize that the individual would think critically before crossing as a form of reducing the additional memory load that comes with "looking both ways." In the real world, however, humans are much more complex. Some have heavier memory loads than others and may follow the cognitive hypothesis of pre-crastination, while others bearing lighter memory loads may follow the behavioral hypothesis of pre-crastination. Therefore, in order to further study such nuances of pre-crastination and its impact in decision-making, we must continue to explore this modest field of psychological research.

FUTURE DIRECTION, LIMITATIONS, AND CONCLUSION

Due to the COVID-19 pandemic, Experiment 3 was conducted via zoom. This caused some technical difficulties, e.g. losing internet connection in the middle of the experiment. There existed further limitations, which are further detailed in *Think Then Act, or Act Then Think*?, a publication currently in press to be published (Rosenbaum et al., 2022). In order to replicate this study in the future, testing a larger pool of participants may be necessary in validating our findings, as this current study overall had a small sample size (N=89) which may have increased the margin of error in our results. Ultimately, we hope results from our experiments will help bring more awareness to the study of our new theory of pre-crastination, which in turn may bring new discoveries regarding how decision-making is prioritized.

Research outlining pre-crastination and implications in cognitive-behavioral psychology have led researchers to extend experiments beyond human subjects and observe how animals behaviorally make decisions in their primitive form. In a 2015 study, researchers found that pigeons unequivocally would move locations sooner rather than later in order to be rewarded with food while following the double-response method (Wasserman and Brzykcy, 2015). Thus, similar outcomes regarding pre-crastination were observed in non-human subjects, albeit through the behavioral hypothesis. Such innovative experiments on animals' decision-making may be progressed in attempts to further understand decision-making at the elementary level, or perhaps even build on Wasserman's experiment to study animals through the cognitive hypothesis, yielding the bigger question – how do animals as a whole think?

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Evaluating Poly(anhydride-ester) Encapsulation Characteristics for Delivery of Hydrophobic Small Molecules

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ABSTRACT

Biodegradable salicylic acid-based poly(anhydride-ester)s (SAPAE) have proven to be effective in many biomedical applications including controlling inflammation, promoting bone growth, and preventing biofilm formation due to the release of salicylic acid upon hydrolysis of the polymer anhydride and ester bonds. Microspheres of SAPAE polymer are one fabrication option available for the encapsulation and controlled release of hydrophobic small molecules. This project aims to evaluate and characterize the ability for SAPAE microspheres to encapsulate, protect, and deliver retinol, a small hydrophobic molecule which is highly used in dermatological and cosmetic products for anti-aging purposes. The SAPAE of interest is a copolymer of salicylic acid (SA), adipic acid, and a diphenylene acetic acid (PAA). Due to supply chain limitations, the polymers used to form microspheres were of two variations, low molecular weight and high molecular weight. Nonetheless, this allowed for comparison of microspheres characteristics including size, morphology, and retinol loading efficiency. Through scanning electron microscopy (SEM), it was confirmed that the unloaded and retinol-loaded microspheres had a spherical shape, and the sizes were similar between the low molecular weight and high molecular weight polymer versions. Residual methylene chloride solvent was successfully reduced in all samples which increases the viability for biological applications. Finally, ultraviolet-visible spectroscopy detected a maximum of 4% w/v loading of retinol in the microspheres.

KEYWORDS: microspheres, salicylic acid, retinol

FACULTY MENTOR - Dr. Kathryn Uhrich, Department of Chemistry

Dr. Uhrich, a distinguished polymer chemist, is a Professor of Chemistry and the Dean of the College of Natural and Agricultural Sciences at UCR. She received her PhD in organic chemistry at Cornell University. Her work focuses on creating new materials and delivery systems with biodegradable polymers for various applications including drug delivery, food safety, and personal care. Currently in her lab, she has collaborations within the university, across the nation, and spanning the globe including partnerships with BASF through the California Research Alliance program. She has been issued over 70 U.S. and international patents and has been elected fellow of AAAS, ACS, AIMBE, CRS and NAI. She is also editor-in-chief of Journal of Bioactive and Compatible Polymers.

Kaitlyn Ngo

Kaitlyn Ngo is a fourth-year Biology major. She studied biodegradable polymers for various applications including medicine as a sustainable bioactive. Her research was conducted under the direction of Dr. Kathryn Uhrich and in collaboration with BASF and the California Research Alliance (CARA). Currently co-president of the Mustard Seed Project at UCR, a medical scribe in Loma Linda, and mentor for Big Brothers and Big Sisters of the IE. She plans to pursue a career as a pediatric physician.

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INTRODUCTION

Retinol, a naturally occurring derivative of Vitamin A, is widely used in cosmetic and dermatologic treatments for skin conditions including acne and psoriasis **(Figure 1)**. 1 This small, hydrophobic molecule is particularly effective as an anti-aging agent by minimizing the appearance of wrinkles and reducing hyperpigmentation on skin. [2–6] However, retinol's therapeutic potential is limited by its tendency to degrade upon exposure to ultraviolet light, heat, and oxygen. [7] This work highlights a biodegradable and environmentally friendly delivery system for retinol that would avoid this complication.

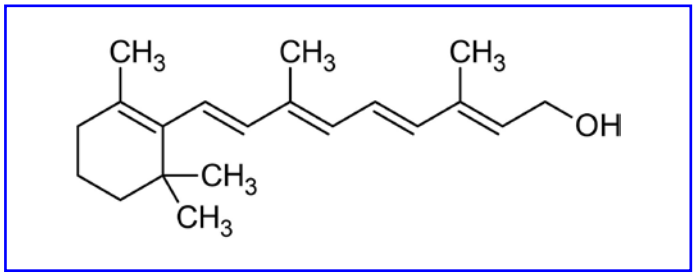

Figure 1. Chemical Structure of Retinol

Previously, our laboratory has reported the synthesis of salicylate-based poly(anhydride-ester)s (SAPAEs) homopolymer that release two naturally occurring compounds upon degradation, adipic acid and salicylic acid **(Figure 2)**.[8] Adipic acid is a biocompatible and biodegradable moiety widely utilized in biopolymers.[9–11] Salicylic acid imparts this material with important antipyretic, anti-inflammatory, and analgesic properties for use in a variety of clinical applications. [12]

Salicylate-based poly(anhydride-esters) (SAPAEs) can be copolymerized and formed into microspheres, which are of particular interest for drug delivery and controlled release of sensitive therapeutic agents such as retinol. The copolymer of SAPAE was synthesized in a 4:1 ratio of salicylic diacid monomer and phenylene diacetic acid linker **(Figure 3)**. In theory, the reduced steric hindrance surrounding the anhydride bonds in the copolymer should make the material more susceptible to degradation versus the homopolymer. The ability to alter the rate of degradation for SAPAEs provides a method for creating SAPAE microspheres that can be customized to release bioactive agents within various time frames based on the desired application.

Overall, the aim of this study is to encapsulate retinol and expand on what is known about SAPAE copolymers as a drug delivery system. It is hypothesized that microspheres made from the SAPAE copolymer will successfully encapsulate retinol while maintaining the characteristic smooth surface morphology of microspheres.

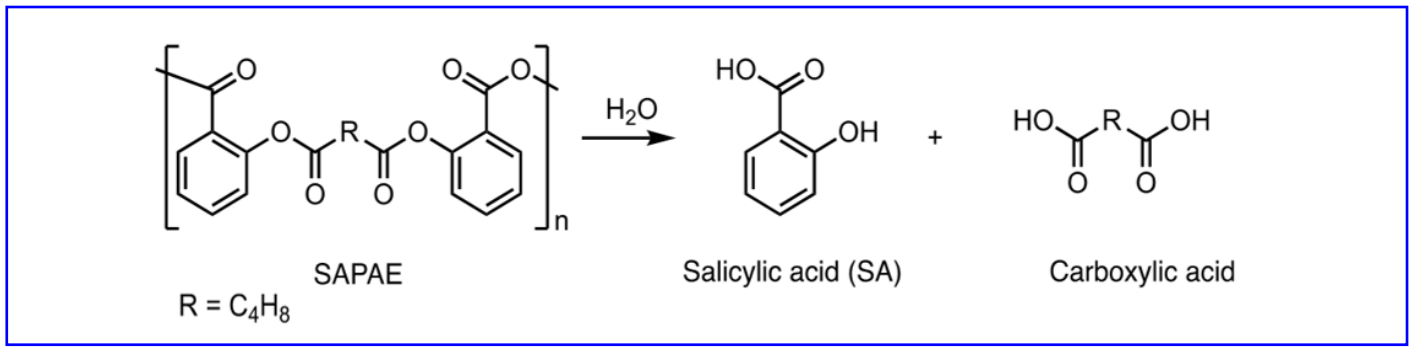

Figure 2. Degradation of SAPAE by Hydrolysis

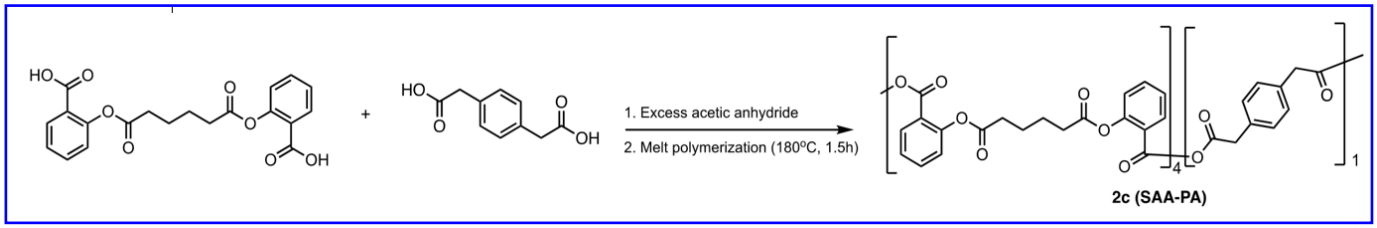

Figure 3. SAPAE copolymer with phenylene diacetic acid (SAA-PA) linker

MATERIALS:

Salicylic acid (Reagentplus®, ≥99%), acetic anhydride with high purity (Reagentplus®, ≥99%), anion traces (GR ACS, 97%), and higher purity (99.5%), poly(vinyl alcohol) (87-90% purity hydrolyzed, powder), anhydrous dichloromethane (>99.8% purity), and retinol (CAS 68-26-8, synthetic, >95% purity by HPLC) were purchased from Sigma-Aldrich. Acetic Anhydride (CAS 108-24-7) with 99+% purity was purchased from Acros Organics. Whatman™ filters Grade 40, 12.7mm) were purchased from GE Healthcare Life Sciences. Dimethyl Sulfoxide (>99.9% purity) was purchased from Fisher Scientific.

METHOD

Polymer Synthesis:

The SAPAE-phenylene acetic acid copolymer (SAPAE-PAA) were prepared based on a previously published method. [8] In brief, a salicylate-based diacid (MW 386.36, 2.0g) and with the addition of p-phenylene diacetic acid (MW 194.18, 0.25g) were added to an excess of acetic anhydride (40mL). The reaction mixture was stirred in a round-bottom flask in an oil bath, cooled, and then reheated while being mixed by an overhead stirrer under vacuum. Polymerization was complete once the viscosity of the melt increased and remained constant. A dark caramel color was also characteristic of a completed reaction. The polymer was cooled to room temperature, stirred with in a minimal amount of DCM to dissolve (~8mL), and precipitated in an excess of diethyl ether (~50mL) isolated, and dried overnight under vacuum.

Molecular Weight Determination:

Gel permeation chromatography (GPC) was used to determine the weight-averaged molecular weight (Mw) of the polymer prior to formulation. The TOSOH EcoSEC HLC-8320 GPC system contains a dual-flow refractive index detector and a porous stationary phase to allow for size exclusion of the polymer samples. The EcoSEC System Control and Analysis software was used to collect and analyze the size distribution of the polymer. The samples of polymer were dissolved in DCM (5-20mg/mL) and the solution was passed through syringe filters with 0.45um pore size. The elution rate was 0.6 mL/min for a total run time of 50 min.

Microsphere Preparation:

Microspheres preparation were based on previously published methods. [13] An oil-in-water emulsion solvent evaporation technique was used to prepare retinol-loaded microspheres with SAPAE-PAA. A retinol/SAPAE/DCM/PVA mixture was homogenized and the resulting emulsion stirred for 1 hour and 30 minutes at room temperature to evaporate DCM. The microspheres were collected by centrifugation at 1500 rpm for 2 minutes and washed three times with deionized water to remove residual PVA before filtering the solution to collect the solids. The samples were stored at -18ºC between further studies to minimize exposure to the environment including moisture. Non-loaded microspheres were prepared as a negative control via

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the same protocol without the initial addition of retinol.

Microsphere Size and Surface Morphology:

A minuscule sample of microspheres were distributed on aluminum studs, sputter-coated with gold-palladium for 20 seconds using a Cressington 108 Manual Sputter Coater and imaged with a TESCAN Vega3 SBH scanning electron microscope (SEM). The acquired images were useful for visualizing the surface morphology and size of the spheres. SEM images were analyzed with NIH ImageJ Software to measure the area of the microspheres. Subsequently, the mean particle diameter in the SEM image was calculated to compare the unloaded and retinol-loaded microspheres.

Retinol Concentration Determination:

When in a solution with DMSO, retinol had a unique absorbance peak in the presence of salicylic acid **(Figure 4)**. Using the Cary 60 Ultraviolet-visible spectroscopy (UV-Vis) and quartz cuvette, a calibration curve was created by measuring the absorbance of retinol dissolved in DMSO with a starting concentration of 200ug/mL. Nine more subsequent concentrations via serial dilution were also measured. The concentrations of retinol in the loaded microspheres were calculated by applying referencing the calibration curve based on an absorbance of 360nm.

RESULTS AND DISCUSSION

In this work, we demonstrate that SAPAE microspheres are capable of encapsulating retinol. Non-loaded microspheres were used as a negative control to compare the microspheres size and morphology.

Melt condensation polymerization yielded SAPAE's with varying molecular weights. In a batch of acetic anhydride with 99% purity, the molecular weight as measured by GPC was 8.0 kDa. However, a batch made with acetic anhydride with reports anion traces at 97% purity resulted in polymers with lower molecular weights of about 4.6 kDa. The predominant impurity in acetic anhydride is likely acetic acid which can hinder the formation of SAPAE's with the molecular weights that are 8.0 kDa or greater which has

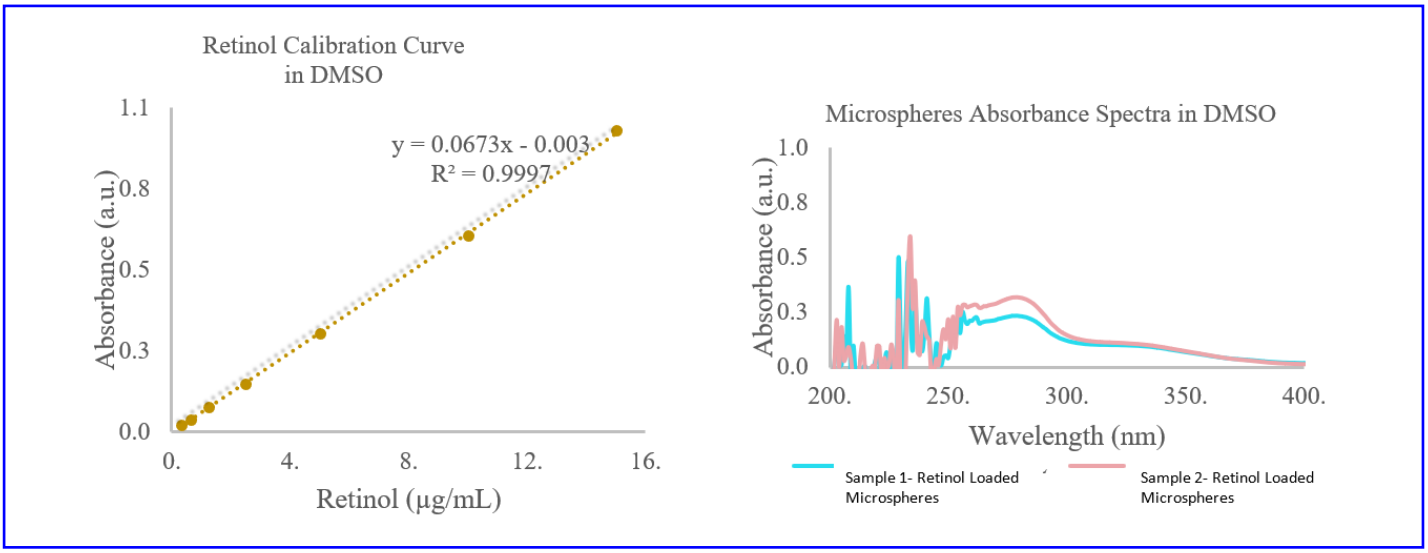

Figure 4. Ultraviolet-visible spectroscopy (UV-Vis) calibration curve for retinol in DMSO at absorbance of 360nm (**left**). Absorbance spectrum of retinol-loaded microspheres (20 mm/mL) in DMSO (**right**).

previously been consistent when using acetic anhydride with higher purity. Due to supply chain issues, only acetic anhydride with 97% purity was available. Nonetheless, the study proceeded with the understanding that the lower purity of acetic anhydride likely has had significant impacts on the quality of SAPAE's and ultimately for the subsequent microsphere studies.

An oil-in-water emulsion solvent evaporation method yielded microspheres with smooth, surfaces **(Figure 5)**. Two separate batches of microspheres, both with loaded and unloaded negative controls, were compared as they were made from SAPAE's with different acetic anhydride purities. Despite differences in the SAPAE molecular weight, the microspheres were similar in morphology and the unloaded microspheres had a greater average diameter than the retinol-loaded microspheres. In addition, both batches of loaded particles were smooth and spherical. On average the low-molecular-weight, unloaded microspheres had a smaller diameter, 9 µm, compared to 15 µm in the sample of high-molecular-weight, unloaded microspheres. The retinol-loaded microspheres were more similar in size between the low molecular-weight and high-molecular-weight microspheres, 7 µm and 6 µm respectively.

The precision of retinol loading studies in DMSO were verified with standards of known retinol and salicylic acid concentrations. Solutions of microspheres dissolved in DMSO were analyzed for retinol loading and the low molecular weight polymer and high molecular weight polymer had similar loading, 4.0% and 4.2% respectively **(Table 1)**. The attempted loading percentage of retinol in the microspheres was 10% by weight thus the experimental encapsulation efficiency is lower than expected. However, since retinol can cause skin irritation at high concentrations, the concentration of this strong bioactive in cosmetic products is between 0.0015% and 0.3%.[1] Thus, the retinol-encapsulating microspheres show potential for formulations at the current encapsulation efficiency. Future analysis could benefit from verifying that all the retinol added into the formulation is accounted for. This would require measuring the amount of retinol that remains in the solution of PVA and residual DCM before the washing step of the microspheres. Changes in the parameters of the microspheres preparation method can be made to achieve higher efficiencies. Even though such modifications have not been explored yet they can be evaluated in the future.

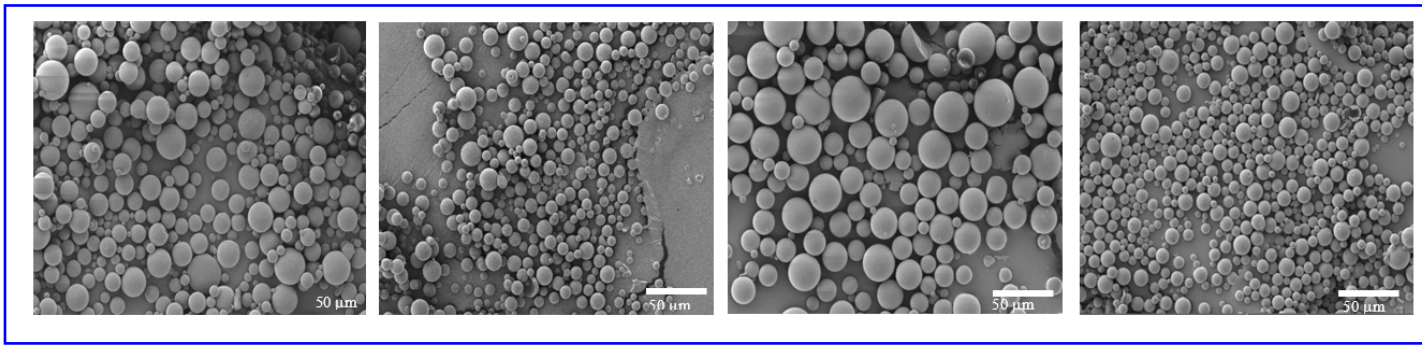

Figure 5. SEM images of microspheres, from left to right, made from SAPAE Mw 4.7kDa, unloaded and loaded with retinol, and SAPAE Mw 8.0kDa, unloaded and loaded with retinol.

Loading Calculation		
Microspheres Preparation	**Sample 1**	**Sample 2**
Abs at 360 nm (a.u.)	0.051	0.054
Calculated [Retinol] (µg/mL)	0.80	0.85
[Microspheres] (µg/mL)	20.0	20.0
Calculate %Loading	4.0	4.2

Table 1. Table of Retinol Loading Calculations including absorbance values by UV-Vis. 10% of retinol by weight was incorporated into the formulation mixture and 3.98% ad 4.24% were the calculated values for loaded retinol from Sample 1 and Sample 2, respectively.

CONCLUSION

In this study, we confirm that 2c SAPAE polymer can successfully form microspheres and encapsulate retinol at 4% encapsulation efficiency. Future experiments are aimed to improve the SAPAEs microspheres encapsulation efficiency. Retinol mass balance can also be done including measuring the amount of retinol left behind in the DCM/PVA solution in the microspheres' formulation mixture before the washing step. Other expansions of this current work include optimizing a standard addition method for quantifying salicylic acid in the retinol-loaded microspheres as well as evaluating the retinol release rate under varying media conditions.

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Transborder Realities: Its Effect on Bordertown Students Pursuing a Higher Education

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ABSTRACT

In this research I dive into the testimonies of five border town students who live on the U.S.-Mexico border in the transborder community composed of sister-cities, Calexico, California, and Mexicali, Baja California Mexico. The goal of this study is to use testimonials to help us understand the flaws within the educational site of Calexico High school as well as the limitations it imposes on transborder or border town students. Transborder Realities is a new type of journalism focusing on the stories of individuals as a way to bring forward the realities of many. This study unveils the intersectionality between social class, residency, and economic status that lead to social hierarchies in school, creating a division between students of different backgrounds. Each of the participants share personal experiences that greatly impacted them academically as transborder students, encounters that have not only led to struggles with their language, mental health, and career and educational endeavors, but also pushed them in search of better opportunities. This study brings to light the reality of being a transborder student in this culturally rich community, what that entails, and the effects it had in their pursuit of a higher education. These testimonials help to reveal the stigma faced within the educational community of Calexico in hopes of decreasing the mistreatment of transborder students pursuing higher education in the United States.

KEYWORDS: Transborder, Education, Mexicali, Calexico, Cross-culture, Bordertown

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"The first and the third world. The border. El bordo. Hell. The other part of the other side. The other side of the other side. The This side of the Other side. The happy world of disenchantment."

-*Gore Capitalism* by Sayak Valencia

INTRODUCTION: WHAT IS THIS TRANSBORDER COMMUNITY LIKE?

When coming to pursue my B.A. at the University of California Riverside, I was continuously asked where I am from. Such a simple question with so many answers. "Well," I'd say, "I'm from Mexicali, but I went to school in Calexico." They'd respond with "Oh, is that in Mexico?" I'd explain that I'm from a border town and that on one side of the border is the city of Mexicali where I resided, and that I would have to cross the border in order to attend school in Calexico, on the U.S. side. I would even explain the intentional play on words of these sister cities with the words California and Mexico, "You know Mexi-Cali and Cal-exico," but they would end up saying "So you're from Tijuana?" I would settle with that, since it would be easier than explaining the complexity of these little border towns on the U.S.-Mexico border. [1]

Mexicali, Baja California Mexico sits south of the border with approximately 1 million residents; a modern progressive city where the maquiladora business thrives (Almaraz, 2002). On the opposite side of the 27-foot border fence, made up of steel slats and barbed wire, is Calexico, California, a 98% Spanish-speaking community, home to 39,949 residents who are of predominantly Mexican or of Hispanic descent (U.S. Census Bureau). Calexico on the other hand, is a small town where agriculture and commerce thrive due to a great influx of workers and consumers coming from Mexico (Gutiérrez & Mabel, 2021). Within these sister cities there's an abundance of people crossing the border from Mexicali to Calexico to work, study, visit family, and shop. Likewise, people cross from Calexico to Mexicali to shop, visit family, as well as to purchase affordable medical services and pharmaceuticals. (Vega Briones, 2016). The relation between both cities can also be seen in their economies, where each depends on the other for their businesses and services to prosper (Gutiérrez & Mabel, 2021). Residents from either side of the border use their location to their advantage in the search of better opportunities and a more affordable life (Villarreal, 2016). Due to this close connection, I will use the term *Transborder* when discussing the border town communities of Calexico and Mexicali. I describe this area as a transborder community due to the manner in which both sister cities merge culturally, socially, and economically. The term borderlands could also represent this community; a *mezcla*[2] of being, neither fully of Mexico nor fully of the United States (Anzaldúa, 1987). I define transborder as the way in which the community is composed of an in-between borders of identities in terms of culture, linguistics, economy, etc., where everyone's identities is a *mezcla* of both cities. Not only are transborder relations used for economic advantages, but also educational. Students along the U.S.-Mexico border cross the border to pursue a higher education or even a better education than the one they would receive in Mexico. I am one of these transborder students.

I grew up in Mexicali, but crossed the border everyday to attend school in Calexico. Both my parents are Mexican citizens and residents who can't legally reside in the U.S., so they decided that it would be in my and my older sister's best interest to be born in the U.S.. My parents wanted us to be U.S. citizens so that we had access to many more opportunities through our dual-citizenship, which included attending school in the U.S.. My transborder experience is a reality for many others whose parents wanted the best possible education and

1 Italicized sections are my personal autobiographical experience.
2 Spanish word for mix.

opportunities for them.

My sister and I had the privilege of having our grandmother who lived in the U.S.. We were able to use her home address as our own for school documents which allowed us to have an education in Calexico, but that is not the case for many other students. Many students I went to school with came from Mexicali and had to find a loophole to this mandatory need of a U.S. home address in order to attend school. It is extremely common for non-resident students to use family or friend's addresses as their own in order to attend school in the U.S.. In extreme cases, parents would pay a monthly fee to known individuals who would allow them to use their home address as their child's own in school documents. Students in these situations would keep this quiet to avoid troubles in school.

According to the U.S. Department of Education and Justice, students must furnish proof of residency within the district in order to attend school. Although using an address where a student is not residing is considered fraudulent, it is frequently done within Calexico. Staff from CHS are aware of this but will turn a blind eye when it comes to hearing students out on issues they encounter as transborder students (for example, tardiness and financial situations). In this article, we will dive into the experiences of five border town students who attended school at Calexico High School (CHS), the only public high school in the city of Calexico, California. These CHS alumni have different migratory, socio-economic, and residential backgrounds and share their experiences within the Calexico educational system as well as their academic journeys. The goal of Transborder Realities is to use these stories to show the limitations that Calexico High school imposes on transborder students in hopes of decreasing the maltreatment of transborder students trying to pursue higher education in the United States. This complicated existence is often simplified by politicians on both sides of the border. By giving these students room to speak, my study helps to restore their lived perspectives to an often fraught debate.

METHODOLOGY

Due to the lack of research on transborder students within this location, I provide a window into their lives to bring exposure to the reality faced by many students of this transborder community on the U.S.-Mexico border. This is a type of new journalism in the style of Rodolfo Walsh and Elena Poniatowska, which focuses on the stories of individuals as a way to bring forward the realities of many. This approach is best suited for the testimony genre because it is "a genre defined by the work of personal witnessing on behalf of a collective struggling against injustice," a pivotal goal of my research (McEnaney, 2020). I interviewed five transborder student alumni with different residential and socio-economic backgrounds, from either Calexico or Mexicali, who experienced injustices within the educational site of Calexico High school. I conducted an auto-ethnographic research which uses the testimony genre to provide a collective voice for students' in this community. By focusing on the stories of these individuals, I hope to restore their voices in a discussion that often silences them. I welcomed these transborder students to share their first-hand experiences in order to explain the reality of residing in such a complex and culturally rich community. The names of the participants involved have been changed to respect their privacy. Being a part of this transborder community myself allows me to voice their lived realities, but to keep a consistent voice throughout the text, my personal experiences and anecdotes will be italicized. The focus of this research is to show the structural issues embedded within the educational institution in Calexico; therefore, all interviews were conducted in-person and participants were encouraged to confide and speak freely in order to make this research as raw and honest as possible.

"MEXICALI KIDS" VS "CALEXICO KIDS"

When interviewing the Calexico alumni on their educational

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experiences, two terms that were repeatedly used when referring to students who came from Mexicali and crossed the border for school and those who resided in Calexico, were "Mexicali kids" and "Calexico kids." To better understand what each of these classifications entail, we will look at testimonials from three students with different residency backgrounds, all U.S. citizens, who attended school at Calexico High school. First we have Miguel and Gustavo, Mexicali residents who had to cross the border everyday in order to attend school, both considered "Mexicali kids." Then we have Camila, a Calexico resident who can be considered a "Calexico kid."

Being a "Mexicali kid" is much harder than many may think. A usual day for a "Mexicali kid" begins early in the morning at 4 or 5 A.M. with a seemingly normal morning routine where they get dressed, brush their teeth, and if they have time grab a bite for breakfast. Then at around 5:30 A.M. they head out to la linea, the U.S.-Mexico port of entry, where one can cross through the vehicle or pedestrian port of entry. "Mexicali kids" commonly cross through the pedestrian port of entry alone since most of their parents may not have papers, time, or a car to cross with them. At the *linea*, wait times range from 30 minutes to 2 or more hours. Upon arrival, students may see familiar faces of other students who are also crossing to get to school, but may also see a large number of field workers, mothers with babies, small children with their parents on their way to school, employees trying to get to their jobs, etc. A variety of different people all trying to get to the same place, across the dreadful *linea*.

Miguel and Gustavo

"Mexicali kids" carry around a stigma of being lazy, dumb, and trouble-making, but Miguel and Gustavo are anything but that. Just like many other "Mexicali kids," Miguel and Gustavo reject this unfair stigma placed upon them since their arrival to the U.S.. Miguel is currently a third year mechatronics engineering student at the California State University, Chico, with a rather positive view of life, and Gustavo is a 22 year-old U.S. reserviced Marine. Miguel and Gustavo both resided in Mexicali throughout their educational journeys, and both managed to go to school in the U.S. with the help of friends or family who allowed them to rent their home addresses as their own for school documents.

I began the interviews by asking them to tell me about this transborder community. Miguel's thoughts on the community are rather positive, "I really enjoy the community aspect, because it has opened many doors for people like me that have been raised in Mexico but can go to school in the United States." He says that although crossing the border comes with an abundance of problems, one can achieve their goals and better themselves by crossing the border daily. Gustavo's opinion is quite different, "When I cross to the U.S., I feel stressed, and when I go back to Mexicali, all that stress goes away." This is something that myself and many other "Mexicali kids" relate to. It is tedious to cross the border and fulfill whatever affairs in the U.S., but being home in Mexicali feels like a breath of fresh air after the journey many of us endure to get to work or school. Gustavo even says that living a transborder lifestyle took a toll on his mental health, "It really does mess with my mental health because there's so many things I stress about because I can't get things done in Mexicali that I have to get done in Calexico."

Miguel and Gustavo talk about the ways in which they adapted and managed their time in order to be able to live a transborder life. Gustavo tells us how his days would begin, "One port of entry opens up at 6 A.M., so what I would do to make sure I got to school on time, is wake up at 3 or 4 A.M., drive to the border and just sleep in my car and wait until the border opens up." He mentions how this routine got so tedious that it unmotivated him to attend community college, and recalls his educational experience being negative due to the fact that he had to cross the border everyday for

school. Miguel's mornings were a bit different and began in Mexicali at 5 A.M. in order to make it to the pedestrian *linea*[3] on time, and school by 8 A.M. Not only did Miguel and many other students wait in line for hours, but once they finally crossed the border, they had to endure a 20 to 30 minute walk to school. Miguel also mentions "La Bestia," a train that goes through Mexico and the U.S. that when static, blocks ongoing traffic, guaranteeing a late arrival to school. Miguel wasn't fond of this train, but recalls finding a way around this issue. He explains that once the train was static, he could jump over or through the train and get across to the other side, allowing him and others to get to school on time. He says "There were times in which your legs would get full of oil from the train but at least you'd get to school on time." Miguel emphasized his priority of making it to school on time so that the school would not call the home phone number on record and bother his 'Tia.' Miguel adds that another worry of his with being late, was the school beginning to suspect that he was living in Mexico; thus, his priority was making it to school on time to avoid troubles. Miguel's after school journey back home was also tough. He recalls missing out on extra curriculars because he would have to be up extra early in order to participate in them, "I couldn't be a part of clubs because I would have to be up at 3 A.M. and sleep at 2 A.M. There wasn't enough time." The only club Miguel participated in was robotics, a club he enjoyed and was willing to make sacrifices for, which also allowed him to discover his career path of choice. Miguel would stay after school in robotics until around 7 or 8 P.M., and at times would have to endure a 3 hour walk back home. Miguel looks on the brighter side and states that although the 3 hour walk back home was arduous, it allowed him to think about his future goals and would make the best of those very long 3 hours.

Miguel and Gustavo acknowledge that their experiences were not unique, and that if they were making 3 hours of line in the morning, it was because many other students were also on their way to school. Miguel mentions that at times he'd see a peer and would allow them to cut in line and vice versa, and that with his charisma, he befriended Custom Border Protection officers who would allow him to cut to the front of the line. He says that throughout those 5 years everyday was different yet the same. He would see the same people/community cross the border with him, which made him feel that in this experience he was never alone.

Miguel also recalls coming to school for the first time in the U.S. in the 8th grade, where his English abilities and class placements were determined by a newspaper. Miguel recalls this experience, "El único examen que requerí para entrar a middle school, fue leer un periódico. Me preguntaron '¿Qué tanto sabes del inglés?' Y el nivel para medir mi inglés fue leer un periódico. Lo leí en Inglés, y me dijeron 'Tú vas a ELA y estas van a ser tus clases.' A partir de ese momento, mis clases eran completamente en Inglés. Un periódico no define cómo iba a entender mis clases, y tuve que desempeñarme para entender bien mis clases en inglés y leer hasta que le agarre.[4]" From word of mouth, this is a common way in which Calexico schools place non-native English speakers in their classes. Shockingly, a single short assessment based on a newspaper was the determining factor on a student's comprehension and ability with the

3 Spanish word for "line." Pedestrian line refers to the way in which crossing the border was done on foot rather than in a car.

4 The English translation of Miguel's testimonial states, " The only exam required to attend middle school was reading a newspaper. They asked me 'How much English do you know?' and the level to which my English was measured was reading a newspaper. I read it in English, and they said, 'Okay, you're going into ELA and these are your classes.' From that moment on, my classes were completely in English. A newspaper could not define how I would understand my classes, and I had to try

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English language.

Miguel described the distinction between the Mexicali and Calexico kids in a way that many other "Mexicali kids" relate to. Miguel recalls taking his first AP class, but what he wasn't expecting was in his words "A whole new world that I did not know due to the fact that I had regular classes. Y vaya sorpresa que me lleve."[5] Miguel realized that within the AP classes were students that were much more entitled than him. He continues, "Ahi note que menospreciaban a mis amigos y a mi solo por ser conocidos como 'Mexicali kids.[6] This is a shared feeling from the "Mexicali kids" towards the "Calexico kids", where "Mexicali kids" can't comprehend where this entitlement from the "Calexico kids" comes from. Miguel points out how ignorant this entitlement is even though we are all the same, Mexican, and in his words "His own blood."

CAMILA

Now we have a common reality for many "Calexico kids" through Camila, a 21 year old nursing student at both Arizona Western College and Grand Canyon University, who resides in Calexico. Camila describes the transborder setting through instances in which she has seen both cities come together, "Both cities unite a lot. On the U.S. side, there's a shopping outlet that holds events and concerts and our sister city Mexicali tends to watch on their side. Since the fence is a little see-through, Mexicali people can watch what's going on our side. There's times where there's people on both sides of the fence listening to music or when they have family members that can't cross you see them speaking through the fence. I also saw that there was a fire on the Mexicali side of the border and the Calexico fire department helped Mexicali firefighters spray down the fire, so I see a lot of unity."

Camila is no stranger to the fact that many students cross the border to attend school in Calexico, but her thoughts regarding "Mexicali kids" were mostly negative, "With all these kids crossing the border you would sit there and think that if our school never allowed these kids to come, our school would have 100 kids because more than half the students were coming from Mexicali. They had a stigma that not everyone else had, that they were lower in test scores and less knowledgeable." She notes that her educational experience was not very memorable due to the environment that the "Mexicali kids" created, "There's a lot of people who aren't from Calexico, so they don't care. They come to our school and do whatever they want…. How they say it here, the 'Mexicali kids' are known for being bad and not caring about school. You were trying to go to school and have this high school experience and go to the sports games and nobody would show up because students are from Mexicali, they're back home in Mexico." Camila also sympathizes with the "Mexicali kids," "I definitely felt bad for all the students that had to walk, especially here, we have summer year round so it's very hot. You'd see kids of all ages walking to the border. Schools out and everyone's headed walking to the border and still have to cross, wait for their parents, and adjust to whatever situation they're in. I felt bad because I got to ride in the car with AC and go straight home. It took me 5 minutes to get home and for all these kids it would take them an hour or 2 to get home across the border. To know you're gonna get home until hours later I bet sucks after having a full day at school."

Camila's experience at a school in a transborder community was very common amongst "Calexico kids." "Calexico kids" can be more easily thought of as regular students in

5 English translation of Miguel's testimonial, "And what a surprise that was."
6 The English translation of Miguel's testimonial states, "That's when I noticed they belittled my friends and I solely because we were known as 'Mexicali kids."

California who just happen to have the benefit of residing next to Mexico. The students interviewed agree that there was a division between both groups, as well as a type of competitive and hostile environment between them. All three "Mexicali kids" interviewed recall instances where they experienced a variety of microaggressions from the "Calexico kids," who had a sort of superiority complex due to the fact that they resided in Calexico. Micro-aggressions towards "Mexicali kids" included being called: beaners, paisa, tecolin, naco, etc., regardless if we were all Mexican. When looking at this division between both parties, I come to the conclusion that it ultimately comes down to the intersectionality of both socio-economic status and residency that causes this division to be reinforced. By intersectionality, I refer to Kimberle Crenshaw's (2015) definition, "Intersectionality is an analytic sensibility, a way of thinking about identity and its relationship to power" (Crenshaw, 2015). All in all, "Calexico kids" showcase that they are better than the "Mexicali kids" because they have access to U.S. resources, which may include a home and transportation, both commodities representing socio-economic status. "Calexico kids"' identified as superior and held pride in the fact that they had access to a home and better life in the U.S., something the "Mexicali kids" didn't have access to. These advantages lead to a sort of power relation between both groups, as well as the demeaning of "Mexicali kids." All things considered, there's a clear differentiation within both of these groups which to this day persists at CHS.

EXPERIENCES IN THE EDUCATIONAL SYSTEM OF CALEXICO

When doing these interviews, I asked the participants to tell me about a positive or negative experience that they have had within the Calexico educational system, and not surprisingly, most could only recall negative experiences. Students agreed on the low quality of teaching from staff at CHS and had negative anecdotes about school staff ranging from advisors, to teachers, to even principals. Camila expresses what it felt like to try and thrive academically in Calexico, "You do good here and they don't care, but when one would do bad, teachers would say 'I knew it' because that's what they expect out of us. It's an ongoing cycle where the staff doesn't care, so the kids don't care, and since the kids don't care, the staff cares even less." A shared negative experience between students, was CHS's push to solely apply to the community college in the valley, Imperial Valley College. Every student interviewed, including myself, were forced to apply to this community college, and when mentioning applying to other schools, they were discouraged. Valeria is a 4th year student at the University of California San Diego majoring in developmental psychology, and shares a negative experience with a member of the Talent Search Program, whose job is to encourage students to pursue a higher education. When receiving her acceptance letter, Valeria was torn on going to a UC or staying in Calexico, and because of this she sought advice from one of the Talent Search advisors. When telling her about her acceptance and indecisiveness on her next step, the advisor told Valeria to stay in Calexico, "Because 'If you stay it's easy. You can live with your parents and do volunteer work. When you graduate here you're basically gonna get out with a job, but if you go to a UC or farther, you're gonna struggle a lot because most institutions, especially this one, are research related, and you're a psychology major'…. She said that if I attended that school and came back, I wouldn't find a job here since I wouldn't know anybody."

This is a common experience at CHS and I experienced something similar my junior year. I was called into my counselors office and he asked what school I wanted to attend after high school. I had a 3.5 GPA at the time which would allow me to apply to 4- year institutions, so I told him I was interested in UC's. This counselor laughed and told me that with my "extremely low GPA" that was

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impossible, and to just go to IVC. I was devastated and set on going to community college because of this; but thankfully, I attended a field trip to UCLA hosted by UCLA students who were also Imperial Valley alumni. This trip allowed me to understand that going to a 4-year institution was more than possible for me, and here I am about to graduate from a 4-year university 4 years later.

CHS staff exclusively promoted attending Imperial Valley College, and kept students within an educational box which often led to us second guessing our decision to pursue a higher education. In the Calexico academic environment, staff would rather tell students what to do and where to go when choosing the next step in their educational endeavors, rather than presenting them with an array of different career choices and universities where their career paths could take place and allowing them to decide what to do based on the options out there. Calexico students weren't allowed to even fathom the idea of going to a 4-year institution, and instead were presented with an easier and more limiting option of attending community college. Although community college is not a bad path to take, when it comes to Imperial Valley College, it is a very limited one. With a limited number of Associate Degree areas of studies, IVC could be considered more of a stepping stone to furthering one's education. Students interviewed agree that IVC has a bad reputation in general where students who attend, take classes primarily for the large financial aid check they receive. There's also the San Diego State University Imperial Valley campus located in Calexico, where students can have dual enrollment in both IVC and SDSU and graduate with a B.A. The only downside is the limited majors available and the lack of STEM related majors. The lack of STEM related majors isn't abnormal, since STEM related career paths aren't common in the area, thus would not thrive.

LANGUAGE IN CALEXICO

> "The town's like a mirror twin of our own, With Spanish spoken everywhere just the same but English mostly missing till it pops up Like grains of sugar on a spicy pepper."
>
> -*They Call Me Guero*: A Border Kids Poems, by David Bowles

Calexico is a predominantly Spanish-speaking community where students and staff speak Spanish or understand it. Camila mentions the abnormality of not knowing how to speak Spanish in Calexico, "Everyone here is Hispanic, if you don't know Spanish here, even though we live in the U.S., people are like 'What? You don't know Spanish?'" CHS staff is even praised for their bilingual abilities, although being a bilingual teacher in this community should be necessary in order to equally communicate with all students (Lockwood, 1996). Code-switching is also very common amongst this community, and is seen when one alternates between Spanish and English at the same time within a casual conversation. (Holguín, 2018) One would think that bilingualism within this community would be praised and welcomed; however, it is a shared experience for students to be told to not speak Spanish because "We are in the U.S.." Students with accents or mispronunciations, specifically "Mexicali kids," were mocked and ridiculed by peers and staff, which often led to students becoming embarrassed when speaking English.

Majority of microaggressions were done between students, but also by staff in school. By microaggressions I refer to racial microaggressions which are "Brief and commonplace daily verbal, behavioral, or environmental indignities, whether intentional or unintentional, that communicate hostile, derogatory, or negative racial slights and insults toward people of color," or in this case minorities being the "Mexicali kids," since Calexico is predominantly

Mexican and fully a community of minorities and P.O.C. (Sue, 2007). I consider this a relational power dynamic in which we can see that within the same group of minorities, these students differentiate themselves through their abilities and pronunciations of the English language, and all become a part of these social hierarchies among themselves by doing so.

With that being said, Daniel's story is particularly adequate to explain the way in which Spanish is shamed at CHS. Daniel, a 21 year-old fourth year student majoring in structural engineering at the University of California San Diego, tells us: "During my senior year of high school, I was one of three people selected to audition to give the graduation speech at commencement. I decided to do my speech in Spanish because we live in a community where 99% is Hispanic or Mexican, where not even 50% of the people here speak English, so it would be ignorant to say it in English when majority of the public wasn't gonna be able to understand it." Daniel auditioned in front of 3 CHS staff, and states, "During my audition I instantly got a feeling that everyone was judging me, and at the end I got rejected and the other two who presented their speech in English got selected. After the audition, the only question asked by the staff members was why I decided to make my speech in Spanish, which was weird since the other auditions were quick with no questions asked. After that, I talked to one of the judges and she told me that I was a *chicalon*[7] and I should stick to what chicalones do, which is not pursue a higher education, since we 'chicalones' aren't able to fit into that environment." In a predominantly Spanish-speaking community where the Spanish language should be praised, it is instead ridiculed and shamed. The person who made this comment was the vice principal of the school, with a last name of Mexican origin whom we will call Mrs. X. It is incredible to think that one of our own would harass a student for the use of their native language.

Experiences with authoritative figures like these can have a detrimental effect on students' self-esteem, self-worth, and also follow them throughout their lives, something Daniel can tell us more about, "My last year in high school made me feel like I was not ready to go out into the world. During my transition to college it caused many problems. It lowered my self-esteem in regards to my language, caused me mental health problems, including depression and anxiety. Those comments still haunt me." Instances like these also make students feel unworthy, Daniel recalls how this incident made him feel embarrassed about his language during job interviews, "Everytime I tried to talk to professional people, I second guessed myself, my language, and everything I was going to say. I saw the employers and connected that professional image to Mrs. X and I thought I was gonna be judged on my language as well from them, which was thankfully not the case." Daniel's story shows the severity and impact that comments made by staff at CHS have on their students. If this happened to Daniel and went unnoticed, how many other students have endured similar experiences like this one?

CONCLUSION: WHAT DO STUDENTS TAKE FROM THESE EXPERIENCES?

Transborder students can come to a collective agreement that living in a transborder community is culturally beneficial. Looking at the community as a whole led me to a deeper understanding of how socially knit these sister-cities are. There's a significant influence from both sides of the border to each other, and looking at the bigger picture, the relationship between them is incomparable. The play on words of both sister-cities' names couldn't be a more perfect way of representing the relationship between them. So when

7 The word "chicalon" is a derogatory term for an unrefined Mexicali, Baja California Mexico resident. This word is not to be confused with the word "Chicano."

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finalizing interviews I asked everyone the same question: Would you return or raise your children in Calexico taking into consideration your educational upbringing here? To my surprise, every participant responded with a no. They all said that they enjoyed the close relation to their Mexican roots, but that due to their educational experience there, they would have to say no. Daniel states, "I would love that my kids have the cross-cultural or the Mexican culture involved in their lives; however, this would be a big no for me since I've been exposed to how corrupt and cruel the educational system here is. I don't think I will ever come back to Calexico." Miguel states, "Regresaría porque me gustaría que, si yo tuviera hijos, ellos realizaran que el mundo esta cabron y estuvo pesado este journey."[8] Valeria shares a similar answer by saying, "The only reason why I would raise my kids here would be for them to experience what I did, which is to have their family close, but I would really raise them somewhere else." Gustavo stands his ground saying, "No because I've lived here and I know the struggle. I want my kids to be successful, which is clearly not something they can acquire here." Lastly Camila explains how she is torn in her decision but ultimately says, "No, but this is something I think about all the time. Your heart feels at home here, but you grew up here and saw how toxic this place was and how there's no opportunities. I wish I could reside somewhere more beneficial to me." Students choose not to return to Calexico due to negative educational experiences, but a positive aspect that comes from these negative experiences, is the desire for all these students to better themselves. The participants shared how these experiences make them never want to return to Calexico, which has helped them reach for the stars when it comes to their current academic and career paths. In a community where academic goals are limited and belittled, Calexico students choose to better themselves in order to reject the stigmas that come with being a transborder student.

Reflecting on the methodology used in this study, I look at the individuals who participated in this research. A goal of mine was to not only include students who resided in Mexicali and went to school in the United States, but to present those who have resided in Calexico their whole life. By including students with different residential and socio-economic backgrounds, we can compare the educational experiences of both groups of students and acknowledge the major differences between their experiences at Calexico High school. The gaps of information regarding transborder students' experiences in Calexico motivated me to show the reality of their lives and the obstacles they had to endure to get to where they are now. The lack of research on students in the area has, in a way, aided the persistence of negative treatment towards them. Therefore, I have presented their stories and voices as a way to unveil the negative treatment present within the educational community of Calexico, in hopes of validating their experiences and decreasing the mistreatment of transborder students trying to pursue a higher education.

To conclude, I return to the term transborder. This concept of being in-between borders is a foreign concept to many, but to us and many others, it is our reality. When thinking about this community I welcome you to reimagine that 27 foot fence made up of steel slats and barbed wire. For transborder students that fence is not a restriction, but rather an open door to many opportunities. This fence has large gaps in between itself, through which one can see right through either side. Each of these gaps allows a hand to touch a family member on the opposite side. A gap through which music from either side flows smoothly to the other. A gap through which opportunities flow through and allow

8 English translation of Miguel's testimonial: "I would return because I would like, if I had kids, for them to realize that the world is tough and that this journey was hard."

us to become who we are. Gaps that allow both communities to merge culturally, socially, and even economically, rather than separating us. Gaps through which students have the opportunity to attend school on the other side of the border. We transborder students reached through those gaps. Rather than being blocked by barriers, we walked right through them and chose to sacrifice for an incredible result. Transborder students are living proof of surpassing any fence or border in our way in order to better ourselves.

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Effects of Parenthood on Neural Responses to Pup-Related Cues

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ABSTRACT

The onset of parental care in female mammals is associated with plasticity in neural processing of infant-related sensory stimuli, which enhances mothers' ability to detect and care for their offspring; however, little is known about sensory plasticity in fathers. We tested the hypothesis that parenthood alters neural responses to olfactory and auditory stimuli from infants in male and female California mice (*Peromyscus californicus*), a biparental rodent. Virgins and new parents of both sexes were exposed to a combination of a chemosensory stimulus (pup-scented or unscented cotton [control]) and an auditory stimulus (pup vocalizations or white noise [control]). Brains were collected one hour later and stained immunohistochemically for Fos, an index of neural activity. We quantified Fos in the main olfactory bulbs (MOB), a region essential to receiving olfactory information, and medial preoptic area (MPOA), a region critical for parental behavior. We predicted that Fos in MOB and MPOA would be greater in parents than virgins, especially after exposure to pup stimuli. We found that in females, MPOA and MOB Fos did not differ between virgins and mothers or across treatment groups. In contrast, fathers had lower expression of Fos in MOB but higher expression in MPOA, compared to virgin males. Moreover, Fos in MPOA was higher in males exposed to pup vocalizations and pup scent compared to those exposed exclusively to pup vocalizations. Fos in MPOA was also higher in males exposed to scent or both scent and vocalization stimuli compared to males exposed to control stimuli. These findings suggest that the onset of parenthood alters activity in the MOB and MPOA, especially in response to pup vocalizations and scents, in males but not females in this biparental rodent.

KEYWORDS: audition, biparental care, brain, California mouse, neural plasticity, olfaction, parental behavior

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INTRODUCTION

Maternal care, or the care of young by their mother, is essential for offspring survival in all mammalian species. The onset of maternal care is associated with neural plasticity in the mother's brain, mediated by hormonal changes that mothers experience during pregnancy, parturition and lactation (Horrell et al., 2021). Neural plasticity refers to the reorganization of neural pathways in the brain and can include changes in the production, survival, morphology, and activity of neurons and synapses. These structural and functional changes in the brains of new mothers can enable mothers to behave appropriately toward their offspring.

Paternal care – i.e., the care of offspring by fathers – is also important for offspring survival and development in some mammals, including humans; however, relatively little is known about the neural mechanisms underlying the onset of paternal behavior. California mice (*Peromyscus californicus*) are one of the few mammals that are biparental, meaning that both parents provide care for their offspring (Gubernick et al., 1987); thus, they are a useful model for examining the neural mechanisms underlying the onset of parental care in both sexes. Both mothers and fathers in this species are strongly attracted to their offspring and begin to huddle, lick, and carry pups shortly after parturition. Parents are also strongly attracted to and nurturant toward unrelated pups (de Jong et al., 2009; Perea-Rodriguez et al., 2015; Horrell et al., 2017; Perea-Rodriguez et al., 2018). In contrast, virgin adult males and females often avoid or attack experimentally presented pups (Gubernick et al., 1994; de Jong et al., 2009; Horrell et al., 2017; Nguyen et al., 2020).

One type of neural plasticity associated with the onset of offspring care by mothers is sensory plasticity, which refers to neural changes in pathways involved in detecting and processing sensory stimuli. For example, in CBA/CaJ house mice (*Mus musculus*), new mothers undergo changes in the brain's auditory pathway that enhance their ability to detect pup vocalizations (Dunlap et al., 2020). In addition, during the onset of motherhood, some mammals, including house mice, have a significant increase in neurogenesis (i.e., production of new neurons) in the olfactory bulbs, the first brain regions that receive information about olfactory stimuli (Medina & Workman, 2020). Some evidence suggests that fathers, too, undergo plasticity in sensory systems. For example, increased neurogenesis in the olfactory bulb has been found in C57BL6 house mouse fathers (Mak & Weiss, 2010), similar to mothers. However, sensory plasticity in fathers has received very little attention, especially in biparental species. Characterizing this plasticity in fathers and elucidating the underlying neural and neuroendocrine mechanisms would enhance our understanding of both the effects of fatherhood on the brain and, conversely, the neural processes underlying the onset of paternal care.

This study investigated the neural pathways that are activated in response to pup-related sensory stimuli – pup scents and vocalizations – in male and female California mice, as well as the effects of parenthood on these neural responses. Neural pathways involved in olfactory and auditory processing interact with pathways involved in the onset of parental care, suggesting that olfaction and audition may be important sensory modalities for parental care, at least in rodents (Kuroda et al., 2011; Horrell et al., 2019; Wilson et al., 2022). The main olfactory bulbs (MOB) are essential for the detection of chemosensory stimuli and may have downstream effects on parental behavior by sending information to other brain regions. One such region is the medial preoptic area (MPOA), which is crucial for parental behavior in both males and females (Numan et al., 2005; Akther et al., 2014; Bales & Saltzman, 2016; Horrell et al., 2019). The MPOA also receives information from the auditory system, suggesting

that sounds, in addition to scents, can influence parental care. Therefore, we tested the hypotheses that the MOB and MPOA of male and female California mice have greater neural responses to pup sensory stimuli compared to control stimuli, that parents have greater neural responses to pup sensory stimuli compared to virgins, and that new parents exposed simultaneously to auditory and olfactory stimuli have greater neural responses compared to those exposed to an isolated stimulus.

METHODS

Animals

Subjects were descended from California mice purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA) and were bred at the University of California, Riverside (UCR). Mice were housed in polycarbonate cages (44 × 24 × 20 cm) under standard laboratory conditions (Nguyen et al., 2020). At weaning age (27–31 days), before the birth of younger siblings, animals were removed from their parents' cages and housed in groups of 3-4 same-sex, age-matched mice until used in the study.

We used 32 breeding pairs housed with their first litter of pups and 29 virgin pairs consisting of a reproductively inexperienced male and an ovariectomized female. Ovariectomies (i.e., surgical removal of both ovaries) were performed as previously described (Zhao et al., 2018; Andrew et al., 2019), 10 days prior to pair formation. Breeding females underwent sham-ovariectomies at the same time point to control for non-specific effects of surgery.

Stimulus Exposure

Each adult mouse underwent a single stimulus-exposure test (**Table 1**). The subject was placed alone in a clean cage for 110 minutes to allow it to acclimate to the cage. An auditory stimulus and a chemosensory stimulus were then introduced for 10 minutes. The auditory stimulus consisted of either pre-recorded vocalizations from an unrelated pup or white noise (control), and the chemosensory stimulus was either cotton containing the scent of an unrelated pup or fresh cotton (control); the cotton was contained in a wire mesh tea ball (Ø: 8 cm) to prevent the mouse from handling it. To obtain the pup scent, an unrelated 3- to 7-day old pup was wiped with a cotton ball 30 times across its ventrum and anogenital region. Tests were conducted in a sound-attenuated room at 08:00-09:00 h. We performed tests during lights-on, which is the inactive period for nocturnal animals, to reduce the amount of background neural activity. Parents were tested 4-6 days after the birth of their first litter, and virgins were tested at a comparable age and time point. Male and female pair mates were tested on the same day, with the female's test beginning 20 minutes after the beginning of her mate's test.

Pup Stimulus	Stimulus Pair	Male Sample Size	Female Sample Size
Pup Scent	White noise + **Pup cotton**	8 fathers, 8 virgin males	8 mothers, 6 virgin females
Pup Call	**Pup calls** + Fresh cotton	8 fathers, 6 virgin males	8 mothers, 8 virgin females
Pup Call + Pup Scent	**Pup calls** + **Pup cotton**	8 fathers, 8 virgin males	8 mothers, 8 virgin females
Control	White noise + Fresh cotton	8 fathers, 7 virgin males	8 mothers, 8 virgin females

Table 1: Experimental design

Effects of Parenthood on Neural Responses to Pup-Related Cues

Brain Collection and Immunohistochemistry

To determine neural responses to stimuli, we quantified expression of Fos, the protein product of the immediate early gene c-fos, in the MOB and MPOA of male and female subjects. c-fos and other immediate early genes are expressed in neurons during genomic activation, beginning approximately 1 hour after exposure to a stimulus, and their protein products can be used as indicators of neural activity (Kovács, 2008).

One hour after the end of stimulus exposure, mice were deeply anesthetized with pentobarbital and euthanized via transcardial perfusion with cold phosphate-buffered saline (PBS 0.1M) and paraformaldehyde (PFA) (de Jong et al., 2009). Brains were harvested and stored for 48 hours in 4% PFA at 4°C. The tissue was then moved to a 30% sucrose solution until fully saturated, then cryoprotected and stored at -20°C.

Brains were sliced into 40 µm-thick coronal sections using a cryostat. Sections were stained immunohistochemically for Fos using Anti-rabbit c-Fos antibody followed by Alexfluor 555 as the second antibody, allowing for visualization of Fos-positive cells as green in color. Stained tissues were mounted onto slides and imaged with a Zeiss 880 inverted confocal microscope. Lastly, a researcher blind to the stimulus treatment quantified Fos-positive cells using QuPath software (Bankhead et al., 2017). Fos-positive cells were counted in a square section with an area of 200 x 200 μm^2 within the MOB and MPOA.

Statistical Analysis

Fos data were square-root transformed and analyzed by linear mixed-effect models. The model for each brain region included reproductive status, stimulus treatment, and their interaction. Data from males and females were analyzed separately. Significant effects and interactions were further investigated using pairwise post-hoc comparisons. We did not compare the sexes directly because male and female brains were processed, stained, and imaged in different time periods, which might have affected Fos expression.

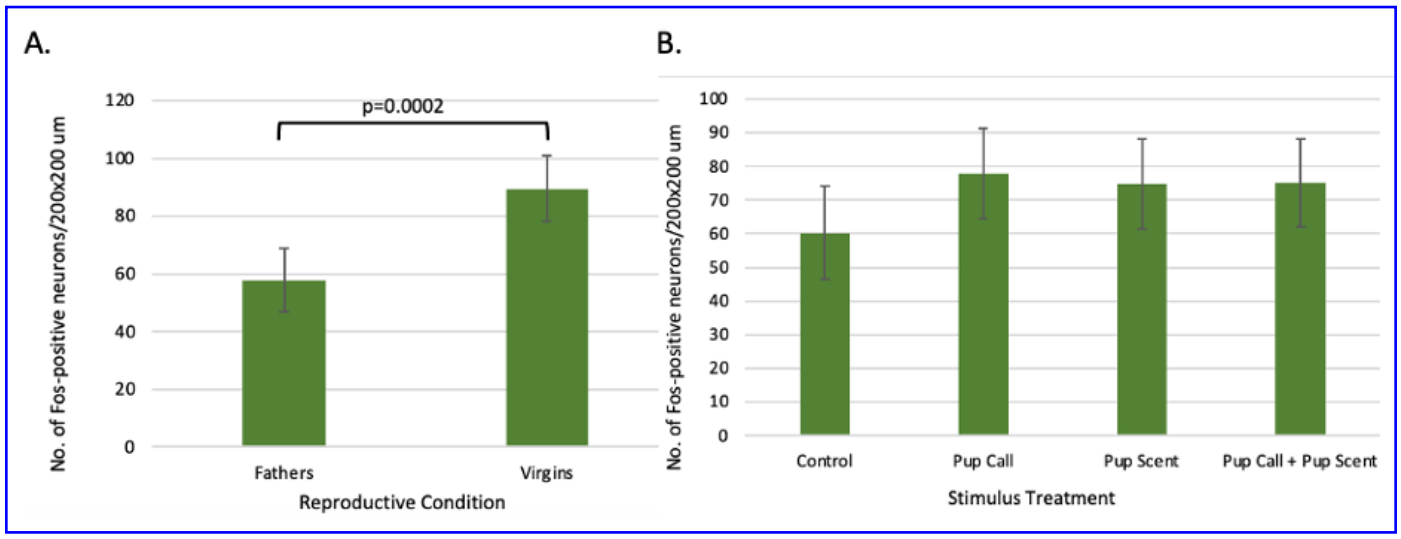

Figure 1: Number of Fos-positive cells (mean ± SE, non-transformed) in the MOB of male California mice. A: Comparison of fathers (N = 30) and virgin males (N = 29) collapsed across the four stimulus treatments. B: Comparison across stimulus treatments for fathers and virgins combined (N = 14 Control, 14 Pup Call, 14 Pup Scent, 17 Pup Scent + Pup Call; P = 0.768).

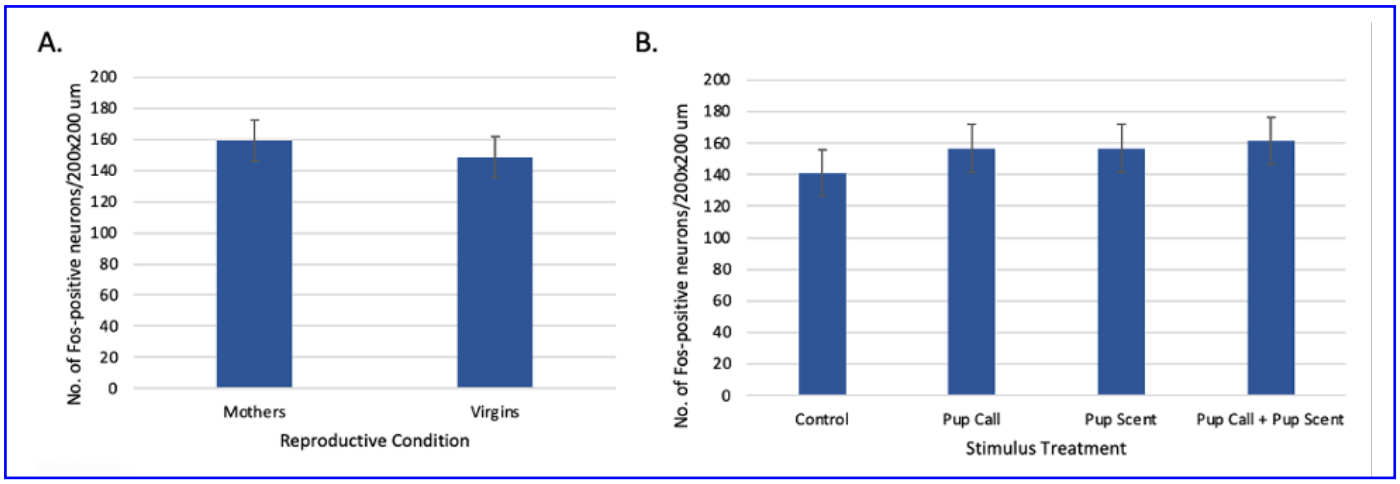

Figure 2: Number of Fos-positive cells (mean ± SE, non-transformed) in the MOB of female California mice. A: Comparison of mothers (N = 32) and virgin females (N = 30) collapsed across the four stimulus treatments (P = 0.433).B: Comparison across stimulus treatments for mothers and virgin females combined (N = 15 Control, 15 Pup Call, 11 Pup Scent, 12 Pup Scent + Pup Call; P = 0.431).

RESULTS

Main Olfactory Bulbs

In male mice, Fos expression in the MOB was significantly higher in virgins than in fathers ($\chi 2 = 13.44$, $P = 0.0002$), but no significant difference was found among stimulus treatments ($\chi 2 = 1.14$, $P = 0.768$) (**Fig. 1**). In females, MOB Fos expression did not differ significantly between virgins and mothers ($\chi 2 = 0.61$, $P = 0.433$) or among stimulus treatments ($\chi 2 = 2.75$, $P = 0.431$) (**Fig. 2**).

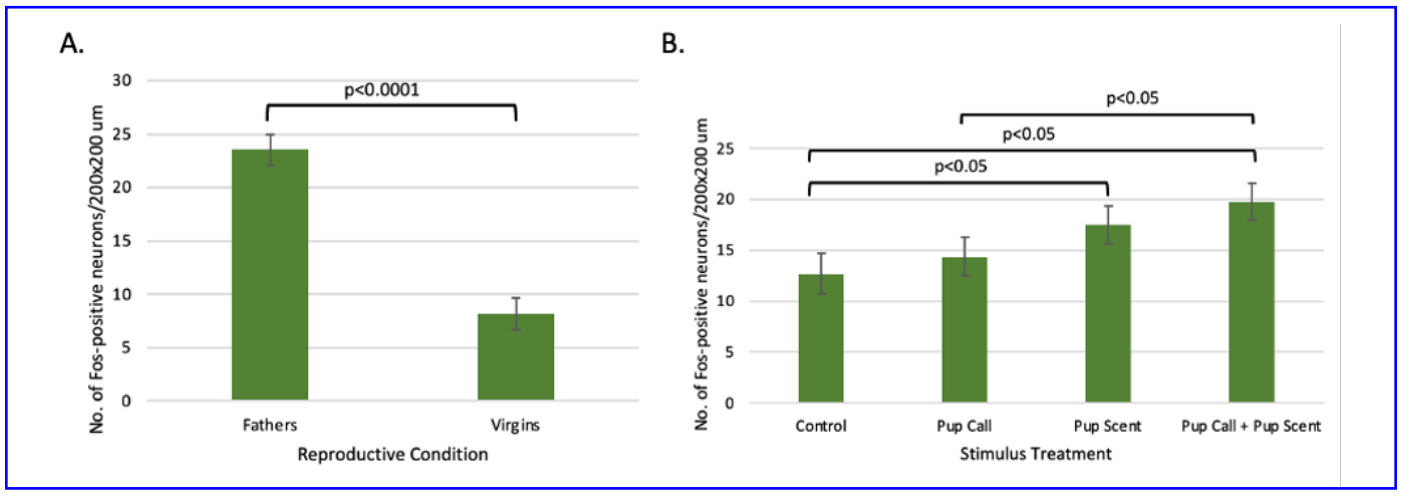

Figure 3: Number of Fos-positive cells (mean ± SE, non-transformed) in the MPOA of male California mice. A: Comparison of fathers (N = 32) and virgin males (N = 29) collapsed across the four stimulus treatments. B: Comparison across stimulus treatments for fathers and virgins combined (N = 15 Control, 14 Pup Call, 16 Pup Scent, 16 Pup Scent + Pup Call; P = 0.010).

Effects of Parenthood on Neural Responses to Pup-Related Cues

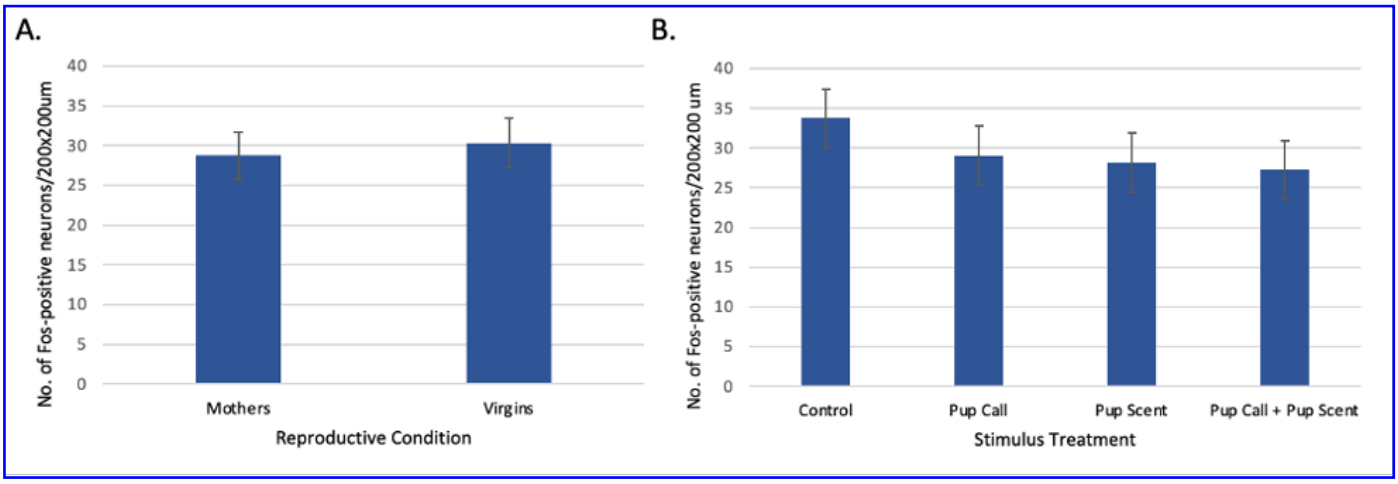

Figure 4: Number of Fos-positive cells (mean ± SE, non-transformed) in the MPOA of female California mice. A: Comparison of mothers (N = 28) and virgin females (N = 25) collapsed across the four stimulus treatments (P = 0.433).B: Comparison across stimulus treatments for mothers and virgin females combined (N = 15 Control, 15 Pup Call, 11 Pup Scent, 12 Pup Scent + Pup Call; P = 0.801).

Medial Preoptic Area

In males, Fos expression in the MPOA was significantly higher in fathers than in virgins ($\chi 2 = 108.70$, $P<0.0001$). Moreover, for fathers and virgin males combined, MPOA Fos differed significantly among treatments ($\chi 2 = 11.32$, $P = 0.010$). Fos expression in the MPOA was significantly higher in males exposed to pup scent only or to both pup calls and pup scent, as compared to males exposed to the control stimuli (P's < 0.05). Additionally, Fos expression was higher in males exposed to both stimuli than in those exposed only to pup calls (**Fig. 3**). In females, Fos expression in the MPOA did not differ significantly between mothers and virgins ($\chi 2 = 1.88$, $P = 0.598$) or among stimulus treatments ($\chi 2 = 0.06$, $P= 0.801$) (**Fig. 4**).

DISCUSSION

The mechanisms underlying the onset of male parental care in biparental mammals are not well understood. Specifically, little is known about the role of sensory plasticity in the onset of paternal behavior. In this study, we show that in male California mice, Fos expression was influenced by both reproductive status (MOB and MPOA) and stimulus treatment (MPOA only), whereas neither of these factors affected Fos expression in the MOB or MPOA in females.

Males

In contrast to our prediction, neural activity in the MOB was lower in fathers than in virgin males but did not differ among stimulus treatments for fathers and virgins combined. Previous studies have investigated the role of MOB in infant-directed behaviors and neurogenesis in male rodents. Kirkpatrick et al. (1994) found that in the biparental prairie vole (*Microtus ochrogaster*), males that underwent olfactory bulbectomy or lesions of the MOB attacked pups more frequently than did control males, suggesting that the MOB is important for inhibiting aggression towards pups. Mak & Weiss (2010) investigated neural plasticity in house mouse fathers and found that neurogenesis in MOB plays a role in offspring recognition. Although these studies implicate the MOB in the expression of pup-directed behavior, we know of no published studies investigating neural responses to pup stimuli in the MOB of males.

In the MPOA, as predicted, Fos expression was higher in fathers than in virgin males. This finding aligns with a previous study in our lab that found higher Fos expression in MPOA of California mouse fathers compared to virgins when males were exposed to a pup (de Jong et al., 2009; Horrell et al., 2017). Lambert et al. (2013) also found that in both the California mouse and the uniparental deer mouse (*Peromyscus maniculatus*), fathers exposed to a pup in distress had higher Fos expression in MPOA than virgin males. Because fathers in our study had higher MPOA Fos expression than virgin males in all stimulus treatments, including the control treatment, our results suggest that activity in MPOA, a region critical for parental behavior in both sexes, is altered by fatherhood and that this effect is not dependent on acute exposure to pup stimuli.

For fathers and virgin males combined, Fos expression in MPOA differed among stimulus treatments. Males exposed to pup scent or pup calls and pup scent combined had higher activation than males exposed to control stimuli, while males exposed to pup calls and pup scent combined had higher Fos than those exposed only to pup calls. These findings suggest that a combination of both olfactory and auditory stimuli results in additive or synergistic effects on neuronal activation in MPOA. Similar results have been found in MPOA of house mouse mothers: mothers had higher neural activity when exposed to both pup vocalizations and scents compared to mothers exposed to pup vocalizations or pup scents alone, suggesting that auditory and olfactory stimuli have synergistic effects (Okabe et al., 2013).

Females

In female California mice, Fos expression did not differ between mothers and virgins or across treatments in either the MOB or MPOA. The onset of parenthood in female house mice is associated with an increase in olfactory bulb neurogenesis (Medina & Workman, 2020). The effect of neurogenesis in the olfactory system on parental behavior seems to differ between house mouse fathers and mothers. Although Mak and Weiss (2010) found that neurogenesis in the MOB is important for offspring recognition in fathers, Feierstein et al. (2010) found that disrupted olfactory bulb neurogenesis via focal irradiation of the subventricular zone had no effect on maternal behaviors such as pup retrieval or offspring discrimination abilities in mothers. Our findings on neural responses in California mice, in conjunction with the previous findings on the function of MOB in house mice, suggest that the MOB might play a more important role in parental care in fathers than in mothers.

As previously described, Okabe et al. (2013) found that pup vocalizations and scents had a synergistic effect on neural activity in the MPOA of house mouse mothers. In contrast, the current study found no difference in neural activity in females exposed to one stimulus or both pup calls and pup scent. The discrepancy in findings could potentially be due to differences between the species studied. Moreover, Okabe et al. (2013) investigated new mothers while we studied new mothers and virgin females. Virgin females may not experience the synergistic effect described.

CONCLUSIONS

In summary, we found that the onset of fatherhood alters activity of the MOB and MPOA and that pup chemosensory and auditory stimuli, especially when presented simultaneously, alter activation in MPOA. In contrast, we found that neural activity in the MOB and MPOA was not influenced by reproductive status or stimulus treatment in females.

Our study might have benefited from a larger sample size in order to increase statistical power. Moreover,

performing immunohistochemistry on male and female brains in the same time period would have allowed us to directly compare Fos expression in males and females. Nonetheless, our findings provide novel insight into plasticity in neural responses to pup sensory stimuli during the onset of parenthood and, potentially, into the neural and sensory mechanisms underlying paternal care. Further research investigating responsiveness of other brain regions implicated in receiving and processing of sensory information and parental behavior in response to pup sensory stimuli would contribute to our understanding of male parenting. Moreover, future studies on responsiveness of MOB and MPOA to repeated exposure to pup sensory stimuli, as well as on the cellular and molecular mechanisms of sensory plasticity would provide valuable insight into potential mechanisms underlying the onset of parental care and role of the MOB and MPOA in biparental rodents.

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Sexualizing Señoritas: Portrayals of Mexican Women during World War I

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ABSTRACT

The obstacles that white women had to face during WWI have been widely documented in books such as Elizabeth Cobbs', Hello Girls and Diane North's California at War: The State and People During World War I. However, less attention has been paid to the obstacles faced by Mexican women. My paper draws on newspaper articles, fictionalized accounts, and recent scholarly work to examine how Mexican women were portrayed in contrast to portrayals of white women during this period. The portrayal of Mexican women in the media as illiterate, ignorant, and in need of white saviors, reinforced the stereotype of a hypersexualized damsel in distress. These portrayals of Mexican women reflected existing racism, sexism, and classism by neglecting/diminishing their accomplishments. Recovering the contributions and lived experiences of Mexican American women during this time are crucial to understanding California history in World War I.

KEYWORDS: feminization, misogyny, racism, stereotype, hypersexualization, Americanization, assimilation

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Sexualizing Señoritas: Portrayals of Mexican Women during World War I

INTRODUCTION: PART 1

Works, such as *Diane North's California at War: The State and People During World War I*, focus on the accomplishments and stories of middle-class white women. This manner of portrayal can also be found in *The Hello Girls: America's First Women Soldiers*, which focused on the importance of women as telephone operators, because men were busy being drafted so they were not as familiar and as talented as women who were able to multitask. Unfortunately, there has been a lack of study on poor women of color during World War I. This paper analyzes the contributions Mexican women made in California during World War I as presented by newspaper articles, books, political cartoons, and fictional works from 1910-1919. The portrayal of Mexican women in the media as illiterate, ignorant, and in need of white saviors, contributed to the stereotype of a hypersexualized damsel in distress. Through comparison of obstacles that white women had to face as opposed to the obstacles of Mexican women, and if race and class affected the successes of women as seen in Elizabeth Cobbs' book, *Hello Girls*. By focusing on a few selected works, I hope to demonstrate the need for increased scholarly engagement with this topic.

Mexican middle-class servant women were placed at the bottom of the socioeconomic ladder, despite multiple attempts to climb it through Americanization and assimilation. Natalia Molina's book, *Fit to Be Citizens?: Public Health and Race in Los Angeles 1879-1939*, discussed Mexicans attempting to be a part of the American mainstream. She argued that the foundation of Los Angeles is credited to white supremacy, due to the racial hierarchies between Spanish, Mexican, and white peoples. These racial hierarchies were determined during the Spanish Mission era in the nineteenth century. Mexicans were at the bottom of the social hierarchy, because those that had previously identified as *Californio*, a term associated with Mexican heritage, were now identifying themselves as Spanish. Molina describes an openly racist magazine, *The Grizzly Bear*, whose "statement of purpose praised the racist Alien Land Laws of the 1910s and encouraged increasing California's white population."[1] The Alien Land Laws were a series of discriminatory laws that prohibited immigrants from owning land and participating in American society. *The Grizzly Bear* was a popular magazine in the mid 20s that elevated the importance of keeping America white and published "restrictionist sentiment towards Mexican and Japanese immigration."[2] By celebrating white supremacy, this magazine was emphasizing discrimination towards minorities.

Mexican mothers were pivotal to the assimilation of Mexican families, because of the influence in implementing American values and culture within their children. In George Sanchez's book, *Becoming Mexican American: Ethnicity, Culture, and Identity in Chicano Los Angeles*, he describes the importance of Mexican women during World War I. "Americanization advocates were interested in the contribution Mexican women could make in transforming their families' habits from those of a rural, pre-industrial lifestyle to a modern American one."[3] Anglo American home teachers focused on Mexican mothers from 1915-1949, this is one of the rare sources that validated the importance of Mexican women that started from within the home.[4] Rather than focusing on the patriarch of the family, the future of an Americanized Mexican family lied with Mexican mothers. Americanization advocates

1 Molina, Natalia. Fit to Be Citizens?, 104.
2 Molina, Natalia. Fit to Be Citizens?, 103.
3 Sanchez, George J.. Becoming Mexican American, 99
4 George J. Sanchez, Becoming Mexican American, 99

focused on Mexican mothers because they wanted to instill American values within second-generation Mexicans, so it was crucial that Mexican mothers were accustomed to the American lifestyle through the knowledge of nursemaids, seamstresses, and laundress.[5] This perspective correlates with the demand for Mexican labor in help wanted ads. By answering these ads for nursemaids and seamstresses, Mexican women were able to close the labor shortage gap in the Southwest, so long as they were able to learn English and pass it on to their children. "During and after World War I, however, English instruction was intended to provide the immigrant with much more than facility with the spoken language of the United States."[6]

In July 1918, a young girl from Los Angeles wanted to teach her Mexican servant, Francisca Munoz, how to read and write English in order to better understand Mexican contemporary and historical events. This led to Francisca paying her mistress for lessons in hopes of increasing her wages.[7] The story, "A Young Teacher," is one of many instances where Mexican women had to rely on white counterparts to assimilate into American society. This anecdote is a common example portraying the celebration of the Americanization of poor Mexican women. Mexican working- and middle-class women were limited to jobs as servants and caretakers, due to a lack of upward mobility for immigrants as opposed to white middle class women. The article, "A Young Teacher" illustrated that white children were offered more opportunities through education than grown Mexican women. Mexican women were entering the American workforce by primarily applying for job titles as servants or housemaids. "Domestic servants in Mexico are, as a rule, very ignorant, scarcely one in a thousand being able to read and write,". During World War I there was also a revolution in Mexico, a possible reason for the lack of emphasis on literacy was that the Mexican government was fearful of the lower and middle class mobilizing. If there was a more educated and politically aware society during the Mexican revolution, it would have dramatically affected the actions of Mexican soldiers and wives. Mexican women could have directly contributed to World War I efforts and the Mexican revolution as white women did with the American Red Cross, rather than indirect participation through middle- and lower-class workforce. If there was a census or chart that compared Mexican wages to white wages, as well as what types of jobs both races of women typically took, that would provide a more concise comparison of economic as well as social opportunities. Had Mexican women been given the same opportunities as white women, they too could have become nurses and purchased war bonds, this is where it becomes a class issue, because there was little to no upward mobility for Mexican and Mexican American women.

There were also significant economic differences between working class Mexican and white women such as job opportunities and requirements. While some upper-class white women were able to pay for housemaids and caregivers of children, there were Mexican women providing childcare and housemaid duties. That is not to suggest that all, or even most white women were able to afford servants, as a majority of the white women were working class. In *Hello Girls*, class background is argued to have shaped voluntarism, "[m]iddle-class women did not need or expect to be paid, and officials were relieved not to fork over money."[8] White women did not have to worry about payment, because they lived comfortable

5 George J. Sanchez, Becoming Mexican American, 100
6 George J. Sanchez, Becoming Mexican American, 100
7 "A Young Teacher" Los Angeles Herald, 8.
8 Elizabeth Cobbs, The Hello Girls, 40.

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lifestyles. However, this sentiment is opposite that of the story described in the article, "This Young Girl Hopes to Solve Servant Girl Problem," where a woman, Mary Rouillet, attempted to correct the stigma of servant girls being uneducated and ignorant, "[b]y making the work an honorable profession she believes it will not only be better, but attract a better class of girls."[9] The article shows Mary Rouillet opening a school to teach social and life skills to young women in order to counteract the stigma surrounding servant girls, however, a majority of servant girls at this time are young Mexican women.

The same day this article was published, President Woodrow Wilson had ratified the 16th Amendment, creating the Federal Reserve, which imposed and collected income tax.[10] In this article, the phrase, "better class" is used but not clearly explained. While it is possible that this is in reference to educated white women, I am left wondering if it could have also been intended for a woman of any racial background. This article could have been created to bring attention to dissatisfied women with servant girls that did not understand social cues or have a grasp on what an American home and upbringing should look like.

It was difficult to find positive accounts of women in databases, because a majority of searches consisted of hypersexualized descriptions of Mexican characters in plays or illiterate maids and/or servants in need of English lessons. Primary and secondary sources are used to support evidence of silencing of minority voices and to explore the trials and tribulations women faced at home and in other states. An issue of economic and cultural discrepancies would be the forced assimilation into American society, because Mexican women needed to learn English in order to be a part of the job market. Some difficulties I encountered were the articles written about the Mexican War which dates conflicted with World War I. Although there were many primary sources, a majority of newspaper articles had obscure or nonexistent authors. As such, the lack of focus on Mexican and Mexican American women stories made it difficult to find clear evidence of lived experiences.

Despite coming from Mexican middle and upper classes and owning property, class structure was not the same in America as some Mexican families had hoped. The article, "Joining the American Mainstream: Texas's Mexican Americans during World War I" describes the hardships Mexican families experienced while assimilating into American society. Although a majority of Mexican assimilation occurred in Texas, slow Mexican migration into southwest America was occurring. World War I was the beginning of assimilation of Mexicans into American society due to participation in civilian and military activities.[11] It was the hope of Mexicans that if they attempted to become a part of mainstream society that they would be accepted by Anglos. Similar to African American veterans, Mexican veterans were not credited after or during military service, and Mexicans were still isolated culturally and politically from American mainstream society.[12] While Mexican men were drafted, Mexican women were fulfilling help wanted ads for nursemaids and nannies. Climbing to the middle class was slim for a majority of lower class and poor Mexican families. Mexican Americans in California and other southwestern states experienced Anglo hostility and social and job discrimination in the nineteenth and

9 "This Young Girl Hopes to Solve Servant Girl Problem", 7
10 "This Month in Business History: Federal Reserve Act Signed,"
11 Carole E. Christian "Joining the American Mainstream" 559
12 Carole E. Christian, "Joining the American Mainstream," 560

twentieth centuries.[13] It appeared that Mexican women were at a predetermined disadvantage due to racial and class factors.

While whites were directly assisting with war efforts, Mexican women were indirectly assisting by entering the lower- and middle-class workforce. White men were drafted and white women were assisting through the American Red Cross and creating dressings and shipping them to soldiers. Examples of prejudice against non-white women was rampant, with documents such as hiring ads making opinions of the time clear. A help wanted ads reads, "WANTED-Washing, also work by day or hour; white woman."[14] The people publishing the ad had a preference for a white woman washer. Another ad reads, "COLORED WOMAN-wants child to care for in own home"[15] The importance of describing herself as a colored woman is because someone that might answer the ad may not work for a colored woman, due to racial tension between white and minority groups.

THE BLENDING OF FICTIONAL AND NONFICTIONAL STEREOTYPES

The line between nonfictional and fictional stereotypes grew increasingly blurred during this time as well. Mexican women were portrayed as illiterate and ignorant in newspaper articles and advertisements as seen in "A Young Teacher" and "This Young Girl Hopes to Solve Servant Problem." White middle-class families placed help wanted ads for young nurses, housekeepers, and cooks. It was typically young Mexican women filling these roles. While fictional stereotypes seen in "Terrwiliger and the Senorita" and "Ysabella" sexualized Mexican women through racist, classist, and misogynist lenses painting Mexican women as damsels in distress.

Representation of Mexican women in the media amplified the stereotype of a sexualized damsel in distress, furthered the cycle of Mexican women in need of a white male savior. The article, "Terrwiliger and the Senorita" is an early California pulp story about a group of men sexualizing a young Mexican woman. Senorita Christobell is in a love triangle between a "half-breed Spaniard" and an American. The American was described as affluent, and the Spaniard was dirty and unintelligent. The difference between the two men is important to note because it creates a division between Americans and immigrants and creates lower class versus middle/higher class. Her appearance is described as "a little off-color among all those smoke-colored relatives."[16] The description "smoke-colored" plays into the stereotype that all Mexicans look alike, and the reason this woman was attractive was due to her European features and lighter skin tone. Several slurs are used to describe Latinos throughout the article, such as "mud-faced half-breed" and the senorita was viewed as property among men. There are several representational issues within this article, the first with Senorita Christobell being sexualized and the second with racial descriptions of her. This is a huge issue leading to the mistreatment and stereotyping of Mexican women as stupid and illiterate, this article was published in 1909 so that sentiment towards Mexicans was shared before the war. Throughout the magazine, The Overland Monthly, there are numerous racist and misogynist stories about Latinas.

Misogynist and racist undertones riddled fictional stories about Mexican women which furthered damaged the integrity of Mexican women in real-life. The story,

13 Carole E. Christian, "Joining the American Mainstream," 565
14 "Female Sit. Wanted", 11
15 "Female Sit. Wanted", 11
16 W.A. Scott, "Terrwiliger and the Senorita", 278-282.

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"Ysabella" is a fictional piece that describes a triangle between a young Mexican woman, Señorita Ysabella Carrillo, American Captain Harry Fitch, and Spanish Governor Enchandia. Ysabella is nineteen years old whereas Captain Fitch is twenty-six., On reflection I believe that it is not a coincidence that Ysabella's character is young as it normalizes older white men preying on young Mexican women. Captain Fitch describes Ysabella,"[h]er eyes are like twin stars, and her lips hath the sweetness of the wine of life. She is modest and discreet withal."[17] The description as modest and discreet is important to note because (within a traditional Mexican society/culture) a desirable woman is meek and has no sense of individualism. Ysabella falls within the stereotypical image surrounding Mexicans because she is treated as property and obedient to all of her male counterparts. Initially, Ysabella is betrothed to Governor Enchandia, however, she meets an Americano and immediately falls in love. Ysabella is the damsel in distress and once again an American is there to save her and steal her away from an overbearing Spanish man. Throughout the story, Ysabella is torn between her cultural duties to Governor Echandia, as an obedient Mexican woman, and her internal desires for Captain Fitch. This story is similar to "Terriwiliger and the Senorita" because Mexican women were objectified by American men, and the Latin American characters are described as eccentric and weak as opposed to the heroic white characters. Ysabella is a prime example of the stereotypical Mexican woman.

While some stories focused on Mexican women being young, other stories focused on the physical appearances of Mexican women amplifying the stereotype that these women were one-dimensional characters that existed solely for pleasure. The article, "The Moreno Earrings" is a fictional story about a man purchasing a pair of earrings at a secondhand shop and imaging a sexual relationship with a fictitious Mexican woman, named Conception, who used to own them. Eventually, this man finds his dream Mexican women in real life, and they marry and live happily ever after. Upon further investigation this work is also filled with stereotypes, when this man was making love to Conception, he states, "the low neck of her gown fell softly from a creamy throat, and only half concealed the sweet roundness of her breast's that pulsed with life and desire."[18] The stereotype of Mexican women as voluminous and sexual creatures is present in his description of Conception. Also, the description of Conception's "creamy throat" exemplifies the desire of light-skinned Mexican women. This is yet another example of how Mexican women were erotically fetishized viewed through fictional pieces. Naming the character Conception was a symbolic representation of the conceptions of

Figure 1: "Just Another Mexican Revolution"

17 "Clarice Garland, "Ysabella", 246
18 Gerald Van Etten. "The Moreno Earrings", 219-224.

Mexican women.

Figure 1, "Just Another Mexican Revolution" is a political cartoon by Clifford Berryman. In it Uncle Sam is sitting at a desk with papers titled League of Nations arguments and peace discussions attempting to find a solution to end World War I. Behind him is a Mexican Pancho Villa jack-in-the-box holding a gun and a knife. Uncle Sam has an amused expression on his face and the text next to him reads, "What again?" At this time, middle-class Mexicans were attempting to break out from under elitist rule due to an imbalance of land ownership. With Uncle Sam's amusement, it appears that America was taking Mexican politics lightly, because next to Uncle Sam a teddy bear states, "Just one after another."[19] Mexicans are viewed as barbaric and uneducated, the jack-in-the-box symbolizes American sentiment towards Mexicans, and this carries on to the treatment of Mexican women as objects and used for entertainment. Sexualizing Mexican women is due in part to Americans viewing Mexican people as weak. The depiction of Mexicans as a jack-in-the-box demonstrates that Mexicans were not included in the war efforts and that they were not to be taken seriously. While people might be aware of the conflict along the border and the United States deemed their issues unimportant.

The feminization of fractured countries was detrimental to the public opinion on the strength of women. Louis A. Perez Jr's book, *Cuba in the American Imagination* describes the political climate of Cuba during the Spanish-American War. Perez argues that white Americans had been using these metaphors to portray Latin American women for decades by the First World War. The feminization of Cuba in Perez's book, as seen in **Figure 2**, uses the metaphor of Cuba as a woman in distress. **Figure 2** is the political cartoon, "The duty of the hour; - to save her not only from Spain, but from a worse fate" which is an image of a woman with a Cuban The feminization of fractured countries was detrimental to the public opinion on the strength of women. Louis A. Perez Jr's book, Cuba in the American Imagination describes the political climate of Cuba during the Spanish-American War. Perez argues that white Americans had been using these metaphors to portray Latin American women for decades by the First World War. The feminization of Cuba in Perez's book, as seen in **Figure 2**, uses the metaphor of Cuba as a woman in distress. **Figure 2** is the political cartoon, "The duty of the hour; - to save her not only from Spain, but from a worse fate" which is an image of a

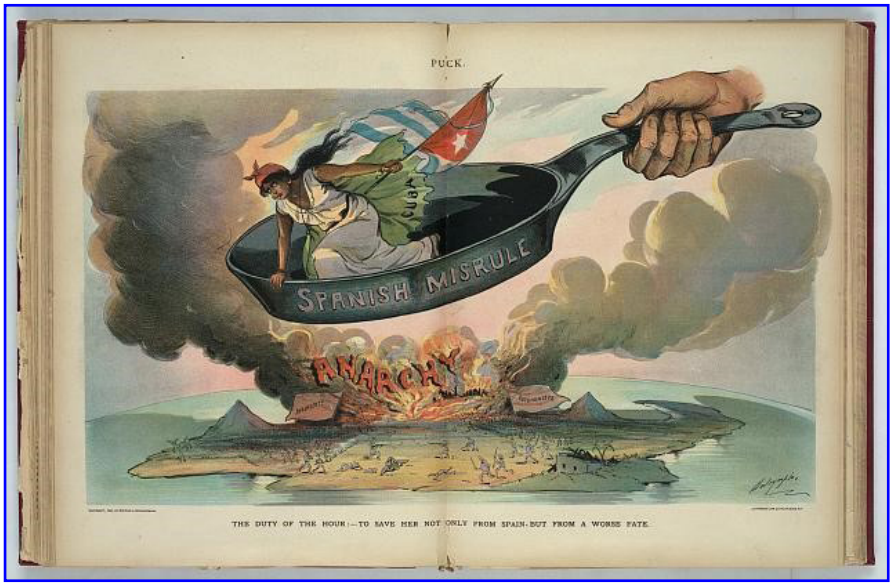

Figure 2: The duty of the hour; - to save her not only from Spain, but from a worse fate

19 Clifford Berryman, "Just Another Mexican Revolution."

woman with a Cuban flag in a frying pan labeled Spanish Misrule. A white hand is holding the pan while attempting to put out a fire labeled Anarchy on the island of Cuba. On both sides of Cuba there are two groups of people fighting, on one side there are the "insurgents" while the other side is the "autonomists." The white hand symbolizes America as the savior and by treating Cuba as a "damsel in distress," it describes Cuba as weak and vulnerable. In fact, this damsel in distress is the primary metaphor used for Latin America by white people and justification for the Spanish-American war effort. This highlights a historical pattern of white attitude towards Latin Americans as feminine and weak. The same sentiment was shared during World War I between America and Mexico. Perez writes, "What made awareness of Cuba particularly significant were the ways that it acted on the formation of the American consciousness of nationhood" [20]

CONCLUSION

Assimilated Mexican women were crucial to the war efforts, because of their impact in the lower-class workforce. Some challenges faced while researching were that many sources were focused on white experiences, and the newspaper clippings were typically from the perspective of what it was like to be a Mexican caretaker of white middle class children. This research hopes to have argued that it is time to look more closely at the experiences of Mexican women in the early twentieth century, with the issues of class, race, and gender being used as lenses for interpretation of their lived experiences. Through the analysis of nonfiction and fiction sources it appears that the stereotype of a damsel in distress applied to Latin American women, and through almost every source there was a white savior. The underlying messages within the media that placed Mexicans at the bottom of the racial hierarchy were detrimental to any attempt to assimilate into American society.

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20 Louis A. Perez, Cuba in the American Imagination: Metaphor and the Imperial Ethos, 3.

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Evaluating Imatinib's Affinities and Specificities for Tyrosine Kinases Using Molecular Dynamics Simulations

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ABSTRACT

Computational chemistry lets us model intermolecular interactions in ways assays cannot. My project focuses on the multi-kinase interactions of the cancer drug, imatinib. Most cancer drugs target one kinase, but some affect multiple kinases. Imatinib treats chronic myeloid leukemia by targeting ABL kinase. Proteomics data reveals it can interact with other kinases, such as KIT to treat gastrointestinal stromal tumors, but the mechanisms are unknown. Imatinib has different affinities for similar kinases, such as a 3000x difference between ABL and SRC, despite sharing 50% structural homology. Here, I investigate the conformational differences between free and imatinib-bound ABL, KIT, and SRC using Molecular Dynamics simulations to understand the key imatinib-kinase interactions. The alignment analysis shows the docked conformations are similar to co-crystal structures in the Protein Data Bank. Root-mean-square-deviation and fluctuation (RMSD and RMSF) analysis show that all simulations converge at 45 ns, with some regions exhibiting differential flexibility. Hydrogen bond analysis across 100 ns simulations show that ABL has one main H-bond, KIT has three main H-bonds, and SRC has no main H-bonds. All the drug-kinase complexes feature at least 15 key salt bridge interactions relevant for structural stability. The dihedral distributions reveal that most residues adopt a single conformation, but some can adopt multiple, increasing the protein flexibility. The entropy results quantify the protein disorder, revealing KIT and SRC favors the apoprotein while ABL favors the complex. This signifies that broad protein similarity does not govern imatinib binding, instead, it is explained by smaller structural details.

KEYWORDS: Drug design, molecular mechanics, kinome, CML, GIST, Off-target

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Dr. Chia-en Chang is a Professor of Chemistry in the Department of Chemistry. She received her Ph.D. from the University of Maryland and conducted post-doctoral research at UC San Diego. Her work focuses on applying computational simulations for biomolecular recognition and drug discovery. She has published over 80 papers on molecular modeling, drug binding, and protein dynamics. She was a previous recipient of the Robert T. Poe Faculty Development award, the Faculty Development Award & Omnibus Travel Award, NSF Career Award, and Chancellor's Award for Excellence in Undergraduate Research. She is also the Vice Chair of the Chemistry Department.

William Troxel

William Troxel is a third-year Biochemistry major. He has conducted computational proteomics research in the Dr. Chia-en Chang lab since January 2020. He is a MARC U STAR Trainee, a former Lead Recruitment Coordinator, and current President of the MARC organization. He delivered an award-winning poster at ABRCMS in November 2021, is a Barry Goldwater Scholarship nominee, and a Phi Beta Kappa invitee. He is conducting an REU at Texas A&M for summer 2022. He will pursue a Ph.D. in Molecular Biophysics to apply computational tools for novel drug discovery.

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INTRODUCTION

Computational protein research allows us to visualize complicated protein-drug interactions in ways conventional protein assays fail to. Drugs designed to target specific signaling proteins may affect other proteins due to shared structures, resulting in unforeseen side-effects and increasing medical applications (Moy et al., 2010). My project focuses on how the cancer drug, imatinib, interacts with ABL, KIT, and SRC kinases. Kinases help reduce the energy needed to transfer phosphate groups from adenosine triphosphate (ATP) to specific acceptor groups on proteins for biochemical reactions. The phosphate charges affect the protein's structure and functions (Koch, 1999). With kinome interaction patterns, kinase profiling data of known inhibitors can predict interactions with help from simulations (Davis et al., 2011).

ABL is a tyrosine kinase involved in cell differentiation, division, and DNA repair, however, overexpression due to ABL-BCR fusion corresponds with chronic myeloid leukemia (CML) for ~95% of reported cases (Aleksandrov and Simonson, 2009) (Ayatollahi, 2018) (Golzarroshan, 2012) (Zagaria, 2015). KIT is a tyrosine kinase involved in cell survival, spread, and differentiation, but overexpression due to KIT mutations corresponds with at least 85% of gastrointestinal stromal tumors (GIST) (Nowain, 2005) (Zhao, 2012). SRC is a tyrosine kinase involved in cell signaling, and while it shares approximately 50% homology with ABL, it has reduced imatinib affinity by 3000x (Aleksandrov and Simonson, 2009) (Ortiz, 2021).

Imatinib is a type II ATP-inhibitor that targets inactive conformations in ABL to treat CML (Smith et al., 2014). It forms six hydrogen bonds (H-bonds) with surrounding residues; E286, T315, M319, I360, H361, and D381 (Dubey, 2011). Van der Waals' interactions on the aromatic ring and a hydrophobic pocket neighboring the piperazinyl-methyl group also contribute to imatinib's ABL affinity (Asaki et al., 2006) (Nagar et al., 2002). Salt bridge interactions have a cumulative effect on protein stability, preventing ATP from binding and suppressing proliferative signals (Eck et al., 2009). This inhibits ABL kinase by reducing phosphorylation of cancer proteins and signal cell transduction in the pathway (Lupino et al., 2014). Researchers later realized imatinib can block PDGF-R and KIT tyrosine kinases, and it is used to treat acute lymphocytic leukemia (ALL), GIST, and dermatofibrosarcoma protuberans (Moy et al., 2010) (Salah et al., 2011) (Seggewiss et al., 2005). This demonstrates the importance of drug and multi-kinase research, as the off-target effects may expand imatinib's purpose and cost-effectiveness. Large-scale proteomics shows imatinib has favorable affinities with many kinases from different families, but the mechanisms remain unknown (Miao et al., 2019).

My objective is to understand imatinib's multi-kinase interactions and selectivities to predict drug efficacy from the bound states and guide structure-based drug design. This will elucidate imatinib's differential affinities and interactions with these kinases based on structural elements and how imatinib's promiscuity changes in different protein environments. If certain kinases share similar structures on the binding site, then the bound complexes should have similar conformations, free-energy, and entropy scores due to their interrelatedness from molecular evolution. If the drug-kinase interactions exhibit different conformations, free-energy, and entropy results, then it would suggest other variables impact drug-kinase binding.

METHODOLOGY

Computational Models

Protein crystal structures are selected from the Protein Data Bank including imatinib-ABL, imatinib-KIT, and imatinib-SRC complexes (PDB: 2HYY, 1T46, 2OIQ). The

disordered residues are resolved using SWISS-MODEL.

MOLECULAR DOCKING

I use molecular docking to study the binding energies and key residue interactions between imatinib and the kinases. Imatinib is removed from the crystal structure using Visual Molecular Dynamics. The imatinib is docked to the three kinases using AutoDockTools with a 22.5x22.5x22.5 cubic angstroms grid box with 20 Lamarckian Genetic Algorithm runs. The energy is reported in kJ/mol, with the average and standard deviation illustrated in table 1.

Molecular Dynamics

I use Molecular Dynamics (MD) simulations to study the conformational changes between the free protein and bound complexes since imatinib interacts with ABL, KIT, and SRC. MD simulations are conducted using the AMBER18 package. The proteins and ligands are parameterized using AMBER ff14sb and Generalized Amber Force Field 2 (GAFF2), respectively (Maier, 2015). They are solvated in a water box using the TIP3P water model with a 12-angstrom buffering distance. To neutralize the systems, 8 sodium ions are added to imatinib-ABL, 5 sodium ions are added to imatinib-SRC, while imatinib-KIT is already neutral. They are minimized starting with hydrogen atoms for 500 steps, then side chains for 5000 steps, then the entire structure for 5000 steps. They are equilibrated in a constant number of molecules, pressure, and temperature (NPT) ensemble, and heated in 50 K increments lasting 200 ps from 100 to 298 K. MD runs are conducted for 100 ns using Langevin thermostat. A 12-angstrom cutoff is used for non-bonded energy calculations, the particle-mesh Ewald method is used for electrostatic interactions, and the SHAKE algorithm is used to constrain covalent bonds involving hydrogen atoms. Trajectories are saved every 2 ps and processed every 20 ps using Amber's cpptraj plug-in. The waters are removed post-production for easier observation. From the MD results, I examine the H-bond and salt bridge frequencies, the dihedral conformations of the residue backbone and side chains, and the entropy for the protein's thermodynamic properties.

Kinase	Docked energy (kJ/mol)
ABL	-10.16 ± 1.13
KIT	-9.29 ± 1.05
SRC	-7.83 ± 1.20

Table 1. Energy results for ABL, KIT, and SRC docking show significant differences for SRC from ABL and KIT.

RESULTS

Molecular Docking Agrees with Experiments

The docking results show that ABL, KIT, and SRC binding have average docking energies of -10.16, -9.29, and -7.83 kJ/mol, respectively. ABL and KIT have statistically similar energies, while SRC is significantly different. The imatinib adopts similar conformations in the binding pocket as the existing crystal structures, demonstrating high accuracy.

RMSD Shows Apoprotein and Complex Stabilization

Figure 1 shows the RMSD for the free apoprotein and imatinib-bound ABL, KIT, and SRC kinases over the 100 ns MD simulation. The ABL equilibrates at 30 ns, KIT at 45 ns, and SRC at 45 ns. The structural stabilization by 45 ns signifies all structures are experimentally appropriate.

RMSF Reveals Flexible Regions of Interest

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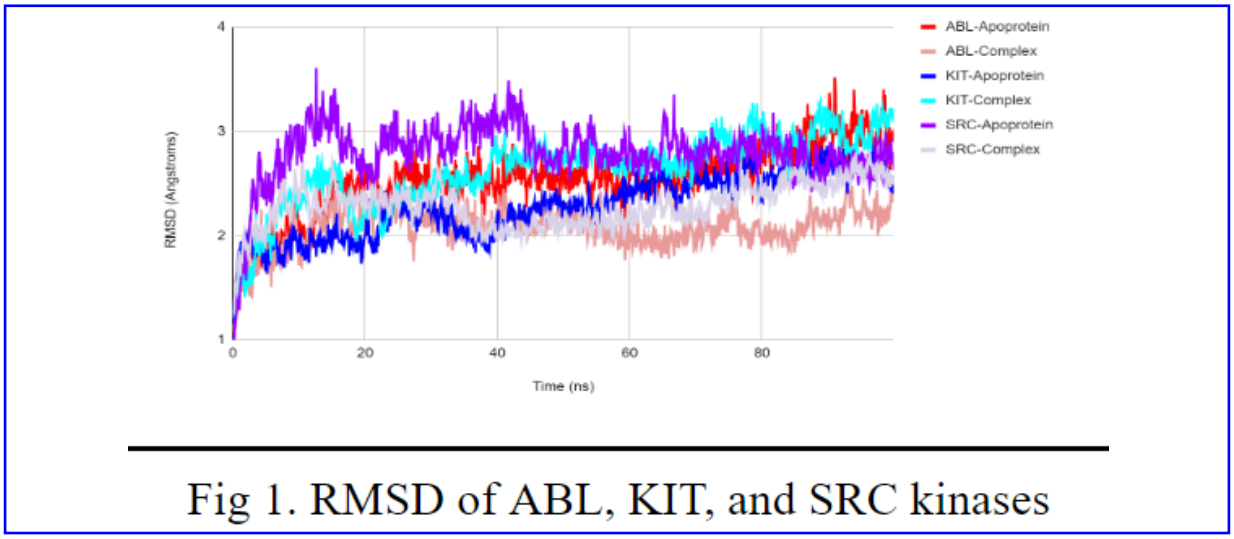

Figure 1. Root-mean-square-deviation for 100 ns ABL, KIT, and SRC apoprotein and imatinib-bound kinases shows stabilization by 45 ns.

The 3D structures for **figures 3, 5, and 7** are colored in accordance with the RMSF plot to reveal the flexible protein regions of interest. **Figure 2** shows broad similarities in the ABL RMSF, except for residues 40-45, which have a greater apoprotein RMSF than the complex. This agrees with the RMSD and ABL's experimentally strong affinity for imatinib. **Figure 4** shows broad similarities in the KIT RMSF, except for residues 127-135 with greater complex RMSF compared to the apoprotein in agreement with the RMSD. **Figure 6** shows that the entire SRC RMSF reveals no significant differences in apoprotein or complex RMSF, aligning with the RMSD and the docking results. This affirms that imatinib has lower binding affinity with SRC.

More H-bonds in ABL Complex and More H-bonds in KIT and SRC Apoprotein

ABL forms 252 intraprotein H-bonds in the complex compared to 240 in the apoprotein. KIT forms 283 intraprotein H-bonds in the apoprotein and 274 in the complex. Finally, SRC forms 243 intraprotein H-bonds in the apoprotein and 237 in the complex. Between the proteins and imatinib, the main H-bond that formed between ABL and imatinib involved M84 for about 20.4 ns. In KIT, there are two main H-bonds between C109 and E76 for 16.8 ns and 11.4 ns, respectively. There are no main H-bonds between SRC and imatinib.

ABL, KIT, and SRC Salt Bridge Role in Apoprotein and Complex Structures

Figure 8 shows that the most substantial salt bridge change forms between E45-R152 in ABL. This salt bridge only forms in the ABL-imatinib complex, but not in the free ABL protein. **Figure 9** shows salt bridges between D143-K129 and E145-K129 in KIT form in the complex but are broken in the apoprotein.

Dihedral analysis reveals residues with multiple rotamers

Most of the dihedrals have a monomodal distribution, meaning the backbone and side chains tend to adopt one conformation. However, several residues express multimodal distributions, representing multiple favorable

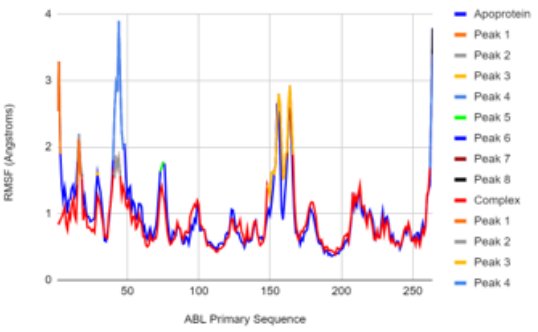

Fig 2. ABL RMSF

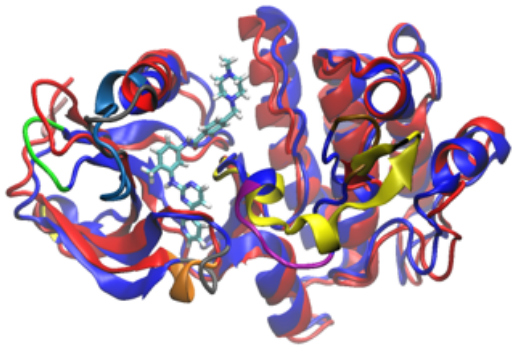

Fig 3. ABL 3D structure

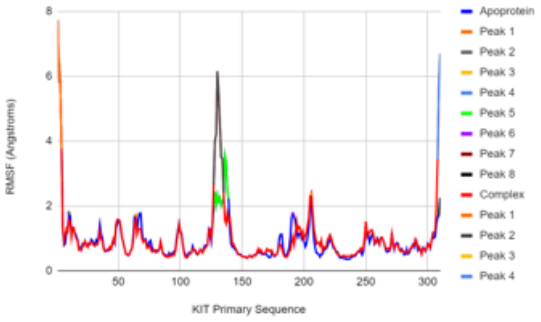

Fig 4. KIT RMSF

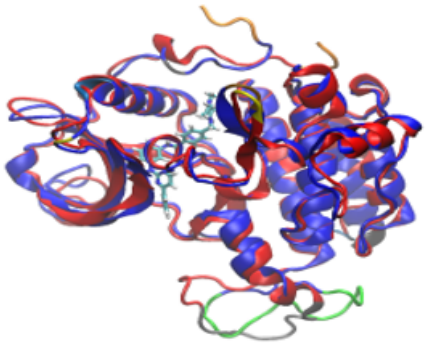

Fig 5. KIT 3D structure

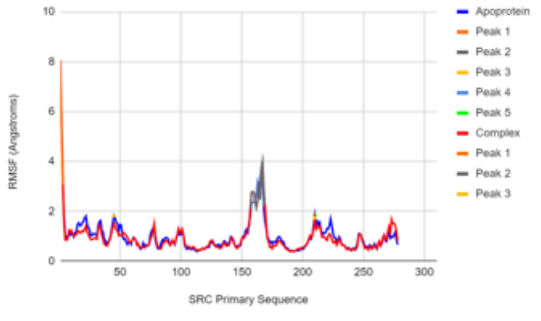

Fig 6. SRC RMSF

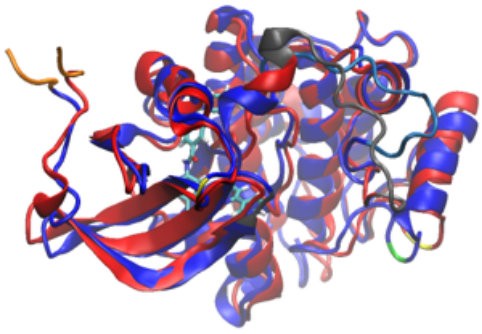

Fig 7. SRC 3D structure

Figures 3, 5, and 7. Superimposed 3D structures for apoprotein (blue) and imatinib-bound complex (red) ABL, KIT, and SRC structures, respectively. **Figures 2, 4, and 6**. Root-mean-square-fluctuation for the ABL, KIT, and SRC apoprotein and imatinib-bound complex revealing flexible protein regions.

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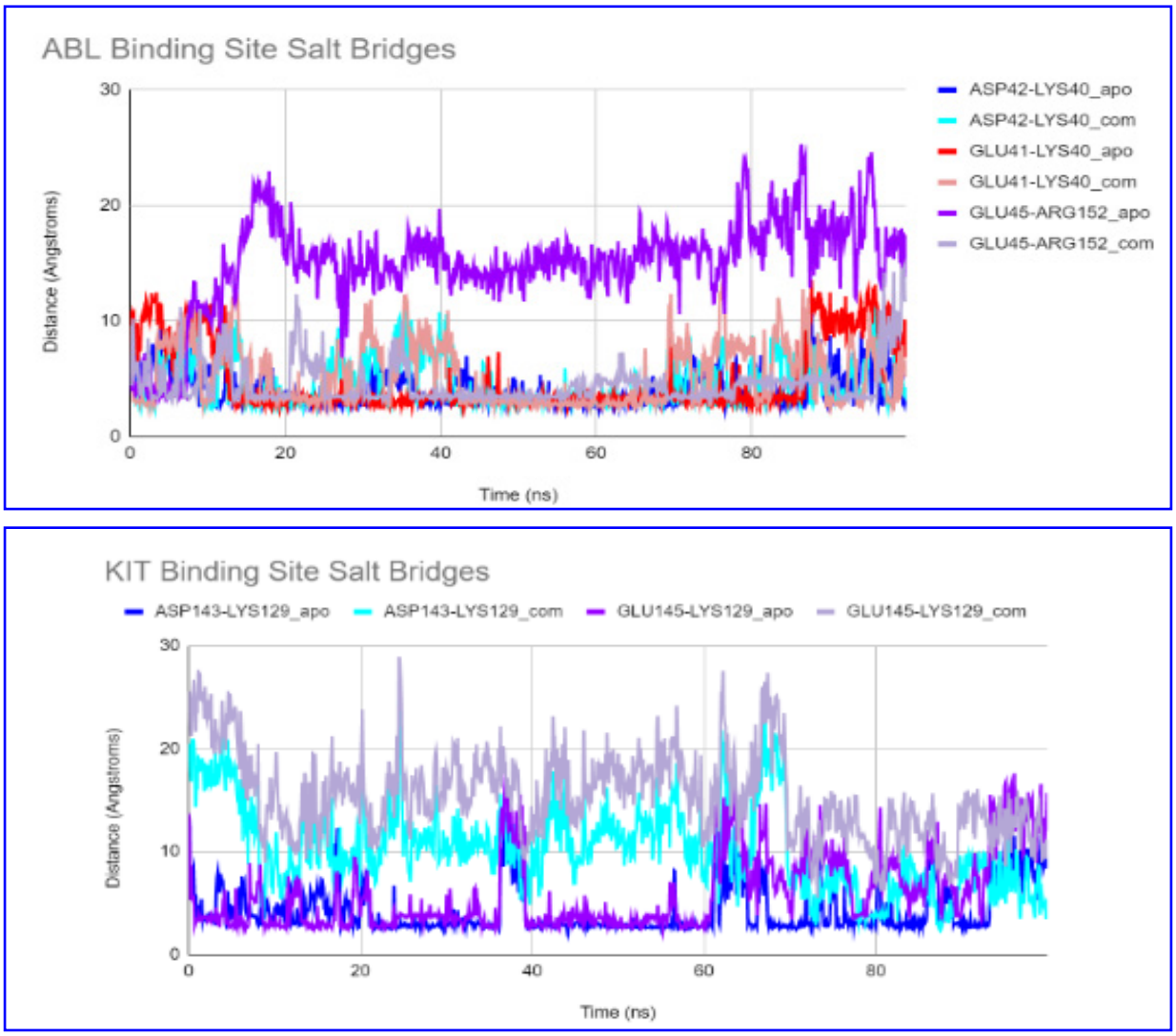

Figure 8 (above) - Salt Bridges on ABL Binding Site. **Figure 9 (left) -** Salt Bridges on KIT Binding Site.**Figures 8 and 9.** Salt bridges that interact with residues within 8 angstroms of imatinib, representing thprotein binding site.

energy wells and conformations the dihedral angle adopts in the protein. This results in higher flexibility, RMSF, and entropy values.

Entropy analysis of the binding site corresponds with RMSF and non-covalent interactions

For the binding site, the most substantial difference is in KIT, with a 2-3 kcal/mol difference favoring the apoprotein structure for the dihedrals. For ABL, the entropy difference is smaller and tends to favor the complex by 0.1-0.3 kcal/mol for the dihedrals. For SRC, the entropy difference favors the apoprotein by 0.4-0.7 kcal/mol for the dihedrals.

DISCUSSION

The purpose of molecular docking is to examine how small molecules bind to larger macromolecules as their interactions are unavailable from the Protein Data Bank. The docking software samples a topological grid to find the global minimum for the drug-protein complex and calculate docking energy values. For the imatinib-kinase trials, the results agree with known results, showing imatinib binds more tightly to ABL and KIT than SRC (Dubey, 2012) (Lin, 2013).

The purpose of RMSD is to show that the structure stabilizes by the end of the simulation. When sampled from the Protein Data Bank, protein structures experience steric hindrance from intramolecular repulsion, so there is an initial increase in the RMSD before it equilibrates. I expect that differences between the bound complex and apoprotein are attributable to the imatinib binding. The RMSD results affirm that imatinib binds tightly with ABL, as it indicates the apoprotein is more flexible than the complex. KIT also exhibits significant change, but with a more flexible apoprotein structure compared to the complex. This is an unusual observation since the apoprotein is typically more flexible. Finally, SRC exhibits similar apoprotein and imatinib-bound RMSD, agreeing with the idea that SRC has a weak affinity for imatinib.

Proteins fold into specific structures to work in the body, so changes in the protein flexibility after ligand binding can affect its bodily function (Teilum et al., 2011). The purpose of RMSF is to discover the flexible residues that contribute most to protein dynamics by examining the positional differences of the structure over the simulation run. ABL and SRC share ~50% of their structure, so it is expected that they share peaks in similar locations. Unlike ABL, SRC has insignificant RMSF differences between the apoprotein and complex structure. This affirms that imatinib's presence in the binding pocket has little effect on SRC's dynamics. KIT peaks in different regions than ABL or SRC, demonstrating that different protein structures can have similar affinities for the same drug while similar structures can have different affinities. This is important as it signifies we cannot rely on the structural similarities alone to judge drug affinities for proteins.

Hydrogen bonds are a crucial non-covalent interaction to maintain protein structures, but they get weaker at longer distances and larger angles. Therefore, I parameterized the H-bond with a maximum length of 3.5 angstroms and angle of 150 degrees for at least 20 ns to categorize it as structurally significant. ABL contains more H-bonds in the complex than the apoprotein, which agrees with the RMSD and RMSF, as more H-bonds help maintain protein rigidity. In contrast, KIT and SRC form more H-bonds in the apoprotein compared to the complex, further agreeing with the RMSD and RMSF. The drug-protein H-bonding shows that ABL and KIT form H-bonds with the imatinib while the SRC has no main H-bonds, explaining the comparatively different docking energies and affirming SRC's lower imatinib affinity compared to KIT or ABL.

Salt bridges weaken as the distance between interacting groups increases. Therefore, I classified a salt bridge when the heavy atoms of D and E are within 4 angstroms distance of K or R residues, where a "significant" salt bridge forms when at least 20 ns of the simulation features a 4-angstrom distance or less (Kumar, 2002). I evaluated the ion pairs that form along the relevant RMSF peaks to investigate how salt bridges change with imatinib binding. Several ion pairs that broke in the apoprotein structure for ABL formed in the complex, and vice versa for KIT. The RMSF for SRC remains similar in the apoprotein and SRC-imatinib complex, meaning the ion pairs likely have little impact on the protein's flexibility after imatinib binds.

I studied the entropy of the binding site residues within 8 angstroms of imatinib for ABL, KIT, and SRC to assess

the relative entropy differences between the apoprotein and bound complex. The KIT and SRC entropy favored the apoprotein structure, with KIT exhibiting a larger difference. For ABL, the entropy difference was also small and favored the complex. The entropy findings are consistent with the observations for protein RMSD, RMSF, and the non-covalent interaction frequencies, and serve as a useful quantitative tool to measure protein flexibility in apoprotein and bound-complex conditions.

FUTURE FOCUS

In the future, I project to simulate the drug-protein interactions of eleven kinases of interest including ABL, KIT, SRC, LCK, p38α, ASK1, AURKA, BRAF, FLT3, CHK1, and GSK3β in the first comprehensive study in atomistic-level details for understanding imatinib binding specificity for future cancer drug design. In addition, I will study the protein structure changes as imatinib unbinds from the kinases. Finally, I will look more closely at the H-bonds and salt bridge interactions that form, break, and reform as imatinib dissociates. From these simulations, I will approximate the affinities based on residence time, the amount of time it takes for the drug to unbind from the protein.

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