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CURJ

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Mitochondrial DNA Mutation

How increased mutation affects macrophage response to *Listeria monocytogenes*

Potassium tetraborate resistance
Improving agricultural storage with salts used in the oil industry

Soundness of a Gradual Verifier
Developing a lightweight tool for catching bugs in Gradual C0 using property based testing

Catfish Commnication
Investigating the effect of fish morphology on acoustic response

CORNELL UNDERGRADUATE RESEARCH JOURNAL

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Letter from the Editor

Dear Reader,

We are incredibly thrilled to present the third issue of the Cornell Undergraduate Research Journal (CURJ). CURJ is Cornell's peer-reviewed bi-annual research publication featuring the exemplary scholarly work of Cornell undergraduates across all disciplines and fields. The goal of this publication is to provide a platform for Cornell undergraduate students to showcase their research articles to the student body and to the general public, and offer the student body a wide range of academic perspectives, provoking intellectual pursuits and collaboration.

This is a bittersweet letter for me to write, as I am graduating this May and this will be my last Letter from the Editor as the Editor-In-Chief of the Cornell Undergraduate Research Journal. I remember how two and half years ago, the idea of this journal seemed so far away and difficult to achieve. The amount of work to create this journal seemed at the time never-ending, and we had to do everything from scratch, from branding, to designing legal processes and forms, to creating processes for article submission and peer review, to financing the journal, to setting up DOIs and ISSNs, to creating the design and layout of the journals, to printing and publishing. Now, our third issue is being released and we have published many articles in these three issues showcasing the exceptional research of undergraduates across a variety of fields. The Cornell Undergraduate Research Journal is operating smoothly from issue to issue, with an amazing team working on it, and I could not be more grateful and delighted with how it has turned out.

All of this has been possible due to our fantastic CURJ team. Building this journal from the ground up required a lot of very difficult work, but our team has worked relentlessly and has risen to the many challenges along the way. I may be graduating and leaving Cornell, but I know that CURJ is in the hands of an incredibly passionate, hard-working, and skillful team and I am positive that they will continue the legacy of this journal for many years to come. I look forward to reading the new CURJ issues each semester and to being CURJ's most enthusiastic fan.

We would like to thank our authors for their exceptional work on their research articles and our graduate student reviewers and faculty advisors for their insightful feedback and contributions that have greatly enriched this publication. We would also like to thank Mike Priehs of the Cornell University Library for hosting and supporting our journal. And of course we would like to express our gratitude to Ellen Hartman, Research Communications Director of Cornell Research and our organization's advisor, without whom this publication would not have been possible.

We are very excited to share this third issue of the Cornell Undergraduate Research Journal and to present the outstanding research of Cornell undergraduates.

Victoria Alkin
Founder and Editor-In-Chief

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Effects of increased mitochondrial DNA mutation on the macrophage response to *Listeria monocytogenes*

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Abstract

Mitochondria are important for cellular function, and as cells divide, their mitochondria also divide by replicating their DNA. The integrity of mitochondria DNA (mtDNA) replication, carried out by Polymerase G (PolG), is critical for the maintenance of mitochondria and their functions. In this study, mice carrying a mutant PolG, $PolG^{D257A}$, were used to determine the effect of increased mtDNA mutations on the macrophage population and polarization in response to bacterial and cytokine challenge. It was hypothesized that increased mtDNA mutations will inhibit pathogen clearance by macrophages. To test this hypothesis, the $PolG^{D257A}$ mice were used, along with *Listeria monocytogenes* (LM) as a model of bacterial infection. Three days post LM infection, the bacterial load and the macrophage population was determined in the spleen and liver of $PolG^{D257A}$ and WT mice. No statistical difference was observed in the bacterial load in the liver or spleen, or in the macrophage population in the spleen of the $PolG^{D257A}$ and WT mice. However, the $PolG^{D257A/D257A}$ mice were associated with a higher percentage of macrophages in the liver during LM infection. Polarization of peritoneal macrophages into classically activated (M1) and alternatively activated (M2) macrophages was also studied in vitro. In a single experiment, increased mtDNA mutations in $PolG^{D257A}$ mice seemed to elicit increased M1 and decreased M2 macrophage polarization. Replication of the experiment is warranted to confirm these results. These experimental findings could lead to a better understanding of the role of the mitochondria and macrophages in infectious disease.

Introduction

1.1 *Listeria monocytogenes*

Listeria monocytogenes (LM) is a facultative intracellular bacterium which is the leading cause of a foodborne infection known as listeriosis. Listeriosis is a relatively rare disease with 0.1 to 10 cases per 1 million people per year, depending on the countries and regions of the world (WHO, 2018). Though rare, listeriosis is associated with a high mortality rate in certain groups of people, therefore making the infection a public health concern. The disease primarily affects pregnant women, the elderly (>65), and the immunocompromised (CDC, 2020). Upon infection through ingesting contaminated food, LM moves through the bloodstream and infects the liver, spleen, lymphoid system, and the brain (Zhang et al., 2017). The macrophage internalizes the bacteria into a phagosome (Abuaita et al., 2018). Proteases in the phagosome of infected cells break down LM proteins which are then recognized by innate immune cells like macrophages (Eitel et al., 2011). Internalization of LM induces the cytosolic surveillance system resulting in the expression of Tumor Necrosis Factor alpha (TNF-α) and Interleukin 12 (IL-12). These cytokines stimulate T cells to produce Interferon gamma (IFN-γ) which drives the clearance of bacteria (Zenewicz et al., 2007). The virulence of LM is enhanced by Listeriolysin O, a cytolysin that mediates the escape of LM from the phagosome to avoid humoral defenses such as antibodies, thus,

enabling cytosolic LM replication and spread through actin polymerization and actin-based motility (Nguyen et al., 2019, Cheng et al., 2018).

1.2 Macrophage polarization

Macrophages play a unique role in phagocytosis and clearance of pathogens. Macrophages produce and secrete anti-microbial peptides, generate protons, and secrete anti-microbial effectors such as ROS and nitric oxide (Ramond et al., 2019). Macrophages are known to exhibit heterogeneity in that they can polarize into two main functional types, M1 and M2 macrophages. During infection, macrophages can differentiate into M1 macrophages under the influence of bacterial Lipopolysaccharide (LPS) and increase tissue inflammation and phagocytic capacity to support pathogen clearance. M1 macrophages can induce an expedited ATP production through glycolysis to provide cell energy for the macrophage during inflammation (Yarbro et al., 2020). On the other hand, M2 activation is directly induced by signals from IL-4, IL-13, and IL-33. M2 macrophages are responsible for inflammation resolution, angiogenesis, and tissue regeneration (Yao et al., 2019). M2 macrophages have lower microbicidal activity, which can present a favorable niche for bacterial replication leading to chronic infections. M1 polarization is dependent on glycolysis and mitochondrial fission whereas M2 polarization is reliant on oxidative phosphorylation, fatty acid oxidation and mitochondrial fusion (Ramond et al., 2019). All the aforementioned cell processes are dependent on mitochondria and contribute to the eradication of bacteria by macrophages.

1.3 Mitochondria, Polymerase G, and mitochondrial DNA replication

The mitochondrion is a double membrane organelle that generates energy through the electron transport chain system. Products from the electron transport chain also fuel an array of signaling pathways that help macrophage activation and function. The mitochondrion genome is approximately 16,500 nucleotide base pairs, which form the circular mitochondrial DNA (mtDNA) and encode 13 proteins (Gahl, 2019). The replication of the mtDNA is essential for cell proliferation and mitochondrial biogenesis (Kujoth et al., 2005). One protein that is important in this process is DNA Polymerase gamma (PolG). PolG replicates mtDNA and eliminates mismatched nucleotides in the mitochondrial genome. Mutations in PolG disrupt the integrity of the mtDNA, thereby making the gene a disease locus, responsible for over 180 human disease mutations for the PolG gene including Alpers' syndrome (Zhang et al., 2011). The PolGD257A mutant mouse model harbors a mutation in the 257th amino acid, a change from Aspartic Acid to Alanine, in the exonuclease domain II of PolG, which results in the polymerase lacking proofreading function in all cell types. The ablation of the PolG proofreading capabilities in these mice results in accelerated aging-associated features such as hair loss, graying and kyphosis (Kujoth et al., 2005). Sequencing revealed that the frequency of mtDNA mutations in the $PolG^{D257A}$ mice was ~3 to 8 times higher than that of the Wildtype mice in various cell tissues including the liver of 5-6 months old mice (Kujoth et al., 2005). This $PolG^{D257A}$ mouse model is used herein to determine the effect of increased mtDNA mutations and the resulting mitochondrial dysfunction on LM infection, induced macrophage recruitment and polarization. It is hypothesized that both LM infection and macrophage polarization will be subdued owing to the increased mtDNA mutations.

Materials and Methods

2.1 Mice

The mice used in this experiment are the wildtype (WT) $PolG^{+/+}$ mice, the heterozygous $PolG^{D257A/+}$ mice and the homozygous $PolG^{D257A/D257}$ mice. The mice were between 4 to 6 months of age. All mice used in this study were bred in the East Campus Research Facility at Cornell University. All studies were performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH guide for Care and Use of Laboratory Animals,

federal and state regulations, and was approved by the Cornell University Institutional Animal Care and Use Committee (IACUC, protocol #2013-0014).

2.2 LM culture and infection

A colony of LM was used to inoculate 3 mL of Brain Heart Infusion (BHI) LM selective media for culture at 37 °C. WT and $PolG^{D257A/D257A}$ mice were infected with 1 x 10^6 CFU of LM via peritoneum injection. Three days post infection, the mice were euthanized, and their spleens were collected. The livers were also harvested after perfusion with 10 mL of Phosphate Buffered Saline (PBS) through the hepatic portal vein. The spleens and livers were homogenized in 4 mL and 3 mL of 1% Triton-X100, respectively. The homogenates were distributed into 1:10 serial dilutions to 10^5 dilutions. 0.1 mL of each dilution was plated onto BHI agar plates and incubated at 37 °C. The LM CFU/organ were counted after 24 to 48 hours of culture.

2.3 In vitro macrophage polarization

The peritoneal fluid from WT, $PolG^{D257A/+}$ and $PolG^{D257A/D257A}$ mice were collected and cultured at 1 x10^6 cells/well as described (Alatery et al. 2008). The cells were cultured in RPMI media with 5 ng/mL macrophage colony stimulating factor (M-CSF), 10% Fetal Bovine Serum (FBS), 1% glutamine and 1% penicillin/streptomycin. The cells were incubated in a humidified incubator with 5% CO2 at 37°C. Three days later, the cells were rinsed twice with 1X PBS and the macrophage stimulating media was replaced with a fresh batch. On Day 7, some of the wells were stimulated towards M1 polarization with 100 ng/mL LPS, and some were stimulated towards M2 with 20 ng/mL IL-4. A third set of the wells were cultured in RPMI media without M-SCF for M0 polarization — 10% FBS, 1% glutamine and 1% penicillin/streptomycin. There were three cell culture replicates for each cell type, cultured at 1 x10^6 cells/well in three different wells. After 48 hours, the cells were detached with 0.25% trypsin in EDTA and washed with 2 mL per well with 10% FBS in PBS (Zhao et al., 2017). The cells were stained for flow cytometric analyses as described in 2.5.

2.4 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total mRNA was collected from peritoneum macrophages in Trizol following in vitro polarization as described (Wellcome, 2007). The cells were separated into an aqueous phase with 200 µL of chloroform per ml of Trizol and the resulting RNA was precipitated in 500 µL 2-propanol. The RNA pellet was washed with 1 mL of 75% ethanol (prepared using RNase-free water), air dried for 10 minutes in the hood and later dissolved in 30 µL of RNase-free water on ice. The concentration of RNA was measured with Quawell Q3000 UV Spectrophotometer. The 260/280 value for each sample was close to 2. The RNA was diluted (1:10) with RNase free water in preparation for Reverse Transcription to cDNA. Each sample received a mixture of 5x iScript Reaction Mix, iScript Reverse Transcriptase and nuclease free water following instructions on the iScript cDNA synthesis kit from Bio-Rad Laboratories, Inc. The resulting cDNA underwent PCR reaction using the following forward and reverse primers respectively; 5'-AAACGGCTACCACATCCAAG-3' and 5'-CCTCCAATGGATCCTCGTTA-3' for the control - 18S rRNA (155 bp) and 5'-GGGCATACCTTTATCCTGAG-3' and 5'-CCACTGAAGTCATCCATGTC-3' for M2 Ym-1 (304 bp) (Mulder et al. 2014). All primers were purchased from Integrated DNA Technologies (Coraville, Iowa). The Denville Scientific Inc DNA marker 100bp ladder (cb3602) was used in the Gel Electrophoresis.

2.5 Flow cytometric analyses

All macrophage populations and phenotypes were determined with the Thermo Fisher Attune NxT Flow cytometer and analyzed using FlowJo. Macrophages were stained directly with fluoro-chrome-labeled antibodies against the following markers: eBioscience AF700 Anti-mouse MHC Class II (I-A/I-E) (clone M5/114.15.2), BioLegend PE/Dazzle Anti-mouse/human CD11b (clone M1/70), eBioscience PE anti-mouse F4/80 (clone BM8),

BioLegend APC/Cyanine7 Anti-mouse CD86 (clone GL-1), BioLegend FITC anti-mouse CD206 (MMR) antibody (clone C068C2), eBioscience Fixable Viability Dye eFlour 506 (clone v506) and eBioscience FC block Anti-Mo CD16/CD32 Purified (clone 93). Macrophages in the spleen and the liver were identified with F4/80+MHCII+CD11b+ markers using flow cytometric analyses.

2.6 Statistics

All data representations shown in this study were illustrated with GraphPad Prism version 9.0. Statistical significance between groups ($P<0.05$, $n>3$) were determined using Student's t-test.

Results

3.1 Increased mtDNA mutations leads to reduced liver weights in LM infected mice

To understand the effect of $PolG^{D257A}$ mutation on the response to LM infection, the weights of mice, livers, and their spleens of WT and $PolG^{D257A/D257A}$ mice were compared 3 days after LM infection. As shown in Figure 1, the WT and $PolG^{D257A/D257A}$ mice had similar weights post infection except the livers, where the $PolG^{D257A/D257A}$ livers weighed less than WT livers ($p<0.05$). There was no difference in weights of the mice and spleens.

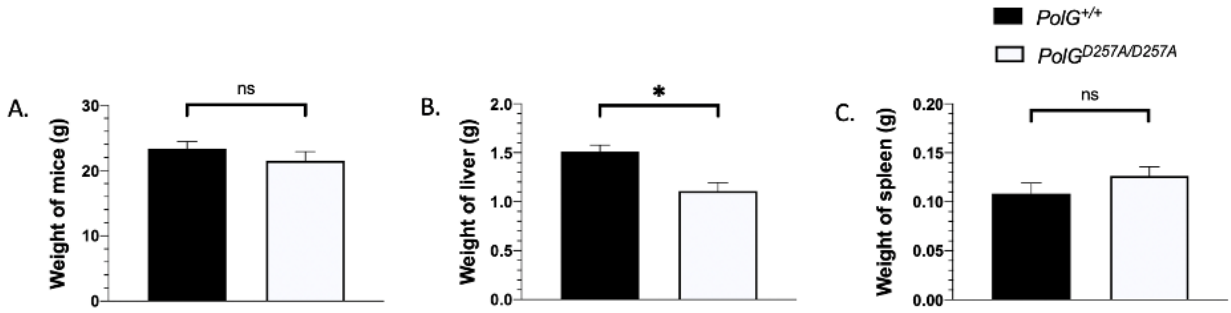

Figure 1: Reduced weights of the liver of PolGD257A/D257A 3 days post LM infection. WT (n=6) and PolGD257A/D257A (n=8) mice were infected with LM and sacrificed after 3 days for analysis of spleen and liver. (A) Weight of the WT and PolGD257A/D257A mice. (B) Weight of liver of WT and PolGD257A/D257A mice ($p<0.05$). (C) Weight of spleen of WT and PolGD257A/D257A mice.

3.2 Increased mtDNA mutations does not affect LM CFUs in the spleen and liver of infected mice

To determine the effects of the $PolG^{D257A}$ mutation on LM colonization 3 days after infection, spleen and liver homogenates were plated on BHI agar plates and the LM colonies were counted. LM CFU was calculated per gram of organ and the results are displayed in Figure 2. Although the difference was not statistically significant, $PolG^{D257A/D257A}$ mice had higher bacterial load in the spleen than the WT mice. However, the reverse was observed in the liver, although again the difference was not statistically significant.

3.3 Increased mtDNA mutations are associated with a higher percentage of macrophages in the liver during LM infection

Three days post LM infection, splenic and liver cells were isolated and stained for macrophages. Viable cells that were positive for F4/80, MHCII and CD11b were gated as macrophages using flow cytometric analyses (Figure 3). The number of macrophages were determined as a product of the percentage of F4/80+MHCII+CD11b+ cells and the total cell counts for each group. There was no statistically significant difference in the percent and number of splenic macrophages (Figure 4). By contrast, the livers of $PolG^{D257A/D257A}$ mice had a higher percent of macrophages when compared to the WT mice ($p<0.05$) (Figure 5). This suggests that increased mtDNA

This suggests that increased mtDNA mutation may be linked to high macrophage recruitment in the liver during LM infection.

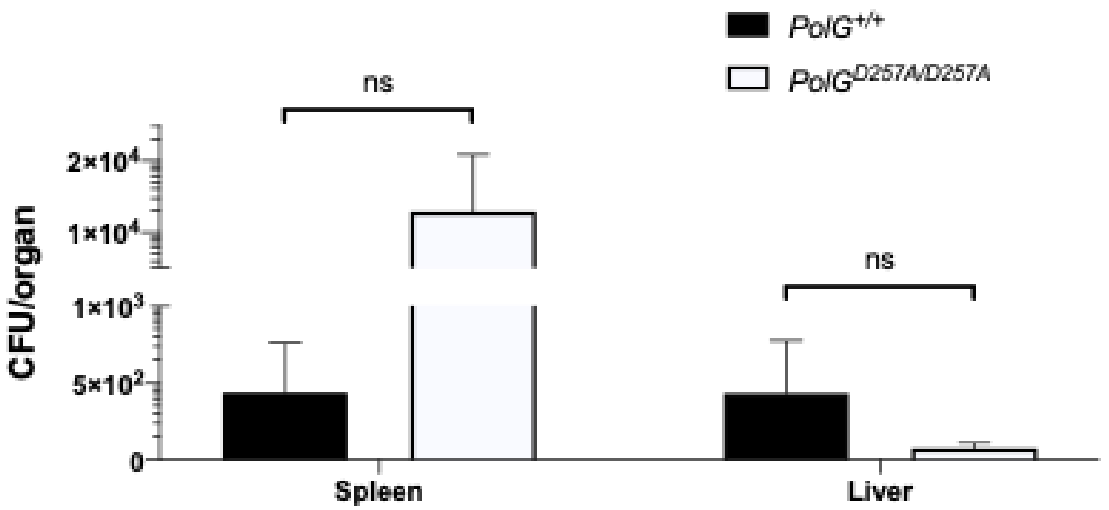

Figure 2: No statistically significant difference in LM CFU/organ in the spleen and liver of WT and PolGD257A/D257A mice 3 days post infection. WT and PolGD257A/D257A mice shown in Figure 1 were analyzed for LM CFUs in the spleen and liver. LM colonies were counted from tissue homogenates 24 to 48 hours after plating. There was no statistical significance in the difference in bacterial load between the WT and PolG$^{D257A/D257A}$ mice. WT (n=6) and PolG$^{D257A/D257A}$ (n=8).

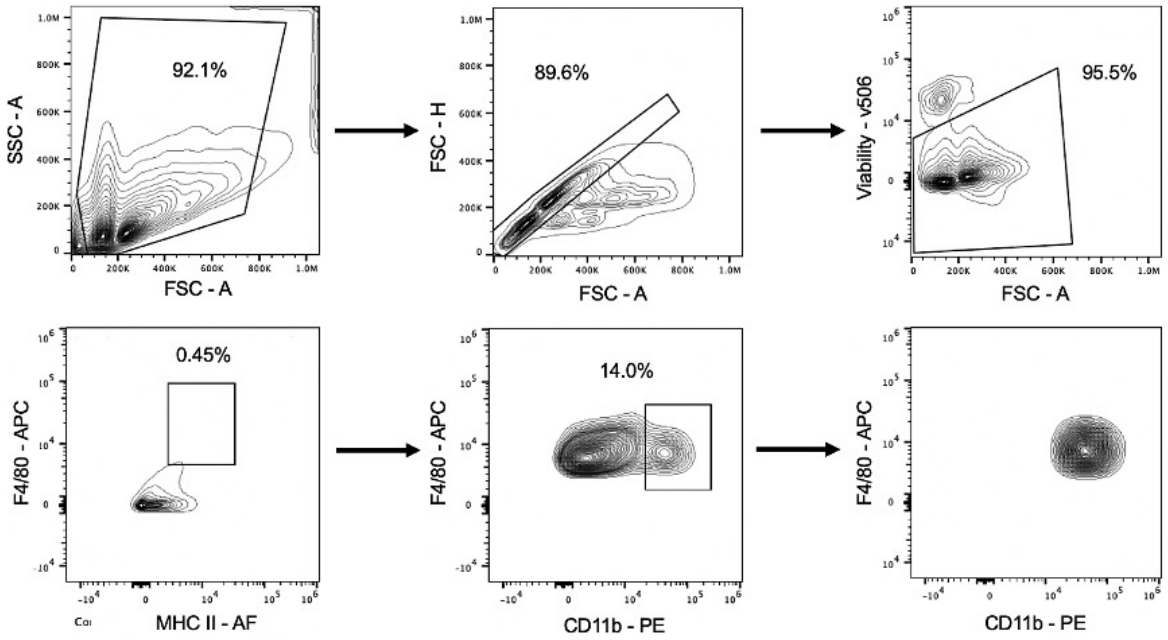

Figure 3: Macrophage Gating Strategy. Macrophages were gated from isolated cells from the spleen and liver of mice infected with LM shown in Figure 1. Viable macrophage populations were detected with F4/80+MHCII+CD11b+ markers using flow cytometric analyses.

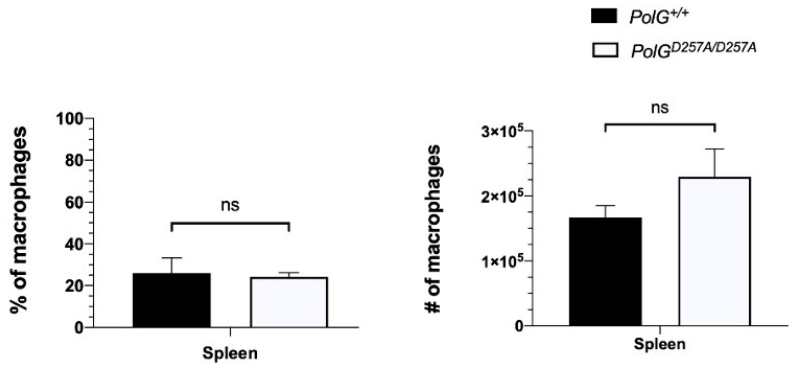

Figure 4: No difference in percent and number of macrophages present in the spleen of WT and PolG$^{D257A/D257A}$ 3 days post LM infection. Macrophages were gated from isolated cells from the spleens of mice infected with LM as shown in Figure 3. Viable macrophage populations were detected with F4/80+MHCII+CD11b+ markers using flow cytometric analyses. There was no significant difference in the percent or number of macrophages between the WT and PolG$^{D257A/D257A}$ mice in the spleen. WT (n=6) and PolG$^{D257A/D257A}$ (n=8).

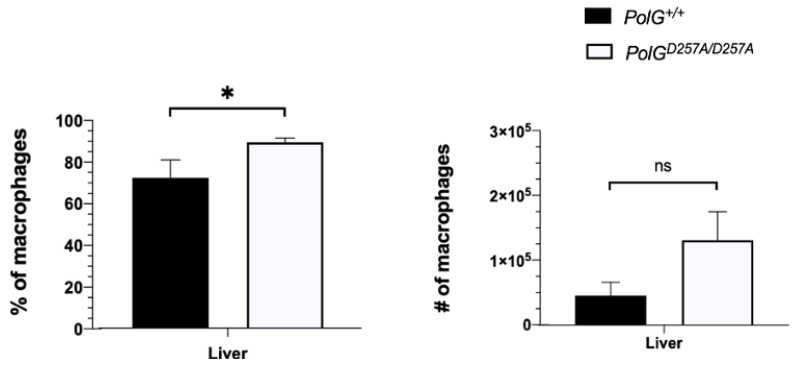

Figure 5: Statistically significant increase in percent, but not number of macrophages in the liver of PolGD257A/D257A mice 3 days post LM infection. WT and PolGD257A/D257A mice shown in Figure 1 were analyzed for liver macrophages. Increased mtDNA mutations were associated with a higher percentage of macrophage in the liver. WT (n=6) and PolG$^{D257A/D257A}$ (n=8) ($p<0.05$).

3.4 Increased M1 and decreased M2 macrophage polarization with increased mtDNA mutations

To investigate the effect of mitochondrial dysfunction due to increased mtDNA mutations on macrophage polarization, classically activated M1 and alternatively activated M2 macrophages from the peritoneum of the WT, heterozygous PolG$^{D257A/+}$ and PolG$^{D257A/D257A}$ mice were evaluated. The peritoneal cavity is a common and easy source of primary macrophages compared to the spleen and liver (Zhao et al., 2017), and so peritoneal macrophages were used for polarization experiments. Cells collected from the peritoneal cavity of the WT, PolG$^{D257A/+}$ and PolG$^{D257A/D257A}$ mice were stimulated with 5 ng/mL M-CSF media to differentiate monocytes into macrophages. After 7 days, the macrophages were treated with no cytokine (M0), 100 ng/mL LPS for M1 polarization and 20 ng/mL IL-4 for M2 polarization. 48 hours after polarization, the macrophages were stained with antibodies for flow cytometric analyses. Viable macrophages were gated as double positive for F4/80 and CD11b markers using the strategy shown in Figure 6. There were 90 to 100% F4/80+CD11b+ cells in both the polarized and non-polarized cell types (Figure 7). This is indicative of the presence of macrophages in all genotypes and testing conditions. Further, the LPS-induced M1 macrophages were detected as F4/80+CD11b+CD86+ cells (Figure 8). CD86 is an antigen surface marker that is highly expressed under inflammatory conditions and is considered an M1 macrophage marker (Zhao et al., 2017). Macrophage M1 wells had a higher percentage of CD86 markers in comparison to the non-polarized M0 macrophages and IL-4-induced M2 macrophages (Figure 8). Statistical significance was not computed for

these results because the data was obtained from the pooling of 2 to 3 mice in one experiment. However, the trend shows a gradual increase in percentage of M1 macrophages as the mtDNA mutations increase (Figure 8). The macrophage mannose receptor, CD206, was used as an M2 gene surface marker (Rőszer, 2015). All macrophages showed elevated percent and number of F4/80+CD11b+CD206+ cells (Figure 9), although more experiments need to be performed to confirm these results. RT-PCR was performed to examine functional M2 markers such as the Ym-1 gene, which is also upregulated by IL-4 in macrophages (Rőszer, 2015). The gel electrophoresis display demonstrates that the Ym-1 gene, unlike the control, was expressed at 304 bp in the IL-4-induced M2 macrophages alone, and not the M0 or M1 macrophages (Figure 10). Also, the brightness of the bands faded with increased PolGD257A mutations. The PolG$^{D257A/D257A}$mice showed no band at all (Figure 10).

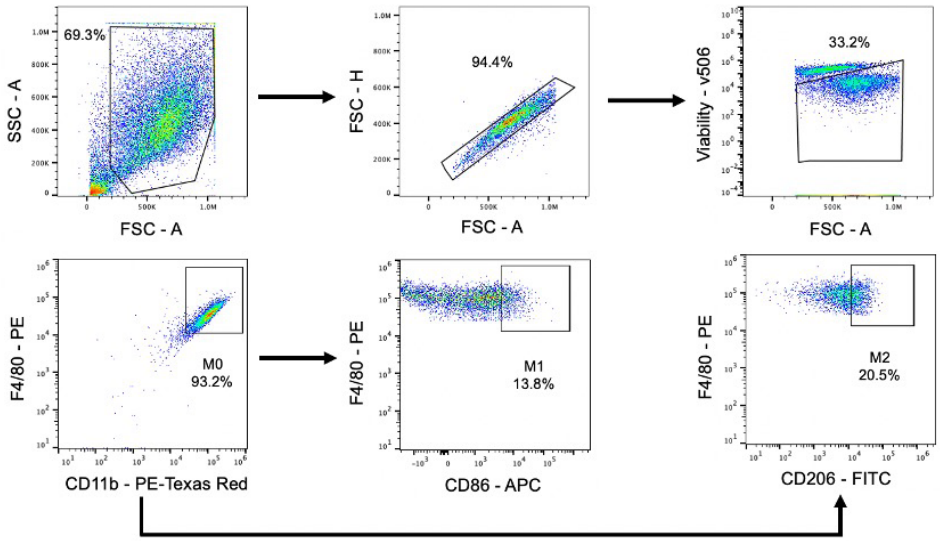

Figure 6: Gating Strategy for Macrophage Polarization. 48 hours after polarization to M0, M1 and M2 peritoneal macrophages, cells were gated using flow cytometric analyses as shown above. Viable macrophage populations were detected as F4/80+CD11b+. LPS-induced M1 macrophages were detected with F4/80+CD11b+CD86+ markers and IL-4 induced M2 macrophages were detected as F4/80+CD11b+CD206+.

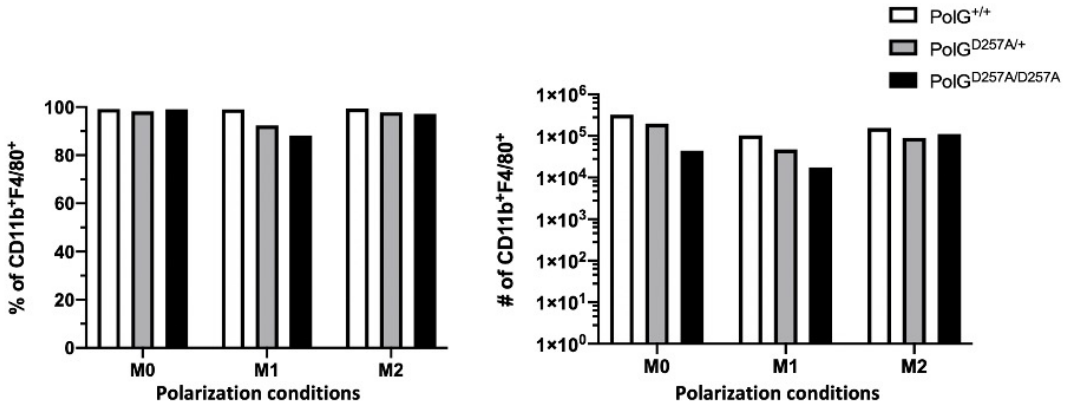

Figure 7: Percent and number of F4/80+CD11b+ macrophages present in the M0, M1 and M2 peritoneal macrophage cultures of WT, PolGD257A/+ and PolGD257A/D257A mice. Peritoneal cells from WT (n=3), PolG$^{D257A/+}$ (n=2), and PolG$^{D257A/+}$ (n=2) were pooled and cultured under M0, M1 and M2 conditions as described in the Methods section. Cells were 90 to 100% F4/80+CD11b+ for macrophage markers after polarization. The number of F4/80+CD11b+ macrophage cells varied from 1x10^4 to 1x10^6.

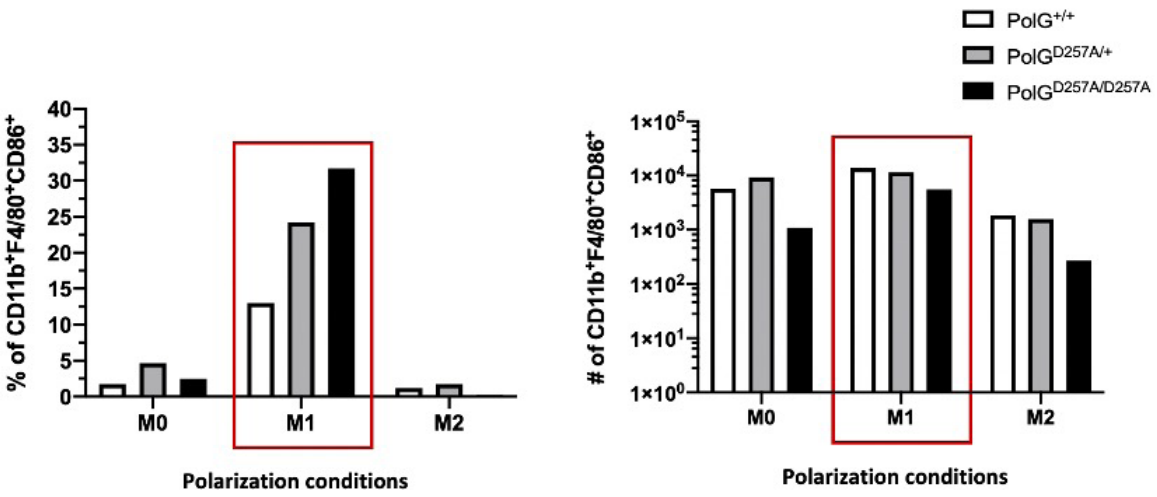

Figure 8: Percent and number of F4/80+CD11b+ CD86+ macrophages present in the M0, M1 and M2 peritoneal macrophage cultures of WT, PolGD257A/+ and PolGD257A/D257A mice.Peritoneal cells from WT (n=3), PolGD257A/+ (n=2), and PolGD257A/+ (n=2) were pooled and cultured under M0, M1 and M2 conditions as described in the Methods section. The red boxes indicate LPS-induced M1 macrophage cultures.

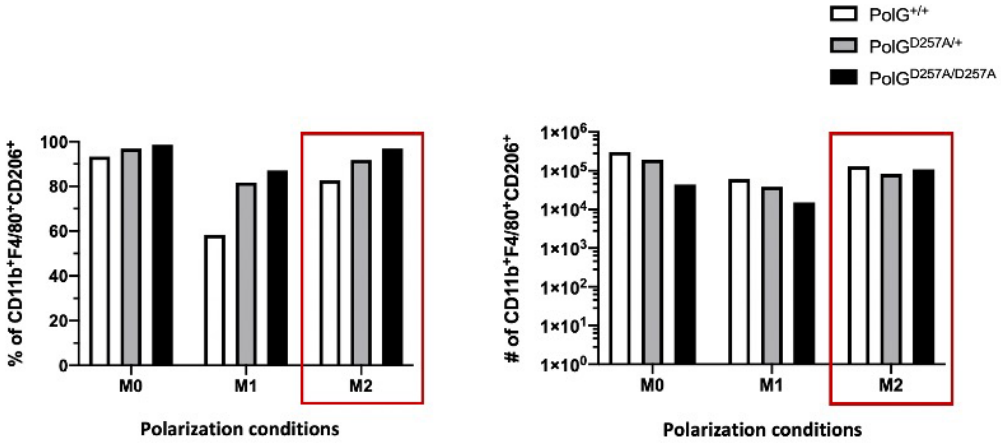

Figure 9: Percent and number of F4/80+CD11b+ CD206+ macrophages present in the M0, M1 and M2 peritoneal macrophage cultures of WT, PolGD257A/+ and PolGD257A/D257A mice. Peritoneal cells from WT (n=3), PolGD257A/+ (n=2), and PolGD257A/+ (n=2) were pooled and cultured under M0, M1 and M2 conditions as described in the Methods section. The red boxes indicate IL4-induced M2 macrophage cultures.

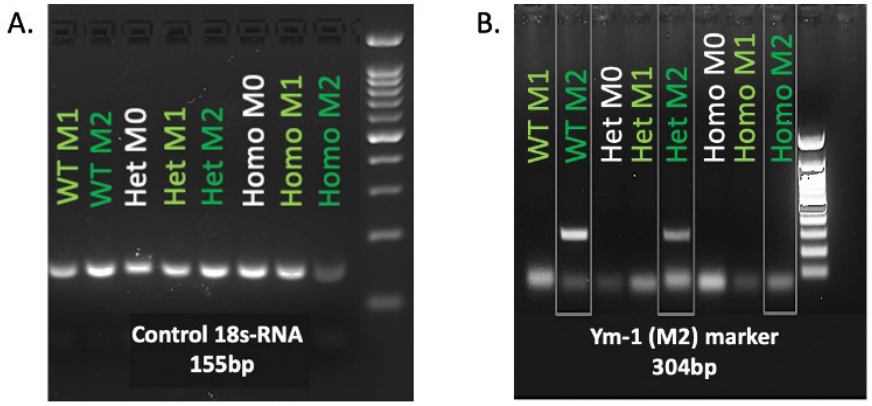

Figure 10: Decreased M2 macrophage polarization in PolGD257A mice. Peritoneal cells from WT (n=3), PolGD257A/+ (n=2), and PolGD257A/+ (n=2) were pooled and cultured under M0, M1 and M2 conditions as described in the Methods section. RT-PCR results of (A) Control 18s-RNA and (B) Ym-1, an M2 associated gene, 48 hours after polarization.

Discussion

Macrophages are among the cells that make up the first line of immune defense and they adopt diverse activation states in response to infectious diseases. While mitochondria have been shown to be important for macrophage function, the effect of increased mtDNA mutations on the macrophage population and polarization in response to bacterial and cytokine challenge is not known. Here, the hypothesis that increased mtDNA mutations and accompanying mitochondrial dysfunction will suppress the ability of macrophages to clear pathogens was tested using LM infection of PolG^{D257A} and WT mice, and polarization of peritoneal macrophages into classically activated (M1) and alternatively activated (M2) macrophages from these mice. The data suggest that increased mtDNA mutations affect the percentage of macrophages in the liver during LM infection and may affect M1 and M2 macrophage polarization in vitro. Replication of the experiment is warranted to confirm these results. The findings from these experiments could lead to a better understanding of the role of the mitochondria and macrophages in infection. Furthermore, as the various phenotypes of macrophages are still being characterized, there remains limited knowledge on how macrophage activation is affected by mitochondrial dysfunction (Anders, 2020). Thus, the identification of macrophage phenotypic differences in mice with increased mtDNA mutations will provide novel insights into the role of macrophages and the mitochondria in pathogen clearance. In addition, the study may improve current drugs and therapeutic interventions for pathologies such as Alzheimer's disease, that are associated with mitochondrial dysfunction (Wang et al., 2020).

In this study, the percent of macrophages was identified as being significantly higher in the livers of LM-infected PolGD257A/D257A mice than LM-infected WT mice. Currently, a direct correlation between macrophage response and mitochondrial dysfunction remains understudied. Increased mtDNA is also associated with accelerated aging, and the PolG$^{D257A/D257A}$ mice have also been shown to exhibit accelerated aging in comparison to the WT mice (Kujoth et al., 2005). Moreover, the process of aging in the liver is associated with degenerative modifications including deficits in mitochondrial function in both macrophages and hepatocytes (Kim et al., 2015). LM induces inflammasome activation that promotes the recruitment and bacterial-killing capacity of hepatic macrophages (Jeong et al., 2020). Several laboratories have demonstrated an age-related increase in hepatic macrophages, as high as threefold greater, in aged mice (Bloomer et al. 2020, Covarrubias et al, 2020, Stahl et al., 2020). Thus, it can be proposed that the increase in macrophages in the liver of PolG$^{D257A/D257A}$ mice conforms to the narrative of the aged mice. Also, the macrophages in the liver PolG$^{D257A/D257A}$ may be actively responding to the LM-induced inflammasome activation leading to the corresponding lower bacterial load in the WT mice, though this was not statistically significant.

In contrast to the liver, both the LM colonization and macrophage population in the spleen showed no differences between the WT and PolG$^{D257A/D257A}$mice. Splenic macrophages from older mice secrete substantially lower amounts of TNF-α and IL-6 cytokines in response to LPS and Toll-like receptor signals (Linehan et al., 2015). Geriatric mice that were gavaged with LM displayed higher LM colonization, higher inflammatory response and produced less IFN-γ in the spleen (Alam et al. 2020). IFN-γ is recognized as a key regulator of macrophage activation that enhances macrophage phagocytosis to fight LM infection (Wu et al., 2014). Thus, low IFN-γ production in aged mice can be linked to reduced phagocytic ability, which could be a consequence of several factors including senescence, defective autophagy, and impairments in mitochondrial and increased ROS production (Yarbro et al., 2020). If replicated, the results from this study would generally agree with these findings in

that the increased macrophages in the LM-infected PolG$^{D257A/D257}$A spleen may have phagocytic defects resulting in the high LM colonization, although not statistically relevant. Replication of the study with more mice and further phagocytosis or ROS production assay may reveal statistical significance in the increase in LM colonies and macrophages observed in the spleen of the PolG$^{D257A/D257A}$ mice.

Additionally, macrophages in the peritoneal cavity of the WT, PolG$^{D257A/+}$ and PolG$^{D257A/D257A}$ mice were examined under polarizing conditions. The success of the in vitro culture of monocytes into macrophages was determined by the higher levels of F4/80+CD11b+ cells in all mouse groups. Large peritoneal macrophages in several mouse strains, including C57Bl/6 used in this study, express high levels of F4/80 and CD11b (Cassado et al., 2015). M1 macrophage activation seemed to increase with and M2 macrophage activation seemed to decrease with homozygous PolG^{D257A} mutations, although the macrophage polarization data needs more replicates. Consistent with these observations, macrophages with reduced mitochondrial content were more likely to be polarized into M1 macrophages and exhibited increased sensitivity to inflammation in response to stimuli (Adesso et al., 2013). Additionally, increased mitochondrial metabolism has been shown as both a requirement and characteristic of M2 macrophage polarization (Van den Bossche et al., 2016 & Tan et al., 2015). In the context of LM infection, LM has been shown to induce M1 macrophage polarization as well as promote repolarization of M2 macrophages into M1 macrophages (Thiriot et al., 2020). Given that mitochondrial dysfunction enhances M1 and impairs M2 polarization, the PolG$^{D257A/D257A}$ mice may activate M1 macrophages in response to LM infection.

A major limitation of this study was the lack of experimental replication and the small sample size used. Thus, repetition of the study with more mice, uninfected controls, and possibly time-course experiments to confirm the results is warranted. Additionally, functional M1 and M2 markers, such as iNOS and Arginase, respectively, will need to be quantified using RT-PCR to help formulate definite conclusions about the phenotypic variations observed, and also, to provide a perspective on the controversy surrounding acceptable M1 and M2 macrophage biological markers. Finally, mitochondrial uncoupling protein 2 has been shown to influence macrophage polarization (Angajala et al., 2018). Hence, future works will identify mitochondrial protein expression in the polarized macrophages using western blot technique to further characterize the players involved in each phenotype. In conclusion, this study used mice with mutated PolG, PolG^{D257A}, to investigate the effects of mitochondrial dysfunction on the macrophage recruitment and polarization following LM infection. A higher percentage of macrophages was observed in the liver of the PolG^{D257A} mice than the wildtype mice. In a single experiment, increased mtDNA mutations were associated with increased M1 and decreased M2 peritoneal macrophages. The findings suggest that mitochondrial dysfunction may lead to increased susceptibility to LM infection, although not statically significant, and increased M1 macrophage recruitment to LM infection. Replication of the experiments is warranted to confirm these findings. This work represents the first study of the potential effects of increased mtDNA mutations in the PolG$^{D257A/D257A}$ mice on the macrophage response to LM infection, and the findings contribute to the understanding of inflammation activation and mitochondrial dysfunction in infectious diseases.

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Evaluating Soundness of a Gradual Verifier with Property Based Testing

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Abstract

Gradual verification supports partial specifications by soundly applying static checking where possible and dynamic checking when necessary. This approach supports incrementality and provides a formal guarantee of verifiability. The first gradual verifier, Gradual C0, supports programs that manipulate recursive, mutable data structures on the heap and minimizes dynamic checks with statically available information. The design of Gradual C0 has been formally proven sound; however, this guarantee does not hold for its implementation.

In this paper, we introduce a lightweight approach to testing the soundness of Gradual C0's implementation. This approach uses Property Based Testing to empirically evaluate soundness by establishing a truthiness property of equivalence. Our approach verifies a test suite of incorrectly written programs and specifications with both Gradual C0 and a fully dynamic verifier for C0, and then asserts an equivalence between the results of the two verifiers using the dynamic verifier as ground truth. Any inconsistency between the results indicates a problem in Gradual C0's implementation. We also show in this paper, as a proof of concept, that this lightweight approach to testing Gradual C0's soundness caught a number of significant implementation bugs from Gradual C0's issue tracker in GitHub. A number of these bugs were only previously caught by human inspections of internal output of the tool. An automated generator for the test suite is our next research step to increase the rigor of our evaluation and catch new bugs.

Introduction

Motivation

Historically, we are familiar with a family of formal verification techniques as providing rigorous approaches to ensure the correctness of computer programs, in particular static verification. These techniques require the programmer to fully specify a program's predicted behavior to give formal assurance that the implementation will abide by what the programmer has in mind: a specification. Unfortunately, this approach does not support incrementality, as it requires the previously mentioned full specification to support quality code coverage. Further, static verification tools cannot provide verification feedback on any partial specifications written on the way to complete static specification. This means that if a user has a program and they only want to verify a particular method, they have to pedantically specify the entire method's annotations for the static verifier to receive with full coverage. This includes completely specifying loop invariants, preconditions, postconditions, etc., none of which can be at the discretion of the verifier, as it does not have enough information to make inferences. On the other hand, dynamic verification techniques focus at asserting specific test cases based on inputs given by a user at run-time. This technique avoids having to specify all information in a program, albeit it experiences massive overhead in regards to run-time costs that limit its practicality. In addition, dynamic verification techniques do not provide full code coverage and are meant to work in tandem with formal verification techniques.

Bader, Aldrich, and Tanter (2018), introduces the idea of *gradual verification*, which soundly

combines both static and dynamic verification techniques to support the incremental specification and verification of programs. Inspired by *gradual typing* (Garcia et al. 2016; Siek & Taha, 2006; Siek & Taha, 2007), with gradual verification the programmer gains control over the trade-offs between static and dynamic checking by way of partial specifications, allowing the behavior of unspecified components to be verified at run-time. Wise et al. (2020) introduces the theory of gradual verification for programs that manipulate recursive heap data structures (trees, graphs, lists, etc.). Wise et al. proved such a gradual verification system sound, and showed its adherence to the *gradual guarantee* property – relaxing specifications does not introduce new static or dynamic verification errors, if it is sound at full static specifications, then it is sound at the relaxation.

DiVincenzo et al. (2022) introduces the design and implementation of *Gradual C0*, the first gradual verifier for recursive heap data structures inspired by Wise et al.'s foundational theory. It is an extension of the pedagogical *C0* language used by Carnegie Mellon University to teach the fundamentals of dynamic verification with a subset of C that supports dynamic specifications, albeit it lacks memory allocation functions well known in C, e.g. *malloc*. Gradual *C0* is built to minimize dynamic checking with statically available information. DiVincenzo et al. (2022) also shows in a performance study that Gradual *C0* reduces run-time overhead by 50-90% on average compared to dynamic verification. This shows that *Gradual C0* is—conservatively—the most optimal alternative to dynamic verifiers, as the programmer can choose to statically specify trivial control flow in their program and remove significant overhead that would otherwise have been computed at runtime. With this relaxation in specifications, the user can also focus on other parts of the program if they verify a more complex aspect.

Introduction

Framework

Separation logic, introduced by Reynolds (2002), supports static verification of the programs that use the heap structure, a particular part in memory with an ordering property which establishes a maximum, greater than or equal to hierarchy, or a minimum, lesser than or equal to, hierarchy. Dealing with the verification of heaps is difficult because we have to make a distinction in permissions given to particular parts of different memory chunks rather than objects in the same memory chunk. Users can make a distinction about the heap's memory chunks with the points-to-predicate operator "$\mapsto$" and the separating conjunction operator "$*$". The arrow operator asserts both ownership of a heap location and its value, e.g. $x.f \mapsto 2$ states that the location *x.f*—accessing the value of field *f* in structure *x*—is uniquely owned and contains the value 2. Further, the separating conjunction works similarly to the logical and, but it's extended to ensure that two chunks of memory—sub-heaps—are distinct in memory. For example, $x.f \mapsto 2 * y.f \mapsto 2$ states that the heap locations *x.f* and *y.f* are distinct (i.e. $x \neq y$), are each owned, and each contain the value 2. This is useful for verification techniques at runtime as it will allow the tool to make a distinction between sub-heaps that would otherwise be indistinguishable because they share the same field value.

A few years later, Smans (2009) presented implicit dynamic frames (IDF) as an alternative to separation logic, which asserts ownership of a heap location and its value separately. Moreover, ownership is ensured through the use of accessibility predicates, e.g. *acc(x.f)*. Then, $acc(x.f.) * x.f == 2$ states that *x.f* is uniquely owned and contains the value 2. Parkinson and Bierman (2009) and Smans et al. (2009) extended separation logic and IDF respectively to support *recursive abstract predicates* and thus recursive heap data structures—trees, lists, graphs, etc. Abstract predicates can be thought of as pure boolean functions. For example, we

consider the following predicate, which specifies that a list is acyclic:

predicate acyclic(Node root) = root == null ? true : acc(root.val) ∗ acc(root.next) ∗ acyclic(root.next).

Acyclic recursively generates accessibility predicates for each node in a list, and joins the predicates with the separating conjunction. Thus, *acyclic(l)* simple denotes that all heap locations in list *l* are distinct—*l* is *acyclic*—providing recursive behavior.

Partial—*imprecise*—specifications contain incomplete static information, which are marked with a question mark "*?*", e.g. *? ∗ x.f == 2* where *x.f == 2* is the static part. Empty specifications are completely imprecise, e.g. ? or ? ∗ *true*. Then, during static verification, imprecise specifications are statically strengthened (in non-contradictory ways) to support proof goals. Wherever strengthening occurs, dynamic assertions are inserted to preserve soundness and complete verification. Bader et al.'s (2018) approach of Gradual Verification smoothly supports the spectrum between static and dynamic verification via the previously mentioned gradual guarantee, *conservative extension*, and pay-as-you-go properties. A gradual verifier is a conservative extension of a static verifier if the two verifiers coincide on fully-precise programs. It also exhibits a pay-as-you-go cost model when users are rewarded with increased static correctness guarantees and decreased dynamic checking, as specifications are refined. Gradual C0 exhibits a static verifier that supports implicit dynamic frames and abstract predicates. The static verifier is extended with *gradual formulas* that support the *?* operator. The entire static verifier is then *lifted*—all imprecise states are *optimistically* processed for later dynamic checking. Wise et al. (2020) proved that their system is sound, is a conservative extension of their static verifier, and adheres to the gradual guarantee. However, we currently have no lightweight techniques to verify the soundness of the implementation of Gradual C0, even if we have reassurance the theory is correct. We propose a *lightweight* technique to incrementally verify the soundness of Gradual C0's implementation and adhere to not just the gradual guarantee, but also a soundness property in regards to Gradual C0 emitting correct dynamic checks.

Introduction

Gradual Verifier Architecture

The following example program shows an overview of the Gradual C0 pipeline:

```
1.    void withdrawFee(Account* account)
2.     /*@ requires acc(account→balance) &&
3.          account → balance >= 5; @*/
4.     /*@ ensures acc(account→balance) &&
5.          account → balance >=0; @*/
6.    {
7.          account → balance -= 5;
8.    }
9.    void monthEnd(Account* account)
10.   /*@ requires ? &&
11.         acc(account→balance); @*/
12.   /*@ ensures ? &&
13.         acc(account→balance) &&
14.         account → balance >= 0; @*/
15.   {
16.         if (account → balance <= 100)
17.               withdrawFee(account);
18.   }
```

This program emulates a machine to withdraw money from a bank account. We want to make sure that the bank account has enough money to withdraw from, never go negative. Line 2 implements how side-effects are reasoned about, using *Implicit Dynamic Frames* (IDF). Access to memory locations is specified using `acc(object-field)`. In line 7, program states are represented by the verifier as formulas in a resource logic. Static information at the end of `withdrawFee` includes the account balance, account balance being greater or equal than zero, and the account balance equaling the old account balance minus five. In line 10 we have our first notation exclusive of Gradual Verification, `?`, which allows the verifier to assume anything necessary to complete proofs. The assumption made in order to satisfy the precondition of the method is that the account balance is greater than five.

Finally, in line 17, we have that wherever specifications are strengthened by the verifier, dynamic checks are inserted into the compiled program to ensure proper behavior at runtime, therefore here the verifier asserts that the account balance is greater than five.

Gradual C0 addresses new technical challenges in gradual verification: Gradual C0's symbolic execution algorithm is responsible for statically verifying programs with imprecise specifications and producing *minimally sufficient* run-time checks for soundness. Achieving these goals with symbolic execution is nuanced. In particular, Gradual C0 tracks the branch conditions created by program statements and specifications to produce run-time checks for corresponding execution paths. At run time, branch conditions are assigned to variables at the branch point that introduced them, which are then used to coordinate the successive checks as required. Further, Gradual C0 creates run-time checks by translating symbolic expressions into specifications—reversing the symbolic execution process by DiVincenzo et al. (2022). The run-time checks produced by Gradual C0 contain branch conditions, simple logical expressions, accessibility predicates, separating conjunctions, and predicates. Each of these constructs are specially translated into source code that can be executed at run-time for dynamic verification. Logical expressions are turned into assertions. Accessibility predicates and separating conjunctions are checked by tracking and updating a set of owned heap locations. Finally, predicates are translated into recursive boolean functions. This is where a lot of soundness bugs in Gradual C0 originate, by not correctly tracking the set of owned heap locations and losing information at run-time.

For example, the verifier experienced a bug in regards to the nature of managing heap permissions demonstrates that an arbitrary method runs when it should not. We assume that a method `assign` assigns the value at an address `x` to be 1. This method is very simple, therefore a user might decide to define an imprecise predicate, `imprecise() = ?`, such that the precondition for the method is left up to dynamic verification. The postcondition just ensures true, and we *unfold* (expanding the abstract predicate to give permission into its body) the imprecise postcondition right before the assignment. If we want to then call the method by *folding* (repacking body) the predicate `imprecise()`, allocating memory for `x`, and declaring `x` to be `0`, when we call the method and assert the postcondition that `x = 0`, the program should error. We already defined in the method for the address value to be 1, therefore asserting the return value to be 0 because we manually allocated the address to 0 manually should not change anything. However, the presence of imprecision allowed for the program to successfully verify in Gradual C0. If we denote impreciseness to represent all heap conditions, the verifier assumed that the permissions represented by iso-recursive predicates won't change, but if they are imprecise, their equi-recursive unrolling includes permission to the entire heap. Their permissions will change even after they are folded. In essence, folding and unfolding a predicate that did nothing, which was set as the precondition for a benign assignment method, displayed unsoundness with the implementation of Gradual C0. Catching an issue such as this one requires heavy lifting when analyzing the formalization of Gradual Verification and provides several roadblocks to maintain a formal methods approach to verify Gradual C0, namely the uncertainty on whether to change the actual semantics of Gradual Verification or utilize the front-end to hack a solution in Gradual C0.

Gradual C0 has two major subsystems: 1) the gradual verification pipeline and 2) the C0 pipeline. The gradual verification pipeline is responsible for statically verifying C0 programs and producing run-time checks for soundness. First, a C0 program is translated into a Gradual Viper (an extension of the Verification Infrastructure for Permission-based Reasoning language) program by Gradual C0's frontend module, GVC0. Next, the Gradual Viper

module uses a symbolic execution approach that handles imprecise formulas to statically verify the Gradual Viper program (Viper comprises a novel intermediate verification language). Wherever imprecise formulas are strengthened in support of proofs, Gradual Viper creates run-time checks in its language to ensure soundness. Finally, GVC0 takes those run-time checks and produces a C0 program from them, in addition to the original C0 program. The C0 pipeline takes this C0 program and feeds it to the C0 compiler, CC0, which executes the program.

An example C0 program implementing logic for a bank account is shown in Figure 3. The `monthEnd` method uses the `withdraw` method to remove 5 units from the account when its balance is less than or equal to 100. Gradual specifications partially define the behavior of both `monthEnd` and `withdraw`. For example, the account balance must be a positive value for a call to `withdraw` to be valid. The postcondition of withdraw is unspecified as indicated by `?`. A `?` in the specifications indicates imprecision, allowing the verifier to optimistically assume information, such as access to the balance field, where necessary.

The C0 program is converted to an intermediate representation (IR), that targets both C0 source output and Viper's intermediate language, Silver. For gradual verification, we need to both convert the semantics of the C0 program into Silver and insert verifier-provided dynamic checks into the program before compilation. Intermediate values (such as complex expressions in a method call's arguments) may need to be verified at run-time, and previous values may need to be examined to determine if a check is necessary at run-time. To meet these requirements, the C0 program's IR is transformed to remove re-assignments, similar to single-static-assignment (SSA) transformations.

Following this transformation, the IR is translated into Silver, which is further translated into a logical formula representation used by Silicon Schwerhoff (2016), the verification engine for Viper. During optimistic static verification, the verifier generates run-time checks wherever an optimistic assumption takes place. Where possible, checks are avoided using static information. Further, some checks are only required for specific execution paths through the program; path information is attached to these checks. All checks are emitted to the frontend, which translates and injects them into the C0 IR.

Figure 4 shows a simple dynamic check. The `withdraw` call in Figure 3 elicits this check before the termination of `monthEnd` in order to ensure a valid account balance, but only for the path denoted by the conditional branch. Wise et al. (2020) extended gradual verification to support heap-allocated data structures using implicit dynamic frames (IDF) (Smans et al., 2009). In addition, Viper uses IDF in its implementation of static verification. IDF imposes constraints on the accessibility of fields in heap-allocated data structures. Since gradual verification may require dynamic verification of specifications, gradual verification using IDF must verify field accessibility at run time. To implement this, an additional argument is added to each method. This argument is used to specify the fields accessible by the method. When calling a fully specified method, the caller passes only the permissions specified in the callee's preconditions. However, for gradually specified methods, all of the caller's permissions are passed. A dynamic check for field access asserts that this set contains a tuple of the field and its parent `struct` reference. This allows the side-effects of fully specified methods to be known during static verification even if they call gradually specified methods where side-effects are not specified.

Approach

Lightweight Verification

While empirically evaluating Gradual C0's performance in DiVincenzo et al. (2022), Gradual *C0* was used to verify thousands of partial

specifications that are correct and approximate the gradual guarantee. A number of bugs were caught and fixed by hand, in which Gradual C0's design was implemented incorrectly. To complement the aforementioned evaluation that only looks at correct specifications and programs, we introduce a *property based testing* (PBT) pipeline that empirically evaluates the correctness of Gradual C0's implementation through incorrect programs and specifications. It has been shown that capturing the *truthiness* of a property's results with lightweight methods provides good coverage for finding implementation bugs (Claessen & Hughes, 2000). In Gradual C0, the truthiness for all programs consists of a pair of outputs: dynamic and gradual verification output message given by Gradual C0. Failed equivalence between this pair of outputs informs us of bugs in Gradual C0's implementation that do not break the gradual guarantee and would not have been caught otherwise.

Unlike classical tools for property based testing, we are not generating input for programs, rather generating programs themselves to input into the pipeline. We implement a three-stage pipeline framework that sequentially gradually verifies a program, stores the output message, either a success or a failure message, followed by pure dynamic verification of the same program, and compares its output to the previously stored gradual output. The three stages are composed of a *reference model language*—Gradual C0's specification language—an *input generator*—test suite of examples that are not supposed to verify correctly which we randomly permute to test on—and a *checker*—compares the output from Gradual C0 and Dynamic C0 (Figure 1). The checker establishes Dynamic C0 as the ground truth, expecting either an error or a pass from Gradual C0 if Dynamic C0's output is a pass, but they should never differ if Dynamic C0's output is an error.

We choose Dynamic C0 as the ground truth because Gradual C0 already has an empirical reassurance of static soundness in the verifier thanks to our benchmarking system, which remedies the issue with Dynamic C0 being complete but not sound: we don't guarantee that a program satisfies a specification if it passes. We evaluate the previously mentioned 50-90% efficiency of the tool by emulating Takikawa et al., (2016)'s performance lattice method, a method to measure run-time cost of gradual typing by testing various configurations of the typed and untyped code, but with relaxed specifications instead. Our benchmark is made up of four fully statically verified algorithms–namely BST (Binary Search Tree), AVL (Adelson-Velsky and Landis BST), Composite, Linked list insertion. If the benchmarking is a success, then we have a pseudo-empirical reassurance of our static verifier because we have passed a fully-precise program. However, dynamic checks while relaxing specifications could be unsound. We therefore use Dynamic C0 as the ground truth, by comparing how the pure dynamic verifier asserts dynamic checks with no static information (it should always emit them correctly, although the program will be very slow to verify), against Gradual C0 which is asserting dynamic checks given optimistic static information.

The input generator is made up of a dozen methods that come from Gradual C0's benchmark test suite. The methods from each test are changed to have incorrect specifications and implementations that do not obey each other. The tests in the input generator also have to maintain certain ways of stating specifications. To prevent a trivial failure of the static verifier in Gradual C0, programs must avoid specifying preconditions and *fold/unfolds* (explicit statements to control the availability of predicate information) that won't be met while running. These folds control the availability of predicate information. Verification tools cannot automatically deal with recursive information in specifications. If a recursive function is referenced in a precondition, for example, the programmer must explicitly fold/unfold the recursive information, similar to specifying loop invariants. These folds/unfolds are considered

an iso-recursive interpretation of predicates. Because static verifiers rely on iso-recursive reasoning, the static verification step in Gradual C0 will trivially fail with the presence of unmet predicate information.

Approach

Inputting to the Input Generator

Any contradictory output regarding the success of Gradual C0 and Dynamic C0's output will result in a reduction of the code to find which method is resulting in the error, informing us where the bug could be in Gradual C0's implementation. For example, the lightweight technique known as *QuickCheck* attempts to write assertions about logical properties that a function should fulfill, and attempts to generate a test case that falsifies such assertions, our reduction follows the same pattern. Once such a test case is found, QC tries to reduce it to a minimal failing subset by removing or simplifying input data that are unneeded to make the test fail. Ideally, however, a fully *fuzzy* tool would auto generate random input to empirically test soundness. Our current tool does not have a way to generate Gradual C0 programs that contradict in specification and implementation. Instead, we take an approach closer to *mutation testing*, in which we slightly modify individual methods in our three of our four benchmarking algorithms. We then have a lower level of property-based testing in which we generate inputs for these methods in association to whatever structure was changed, if we encounter an arithmetic change we have integer literals. These slight modifications search for basic operators in the core logic, e.g. greater than, addition, loop termination, and replaces them with the dual operation, e.g. lesser than, subtraction, different loop termination value, respectively.

We can strengthen the exhaustiveness by including examples which have been caught by hand in the past, as shown in Figure 2. This example begins with an append method with no permissions, and in an imprecise state. When we encounter the while loop on line 10, we correctly emit a check for asserting that we have access to `n->next`. The first iteration of the loop is fine, because we have checked that we have access to `n->next`. Before we begin the next iteration, though, we should check again that we have access to `n->next` (since we might have lost it on the current iteration). If we don't have access, the program shouldn't be able to evaluate the loop condition, and should crash. Gradual C0 did not crash at the time of the bug being found, because it was unsound. This was due to an issue on permissions given to the optimistic heap at a certain time in compilation. Our tool was able to make a distinction between Gradual C0's output and Dynamic C0's resulting error and immediately identify the bug.

Tool Analysis

To test the correctness of our tool, we retrospectively find bugs through Gradual C0's issue tracker on GitHub, and run the tool on our test suite. In addition to previously mentioned programs, our test suite is expanded to include tests that implement failing implementations from each issue in the issue tracker. A particularly interesting and significant issue was originally caught with the formalization of Gradual Verification from Wise et al. (2020) regarding the internal output of Gradual C0 after the behavior had been formalized—*Footprint Splitting*. In this issue, Gradual C0 was not removing information from the *optimistic heap*, framed by an imprecise specification, when it should. Permissions would not be tracked inside precise methods that call imprecise methods or methods with internal precision. In our input generator, the set of examples that trigger this issue come from the binary search tree benchmark. This example fails the property because Gradual C0's output would pass at the creation of the imprecise predicate, *treeRemove*, to delete a binary search tree. This predicate is called in the postcondition of the tree removal function, and recreating run-time permissions after the function is called is incorrect because

it causes an accessibility predicate to be missing. Our tool catches this failing property and returns Dynamic C0's error.

We can further understand the usefulness of our tool by running our test suite on a point of Gradual C0's GitHub commit history. Table 1 shows us which issues were caught by our tool. Our entire test suite ran at a rollback of Gradual C0 to the dates listed under the *Commit found* column and we specify which example in the suite found the bug if any. The custom loop example on April 1st is shown in Figure 2. This analysis helps to prove the efficacy of PBT in Gradual C0, capturing most bugs. The only issues that were not caught were due to a benchmark test that was not implemented in our test suite, the AVL benchmark. A more exhaustive test suite that implements this test could have identified all 7 soundness bugs.

Conclusion

Gradual Verification is a powerful tool that supports the relaxation of static specification without losing any information to create unsoundness issues. It leverages dynamic and static techniques and has been proven to have soundness guarantees. However, there are various bugs in the implementation of Gradual C0, the first gradual verification tool, that cannot currently be addressed with formal methods, due to a lack in consistency when bridging the formal semantics of Gradual Verification and non-theoretical hacks to solve implementation issues in Gradual C0. To combat this uncertainty, we develop a lightweight tool relying on Property Based Testing for finding implementation bugs in Gradual C0 that do not follow Gradual Verification's formal design, instead opting for front-end based solutions. However, there are still challenges that must be addressed to exhaust this lightweight method for a rigorous evaluation of new bugs with arbitrary programs. Currently, the test suite is implemented by hand by iterating through the benchmark examples which all pass the gradual guarantee. The new bugs that have been found are associated with this set of (exhaustive) examples and arbitrary programs might expose bugs which aren't guaranteed to be caught with this tool. We know that testing is enhanced by the specifications that are written at the boundaries – they might even help with generating tests. Conversely, the dynamic verification part of our work is only useful if test cases cover the places where the assertions are.

This implementation has limited applicability due to the restrictive test suite. A promising approach to expand the domain of bugs caught by our tool relies on iterating through all examples in the benchmark test suite and breaking individual methods by generating random inputs that violate each method's specification. Nevertheless, we lay the groundwork for a consistent lightweight tool which is the first automated method for finding implementation bugs in Gradual C0.

Figures/Tables

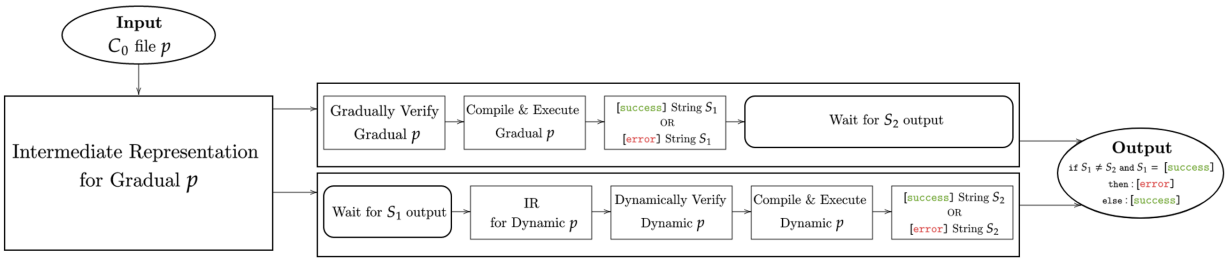

Figure 1. Checker Architecture

```
/*@
predicate list(struct Node *l) =
  ? && (l != NULL ? acc(l->value) && list(l->next) : true);
@*/
void append(Node *root, int value)
  //@requires ?;
  //@ensures ? && list(root);
{
  Node *n = root;
  while (n->next != NULL)
    //@loop_invariant ?;
    n = n->next;
  n->next = alloc(Node);
  n->next->value = value;
}
```

Figure 2. Custom loop example for issue 34

```
void monthEnd(Account *account)
  /*@ requires ? && account->balance >= 0; @*/
  /*@ ensures ? && account->balance >= 0; @*/ {
  if (account->balance <= 100)
    withdraw(account, 5);
}

void withdraw(Account *account, int amount)
    /*@ requires acc(account->balance) &&
        account->balance >= 0; @*/
    /*@ ensures ?; @*/ {
    ...
}
```

Figure 3. Bank account example of a gradually verified program in C0

```
if (previous_account_balance <= 100)
    assert(account->balance >= 0);
```

Figure 4. Runtime check example in Gradual C0

Table 1. Bugs caught with the PBT tool

Commits found	Bugs and test that caught it	Property failure caught
August 16, 2022	Issue 38: AVL (Not implemented)	No
August 29, 2022	Issue 46: AVL (Not implemented)	No
May 13, 2022	Issue 27: BST	Yes
May 12, 2022	Issue 25: BST	Yes
August 15, 2022	Issue 44: AVOL (Not implemented)	No
April 1, 2022	Issue 34: Custom loop example	Yes
March 9, 2022	Issue 24: List insertion	Yes

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Investigation of potassium tetraborate resistance in *Dickeya* spp.

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Abstract

Dickeya spp. are common plant pathogens associated with bacterial soft rot, potato blackleg, and slow wilt, which are plant diseases that account for major losses in the agricultural industry. The diseases caused by these bacterial species are not yet fully managed with existing techniques, and new approaches need to be considered to minimize future crop loss. Previous research has shown that the inorganic salt potassium tetraborate tetrahydrate (PTB) can inhibit the growth of *Dickeya* species; however, disk diffusion assays result in a unique phenotype with two zones of inhibition. This study investigates the effects of PTB on the growth of four *Dickeya* spp.. It was hypothesized that the production of phage is responsible for the two zones of inhibition. Disk diffusion assays and growth curves were used to confirm the impact of PTB on *Dickeya* and attempts were made to directly isolate phage from the strains. To elucidate the mechanism of action of PTB, Tn-Seq libraries were used to determine which genes are required for growth in the presence of PTB. Tn-Seq libraries showed that different *Dickeya* strains shared seven overlapping genes including stress-related genes that increase bacterial resistance to PTB. Gene expression studies were used to determine the changes in gene expression that result from PTB exposure. Preliminary results showed that exposure to PTB induces the expression of stress-related genes in *Dickeya* to increase survival in the presence of the compound. Further research is needed to better understand the implications of observed changes in bacterial gene expression.

Introduction

Members of the *Pectobacteriaceae*, a family of Gram-negative bacteria, are known to cause bacterial soft rot and potato blackleg among other diseases which are destructive to many crops in the fields (Charkowski, 2018; Toth et al., 2021; Toth et al., 2011). It has been reported that bacterial soft rot losses after crop harvest account for up to a third of total loss, significantly greater than any other disease (Agrios, 2005). The severe losses in agriculture yield from soft-rot plant pathogens make the study of these soft-rot pathogens crucial to the success of the agricultural industry. Soft-rot diseases are particularly detrimental to potatoes, the fourth most important crop in the world, causing up to two-thirds of losses (Mantsebo et al., 2014). Soft-rot diseases are currently largely managed by good agricultural and handling practices, but better prevention mechanisms are needed to sustain the food needs of a growing population (Olsen & Nolte, 2011). Although there are currently several management options used to control the bacteria, these options are limited, and alternative methods need to be considered. Existing management options include cultivation measures and hygienic practices such as using clean seed and planting in pathogen-free areas, optimizing storage conditions to decrease the development and spread of disease, physical seed treatments with heat and UV radiation, and chemical treatments primarily with antibiotics (van der Wolf et al., 2021). Other chemical treatment options to prevent infection are being explored, including the use of inorganic salts. It has been reported that inorganic salts inhibit the growth of Pectobacterium, a genus within *Pectobacteriaceae*, the same family as *Dickeya* (Yaganza et al., 2009). Not only

does the agricultural industry seek at inhibiting growth of *Dickeya*. An interesting double-ring-of-inhibition phenotype was observed with *Dickeya* in diffusion assays using agar plates (Liu & Filiatrault, 2020). This phenotype has not been previously seen in *Dickeya* or other bacteria. This study explored many questions pertaining to *Dickeya*'s unique response to PTB and PTB's mechanism of action. PTB can be considered for further investigation as a potential chemical treatment option for crops in storage and fields if it is found to be an effective inhibitor of soft rot diseases.

The mechanism of action of PTB is not yet fully known but understanding how the chemical affects bacterial growth is crucial to uncovering its potential for agricultural use. Previous research found that soft-rot *Pectobacteriaceae* including both Pectobacterium and *Dickeya* may have phages (Czajkowski, 2016; Adriaenssens et al., 2012; Resibois et al., 1984) and phage-like elements (Czajkowski, 2019) in their genome that contribute to bacterial virulence. The presence of prophages can be extremely beneficial to bacterial fitness and can introduce new traits into the host (Bondy-Denomy & Davidson, 2014; Nanda et al., 2015). These new traits can alter the bacterial genome in many ways, one of which is by changing the bacteria's pathogenicity and responses to stress (Hacker & Carniel, 2001; Fortier & Sekulovic, 2013; Casjens, 2003). Stress conditions can cause phage induction and kill the bacterial host (Du Toit, 2019), and compounds containing boron such as PTB have been found to increase oxidative stress in pathogens (Qin et al., 2010). Although the details are not well understood, phage present in plant-pathogenic bacteria can carry genes that alter plant pathogenicity (Varani et al., 2013).

The main objective of our research was to investigate the effect of PTB on growth and gene expression in *Dickeya* spp.. Possible reasons for the unique disk diffusion phenotype were explored by testing a larger collection of *Dickeya* strains and exploring four hypotheses tailored to possible reasons for the phenotype displayed by *Dickeya* when exposed to PTB.

Hypothesis 1: The inner ring represents bacteria that are resistant to PTB.

Hypothesis 2: The zone of clearing is the result of phage-induced gene expression.

Hypothesis 3: The genes responsible for sensitivity or resistance in *Dickeya* are different from those found in Pectobacterium.

Hypothesis 4: The presence of PTB induces production of phage by *Dickeya* spp..

Methods and Materials

Bacterial Strains

Dickeya dadantii 3937, *Dickeya dianthicola* ME23, *Dickeya dianthicola* 67-19 and *Dickeya fangzhongdai* 643-a were cultured on lysogeny broth (LB) agar plates or in LB broth (10 g tryptone, 5 g yeast extract, and 10 g NaCl per 1 L) at 28 °C (Bertani, 1951).

Antibacterial Activity of PTB

Disk diffusion assays were used to test the antimicrobial activity of PTB on the four strains of *Dickeya* and to confirm their double-ring phenotype (Liu & Filiatrault, 2020). Triplicate overnight cultures were grown in LB, adjusted to 2×10^8 c.f.u. ml^{-1} (OD_{600} = 0.2), and split into three replicates. The cultures were spread on LB agar plates using sterile cotton swabs, and one sterile paper filter disk (6 mm in diameter; GE Healthcare) loaded with 20 µl filtered 500 mM PTB solution was put on each plate. Plates were incubated at 28°C for 24 hours after which pictures were taken.

Direct Phage Isolation

Several different protocols were used in our attempts to isolate phage from the bacteria in the double zone (Figure 1) and bacteria were grown in liquid culture with PTB. These protocols included a phage genomic DNA extraction (modified Promega Wizard method; Promega, Madison, WI, USA) as well as several adaptations involving chloroform (*Phage genomic DNA extraction*, 2011; *Protocol for Phage DNA Extraction*, 2018; Adams, 1959).

Growth Curve

Growth curves were performed using a Synergy 2 multi-mode microplate reader (Liu & Filiatrault, 2020). Overnight cultures of the four strains of *Dickeya* were grown in LB and adjusted to 2×10^8 c.f.u. ml^{-1} (OD_{600} = 0.2). 200 µl of adjusted overnight culture with PTB concentrations of 0 mM, 1.88 mM, 3.75 mM, 7.5 mM, and 15 mM were measured at approximately 25 °C in for up to 16 h at 30 min intervals. Concentrations were chosen based on growth curves performed on Pectobacterium (Liu & Filiatrault, 2020). Six technical replicates and three biological replicates were performed for each treatment. The vertical lines represent the standard error of the mean.

Tn-Seq Libraries

Previously constructed randomly barcoded Tn-Seq libraries from Helmann et al. (2022) were used to identify genes of interest in three strains of *Dickeya*: *D. dadantii* 3937, *D. dianthicola* ME23, and *D. dianthicola* 67-19. The libraries were recovered in LB with kanamycin and incubated at 28 °C for 7 hours until reaching an OD600 of 0.5-0.7 and split into four replicates. 24-well plates were prepared with LB and PTB concentrations of 0 mM, 1.88 mM, 3.75 mM, and 7.5 mM. The libraries were inoculated in the 24-well plates and grown overnight. Genomic DNA was extracted using the Monarch Genomic DNA Purification Kit (New England Biolabs, Ipswich, MA, USA). Helmann performed the PCR on the extracted DNA to amplify the barcode sequence and index each sample, and then pooled all the samples to submit to the Biotechnology Resource Center (BRC) Genomics Facility at the Cornell Institute of Biotechnology for Illumina sequencing. The libraries had been previously mapped (Helmann et al., 2022). Tn-Seq mapping data was analyzed using the FEBA RB-TnSeq analysis pipeline to identify genes for each strain that are important for growth in PTB (Helmann et al., 2022; Wetmore et al., 2015).

Real-Time Quantitative PCR

Three strains of *Dickeya*, *D. dadantii* 3937, *D. dianthicola* ME23, and *D. dianthicola* 67-19, were grown overnight. The next day, half of the bacteria were exposed to 3.75 mM PTB and cultured for another hour while the other half also were cultured for another hour without PTB. The PTB concentration used was selected based on growth curve results. RNA from bacteria grown with and without PTB were used in the qRT-PCR experiments to determine differences in gene expression. RNA was extracted using the Qiagen RNeasy kit (Hilden, Germany), treated with Ambion DNase I (Thermo Fisher, Waltham, MA, USA), and cleaned using the Zymo Clean and Concentrate kit (Irvine, CA, USA). cDNA was made from RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), and the resulting cDNA was quantified and diluted to 100 ng/µL. The SsoAdvanced Universal SYBR Green Supermix instruction manual (Bio-Rad) was used for real time PCR reactions. Five genes selected from Tn-Seq results were evaluated. For *D. dadantii* 3937, the genes were: Mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase, phosphomannomutase/phosphoglucomutase,GDP-mannose 4,6-dehydratase, WbeA, and sigma E protease regulator RseP. For *D. dianthicola* 67-19, the genes were: GDP-mannose 4,6-dehydratase, phosphomannomutase CpsG, mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase, envelope stress response regulator transcription factor CpxR, and microconductance mechanosensitive channel MscM. The reactions were set up with SYBR Green Supermix at 1x, 200 nM of each primer, and 100 ng of cDNA. The thermal cycling protocol was as follows: denaturation at 95 °C for 10 seconds, annealing and extension at 52 °C for 30 seconds, and cycled through 40 cycles on a Bio-Rad Connect. Due to time constraints, qRT-PCR was performed on RNA isolated from two strains: *D. dadantii* 3937 and *D. dianthicola* 67-19. Three technical replicates and three biological replicates were performed for each treatment. Data was analyzed using the delta delta Ct analysis (Bradburn, 2020), and the vertical lines represent the standard deviation of the mean.

Results

Antibacterial Activity of PTB

Disk diffusion assays were used to test the antimicrobial activity of PTB on the four strains of *Dickeya* to determine whether the phenotype was conserved among species and if colonies growing in the presence of PTB developed resistance. It was found that all strains demonstrated the double-ring phenotype, suggesting that the phenotype is conserved in multiple *Dickeya* species (Figure 1). To determine if the bacteria growing between the two zones of clearing are resistant, bacteria were isolated and used to repeat the same disk diffusion assay. The disk diffusion assay for bacteria taken from the area between the two zones (bacterial growth between red arrow, inner zone, and black arrow, outer zone) gave phenotypically similar results as bacteria not previously exposed to PTB (Figure 2). This assay ruled out the possibility PTB had led to the selection of PTB resistant bacteria and rejected hypothesis 1 – had there been evolution, the bacteria between the two zones would all be resistant to PTB in the disk diffusion assay. No colonies were found in the zone of clearing. Since the same phenotype with two zones of inhibition was observed even when using the bacteria grown between the two zones of the original assay, it was concluded that the bacteria between the two zones were still sensitive to PTB and that the PTB was not selecting for resistant bacteria.

Direct Phage Isolation

Attempts were made to directly isolate phage from all four *Dickeya* strains to test Hypothesis 2 and determine whether the presence of PTB induces production of phage by *Dickeya* spp.. A phage genomic DNA extraction (modified Promega Wizard method; Promega, Madison, WI, USA) and several adaptations (*Phage genomic DNA extraction*, 2011; *Protocol for Phage DNA Extraction*, 2018; Adams, 1959) were used, all of which failed to produce any sort of pellet after centrifugation. Phage isolation attempts were unsuccessful, and phage DNA was unable to be extracted using any of the protocols. Many variables were in play with direct phage isolation. It was possible that the phage was not released from the bacteria used, and gene expression experiments (qRT-PCR) and Tn-Seq were instead used to investigate phage presence.

Growth Curve

Growth curves were performed to determine the minimal inhibitory concentration (MIC) of PTB at which each *Dickeya* strain cannot grow. All strains grew in cultures lacking PTB, and all four *Dickeya* strains were found to be sensitive to PTB depending on the concentration (Figure 3). 15 mM PTB showed to completely inhibit the growth of *D. dadantii* 3937 and *D. fangzhongdai* 643-a while 7.5 mM PTB was sufficient to inhibit the growth of *D. dianthicola* ME23 and *D. dianthicola* 67-19 (Figure 3). While the double-ring phenotype of interest cannot be observed using growth curves, the MIC was used to guide the concentrations tested in the Tn-Seq experiments.

Tn-Seq Libraries

Tn-Seq libraries were used to identify genes in *Dickeya* important for growth in PTB. The genes identified were compared to genes found in PTB-resistant Pectobacterium (Liu & Filiatrault, 2020) and were used in qRT-PCR to evaluate changes in gene expression. The libraries revealed several genes that when disrupted improve growth in PTB and several genes that when disrupted are detrimental to growth in PTB (Table 1). All experiments passed established quality check parameters (Helmann et al., 2022; Wetmore et al., 2015) and the genes shown in Table 1 were all statistically significant with $|fit| > 1$ and $|t| > 4$. Normalized gene fitness (fit) values were used to sort the genes. A positive fit value would indicate that the strain is more resistant to PTB, and the disruption in the gene improved survival. A negative fit value would indicate that the strain is more sensitive to PTB, and the disruption in the gene was detrimental to the bacterial strain. In *D. dadantii* 3937, nine genes improved growth in PTB when disrupted, and 14 genes were detrimental to growth in PTB

when disrupted; in *D. dianthicola* ME23, six genes improved growth in PTB when disrupted, and nine genes were detrimental to growth in PTB when disrupted; in *D. dianthicola* 67-19, five genes improved growth in PTB when disrupted, and five genes were detrimental to growth in PTB when disrupted (Table 1). Seven genes were shared between at least two of the three strains tested including mannose-1 phosphate guanylyltransferase/mannose 6-phosphate isomerase, phosphomannomutase/phosphoglucomutase,GDP-mannose4,6-dehydratase, microconductance mechanosensitive channel MscM, and envelope stress response regulator transcription factor CpxR (Figure 4). The genes related to sensitivity and resistance in *Dickeya* are different from those found in Pectobacterium (Liu & Filiatrault, 2020) which confirms Hypothesis 3. *Dickeya* and Pectobacterium respond to PTB through different mechanisms – the genes found in *Dickeya* can then be used to study differences in gene expression and potential overlap with phage-related genes.

Real-Time Quantitative PCR

To track the differences in gene expression between PTB-exposed bacteria and non-exposed bacteria, qRT-PCR was performed using the genes that were identified using Tn-Seq (Table 1). Five genes were selected from the Tn-Seq experiments to use as targets for qRT-PCR, and the resulting data was analyzed using the delta delta Ct analysis (Bradburn, 2020). Due to time constraints, each experiment was run once and for only two strains of *Dickeya*. *D. dadantii* 3937 and *D. dianthicola* 67-19 varied greatly in gene expression. Unexpectedly, all five genes tested for *D. dianthicola* 67-19 had fold changes in gene expression < 1, which would mean that all five genes demonstrated reduced expression in PTB-treated samples (Figure 5). This result did not agree with our expectations since three of the genes tested are detrimental to growth in PTB when disrupted (Table 1).

Discussion

Dickeya displayed a unique response to PTB in disk diffusion assays by showing two distinct rings of clearing (Figure 1). The observed phenotype was hypothesized to potentially be the result of phage production as a response to PTB exposure. Previous research has found *Dickeya* to contain phages (Czajkowski, 2016; Adriaenssens et al., 2012; Resibois et al., 1984) and phage-like elements (Czajkowski, 2019) which could impact bacterial fitness when exposed to a stressor like PTB. All four strains reacted similarly in the disk diffusion assays and were sensitive to PTB in LB, which suggests that response to PTB may be a conserved mechanism.Through multiple disk diffusion assays and the Tn-Seq experiments, it was determined that PTB exposure did not select for resistant *Dickeya*, and the genes responsible for PTB sensitivity or resistance in *Dickeya* did not overlap with mutated Pectobacterium genes previously found (Liu & Filiatrault, 2020). Further research can be done to more closely investigate these differences.The Tn-Seq experiments provided important information about genes involved in *Dickeya* resistance and sensitivity to PTB. Tn-Seq experiments showed that *Dickeya* strains shared several genes of interest, many of which were stress-related genes that may help increase bacterial resistance to PTB. The double-ring phenotype could have been caused by changes in gene expression at different PTB concentrations, but more research is needed to draw conclusions. Stress-related genes are likely required for growth in PTB; borate has been shown to inhibit growth and induce stress responses of certain pathogens (He et al., 2019). Bacteria respond to stress in a variety of ways, which can include inducing of expression of different genes relating to survival and virulence. Changes in gene expression related to mannose-metabolism have been previously observed in pathogenic bacteria as a response to osmotic stress (Chowdhury et al., 1996). The disturbance of mannose-metabolism in response to salinity is consistent with the observed importance of mannose-related

genes for growth in PTB (Table 1). The genes disrupted in *Dickeya* were different than the mutated genes found through whole-genome sequencing of PTB-resistant Pectobacterium mutants which affected peptide chain release from the ribosome (Liu & Filiatrault, 2020). The difference between responses to PTB in *Dickeya* and Pectobacterium may indicate that even though both bacteria are part of the same family of *Pectobacteriaceae*, they may not react to compounds in the same way and PTB may be effective on one genus but not the other. Further research is necessary to elucidate the differences in response to PTB between the two genuses and to determine whether responses are consistent within different species of the genus. We hypothesized that phage was responsible for *Dickeya*'s response to PTB exposure but attempts to directly isolate phage were unsuccessful. Phage isolation can be heavily timing dependent – it is possible that our isolation attempts did not align with the timing of phage release or that phage was not released from the bacteria. Further research would be necessary to thoroughly explore phage-related responses of *Dickeya* to PTB exposure. No firm conclusions were made regarding phage production due to time constraints. If a suitable phage-sensitive host can be found, plaque assays can be used to identify phage plaques. Otherwise, continued exploration of gene expression would likely be the preferred route. The Tn-Seq experiments revealed genes related to lipopolysaccharide (LPS) production, which has been found to interact with phage (Andres et al., 2010), reinforcing the likelihood of phage involvement. An alternative explanation for LPS production in PTB could be that LPS production is stimulated during the bacterial stress response due to its role as a barrier molecule (Poole, 2012). A potential next step would be to sequence the bacteria grown between the two zones of clearing using both whole genome sequencing and RNA-Seq to examine any mutations or differences in gene expression from the freezer stock. Another possibility would be to directly locate the phage genes in *Dickeya* and track expression of specific known phage genes such as that of the transducing phage φEC2 (Adriaenssens et al., 2012). Overall, more work is necessary to examine PTB as a potential chemical treatment for bacterial infection of crops. Understanding *Dickeya*'s response to PTB would clarify the double-ring phenotype and help evaluate efficacy of PTB treatment and the ideal conditions under which treatment should be applied. Although present findings showed that all four *Dickeya* remained sensitive to PTB after treatment (Figure 2), further experiments should investigate the possibility of developing bacterial resistance after continued exposure to PTB to ensure its success as a plant treatment option.

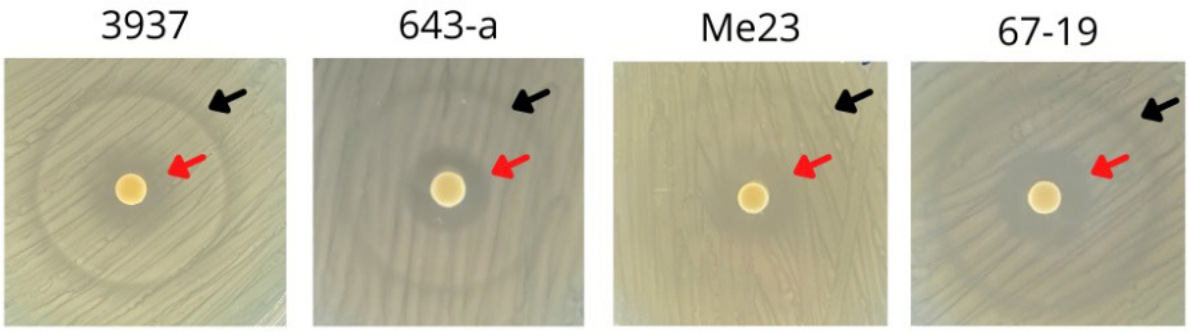

Figure 1: Disk diffusion assay of antibacterial activity of PTB on four strains of *Dickeya* freezer stocks:, *D. dadantii* 3937, *D. dianthicola* ME23, *D. dianthicola* 67-19, and *D. fangzhongdai* 643-a.

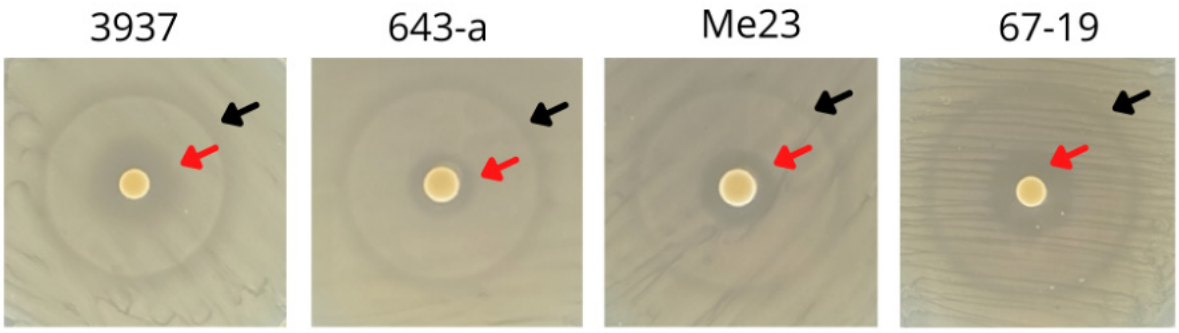

Figure 2: Disk diffusion assay of antibacterial activity of PTB on four strains of *Dickeya* re-streaked from the middle zone of growth between the two zones of clearing in Figure 1.The black arrows point to the outer zone of clearing and the red arrows to the inner zone.

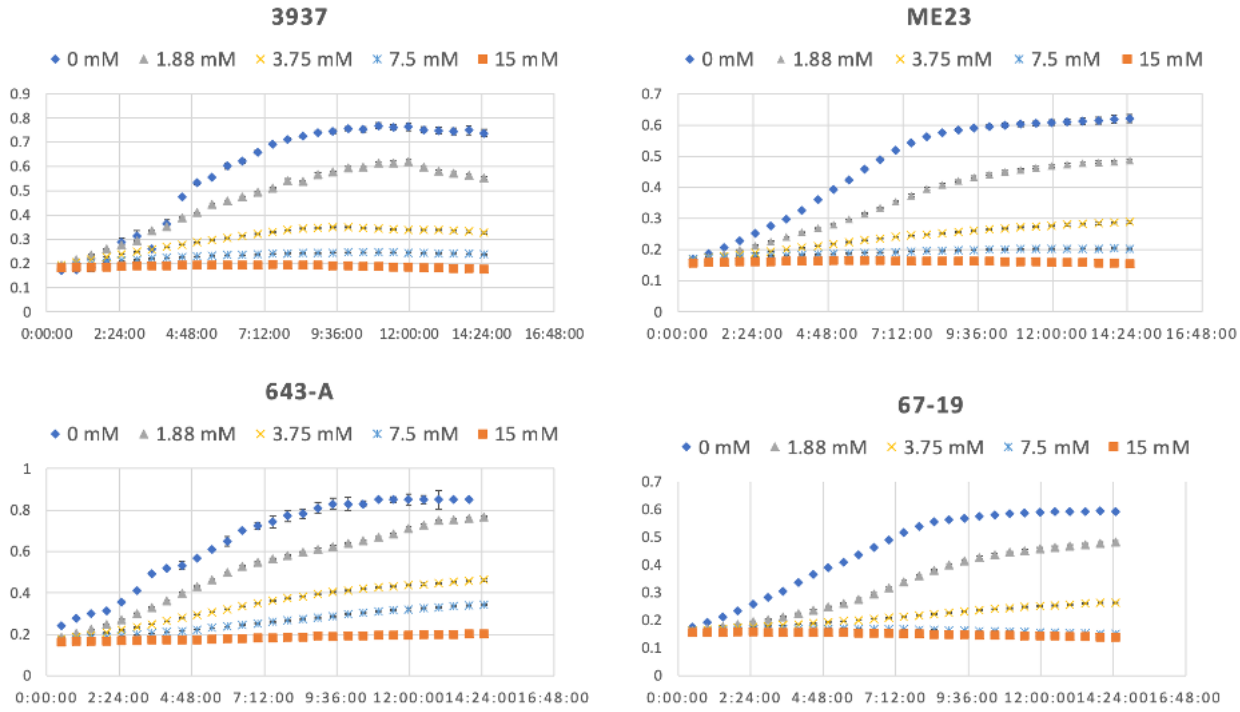

Figure 3: The growth (OD600) of four strains of *Dickeya*: *D. dadantii* 3937, *D. dianthicola* ME23, *D. dianthicola* 67-19 and *D. fangzhongdai* 643-a was measured for up to 16 hours. Each treatment had six replicates and each experiment was repeated at least three times. The most recent (4/30/2021)

Table 1. Genes found in the three *Dickeya* strains constructed for Tn-Seq libraries

Dikeya **dadantii 3937 Tn-Seq Genes**		
Genes that improved growth in PTB when disrupted	**Locus ID**	**Description**
	DDA3937_RS03410	polysaccharide biosynthesis protein
	DDA3937_RS03420	sugar transferase
	DDA3937_RS1865	mannose-1 phosphate guanylyltransferase/mannose 6-phosphate isomerase
	DDA3937_RS18660	phosphomannomutase / phosphoglucomutase
	DDA3937_RS18665	GDP-mannose 4,6-dehydratase
	DDA3937_RS18670	ABC transporter permease
	DDA3937_RS18685	WbeA
	DDA3937_RS18690	Glycosyltransferase
	DDA3937_RS20290	transcription/translation regulatory transformer protein RfaH
Genes that were detrimental to growth in PTB when disrupted	**Locus Id**	**Description**
	DDA3937_RS00900	cell division protein ZapB
	DDA3937_RS01600	outer membrane-stress sensor serine endopeptidase DegS
	DDA3937_RS02810	DedA family protein
	DDA3937_RS19930	uroporphyrinogen-III c-methyltransferase
	DDA3937_RS12215	sodium-potassium/proton antiporter ChaA
	DDA3937_RS05150	sigma E protease regulator RseP
	DDA3937_RS05640	ATP-dependent Clp endopeptidase proteolytic subunit ClpP
	DDA3937_RS05645	ATP-dependent protease ATP-binding subunit ClpX
	DDA3937_RS15765	RNA polymerase sigma factor RpoE
	DDA3937_RS06350	replication initiation negative regulator SeqA
	DDA3937_RS11035	redox-regulated ATPase YchF
	DDA3937_RS11210	septum site-determining protein minD
	DDA3937_RS18610	RNA chaperone Hfq
	DDA3937_RS12490	exoribonuclease II
Dickeya **dianthicola ME23 Tn-Seq Genes**		
Genes that improved growth in PTB when disrupted	**Locus Id**	**Description**
	DZA65_RS19720	mannose-1 phosphate guanylyltransferase / mannose 6-phosphate isomerase
	DZA65_RS19725	phosphomannomutase / phosphoglucomutase
	DZA65_RS19730	GDP-mannose 4,6-dehydratase
	DZA65_RS19755	glycosyltransferase
	DZA65_RS03635	sugar transferase
	DZA65_RS19750	hypothetical protein

Genes that were detrimental to growth in PTB when disrupted	DZA65_RS20165	microconductance mechanosensitive channel MscM
	DZA65_RS20220	glucose-6-phosphate isomerase
	DZA65_RS1610	envelope stress response regulator transcription factor CpxR
	DZA65_RS22635	4-amino-4-deoxy-L-arabinose-phosphoundecaprenol flippase subunit ArnE
	DZA65_RS22640	4-amino-4-deoxy-L-arabinose-phospho-UDP flippase
	DZA65_RS00800	O-antigen ligase family protein
	DZA65_RS01525	LPS export ABC transporter periplasmic protein LptC
	DZA65_RS09455	DUF3413 domain-containing protein
	DZA65_RS04310	Dyp-type peroxidase
Dickeya **dianthicola 67-19 Tn-Seq Genes**		
Genes that improved growth in PTB when disrupted	**Locus Id**	**Description**
	HGI48_RS18585	mannose-1 phosphate guanylyltransferase / mannose 6-phosphate isomerase
	HGI48_RS18590	phosphomannomutase CpsG
	HGI48_RS18595	GDP-mannose 4,6-dehydratase
	HGI48_RS06765	dTDP-4-dehydrorhamnose 3,5-epimerase
	HGI48_RS03520	sugar transferase
Genes that were detrimental to growth in PTB when disrupted	**Locus Id**	**Description**
	HGI48_RS19050	microconductance mechanosensitive channel MscM
	HGI48_RS20595	envelope stress response regulator transcription factor CpxR
	HGI48_RS20045	tyrosine recombinase XerC
	HGI48_RS05730	SmdA family multidrug ABC transporter permease/ ATP-binding protein
	HGI48_RS01595	DnaA initiator-associating protein DiaA

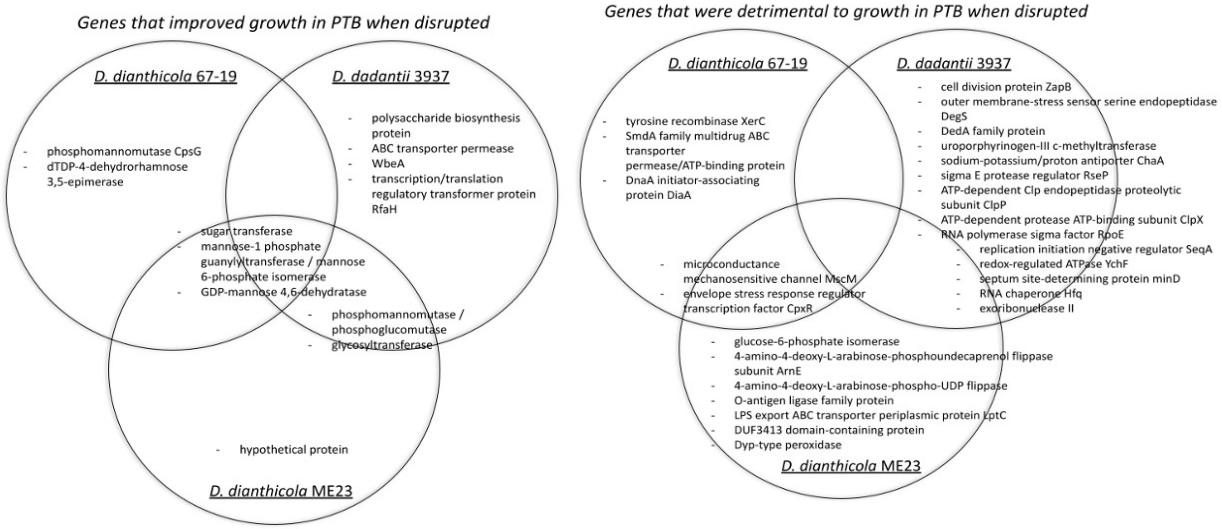

Figure 4: Venn diagrams of genes identified from Tn-Seq experiments. All experiments passed QC. Genes shown are all genes where |fit| > 2 and |t| > 4 and are sorted by fit value into genes that improved growth in PTB when disrupted (top) and genes that were detrimental to growth in PTB when disrupted

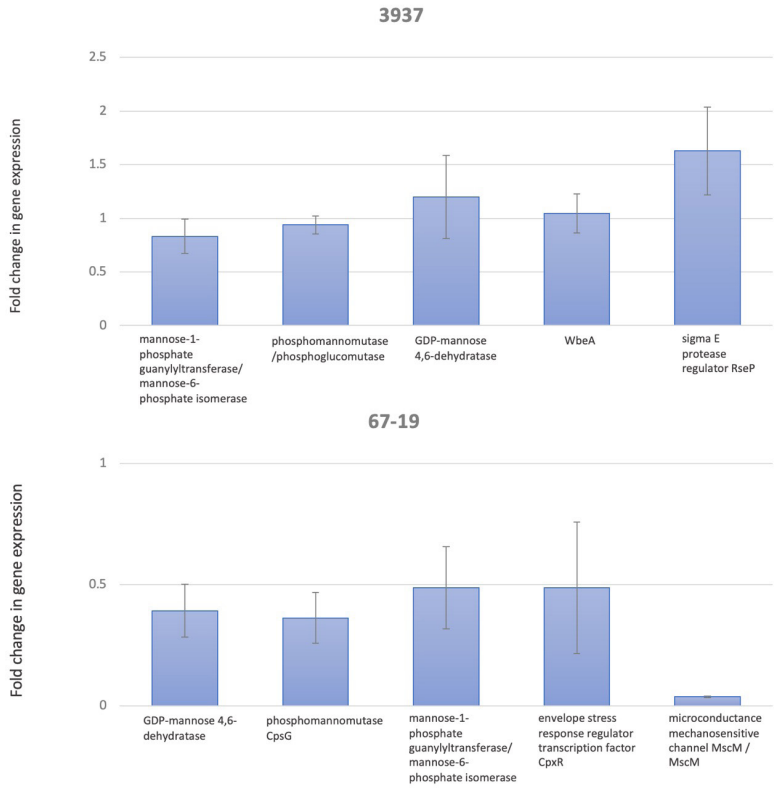

Figure 5: qRT-PCR fold change in gene expression for *D. dadantii* 3937 and *D. dianthicola* 67-19. The fold change in gene expression was calculated as a ratio of the gene expression of PTB-treated samples over the gene expression of non-treated samples. A fold change in gene expression < 1 would indicate that PTB-treated samples decreased expression of the gene of interest whereas a fold change in gene expression > 1 would indicate that PTB-treated samples increased expression of the gene of interest. Error bars indicate standard deviation of the mean.

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Acoustic Variation in Ictalurid Catfishes

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Abstract

More than 35,000 ray-finned fish (Actinopterygii) species are potentially using acoustic communication. However, of the approximately 1200 known soniferous fish species, few include North American freshwater fish. To help fill this knowledge gap in fish acoustic communication, which holds great promise for conservation monitoring, I document acoustic measurements (duration 90%, bandwidth 90%, number of pulses, center frequency, and peak time) across 4 species (Ameiurus nebulosus, Ameiurus natalis, Noturus flavus, Ictalurus punctatus) from 3 genera of the North American catfish family, Ictaluridae. This was done by recording 10 trials of disturbance calls from 28 individuals and analyzing 1294 sounds using Raven Pro 1.6 software. I hypothesized that: 1) more phylogenetically/morphologically related species would have more similar acoustic features, 2) acoustic features would correlate with one another, and 3) acoustic features would correlate with standard length (cm). For hypothesis 1, I instead found that Ameriurus nebulosus was the most acoustically dissimilar, despite having the highest level of phylogenetic/morphological similarity with Ameirus natalis. However, only Ameriurus nebulosus' number of pulses were significantly different from other species. For hypothesis 2, it was found that many acoustic measurements were correlated with one another as predicted. For hypothesis 3, only the number of pulses was found to be significantly correlated with standard length, but minimally so. These findings further support that pulsation measurements may contain a high level of phylogenetic signal, given that it is the most crucial characteristic to differentiate species.

Introduction

The diversity of sound production in animals is vast, yet, many people are largely unaware that fishes produce sound. This is despite the early history of philosophers such as Aristotle describing fish vocalizations (Zograf, 1890). Fish sounds take on various roles, including reproductive, territorial, agonistic, aggressive, social, and feeding (Kasumyan, 2009). Despite more than 35,000 ray-finned fish (Actinopterygii) species potentially using acoustic communication, sounds from only approximately 1200 soniferous fish species are known (Anderson et al., 2011; Rice et al., 2020; Loobey et al., 2022). Of these sounds, only 87 freshwater species in North America and Europe have been recorded (Rountree et al., 2018). Thus, there exists a large gap between the possible number of soniferous fish species that have been recorded and what could be recorded. Predominantly due to human activities, biodiversity is declining at a higher rate in freshwater than in any other environment, thus making conservation efforts particularly important for freshwater habitats (Desjonquères et al., 2019). Understanding which fishes make sound and having quantitative descriptions of these sounds is essential to aquatic Passive Acoustic Monitoring (PAM); a non-invasive conservation technique that allows for autonomous audio recording over long timescales (Rountree et al., 2018; Desjonquères et al., 2019). Data collected from PAM is often cost-effective and can be used for various purposes, such as estimating abundance, species occupancy, population density, community composition, and much more (Browning et al., 2017). Thus, the benefits and implications of understanding fish sounds are far-reaching in the conservation space.

Although sound descriptions are lacking in literature despite the high prevalence of soniferous fishes, phylogenetic comparisons of the evolution of fish acoustic communication are arguably even more sparse, which was recognized by a similar study focused on tetrapods (Chen & Wiens, 2020). A paper that did make phylogenetic comparisons from fish acoustic signals was done for Mediterranean gobies of the Gobius lineage, which compared five acoustic properties across seven species using thirteen individuals for sampling (Horvatić et al., 2016). The degree of acoustic similarity has been found to be related to phylogenetic relationships for insects, birds, mammals, and anurans, but has not been fully explored in fish (Robillard et al., 2006; Tavares et al., 2006; Cap et al., 2008; Gingras et al., 2013b). Since fish are not known to be vocal learners, it would be assumed that their acoustic signals would have a strong genetic component. Thus, there is reason to suspect that phylogeny should be related to fish acoustic signals. I explore this phylogenetic and acoustic relationship in Ictaluridae (Figure 1).

Ictaluridae is the only family of freshwater catfish native to North America. In New York state, there are three closely related catfish genera (Ameiurus, Ictalurus, and Noturus) in the family Ictaluridae, which offer the opportunity to understand the evolution of sound production within this lineage (Acre-H al., 2016). Catfish are a particularly good model for studying acoustic communication, as they are already known to be widely soniferous, using either pectoral stridulation and/or swimbladder drumming as sound production mechanisms (Kaatz et al., 2010). Although capturing reproductive or agonistic sounds may be challenging outside of the natural habitat of ictalurids, disturbance sounds can be readily produced in the laboratory as they occur when a catfish is physically restrained in a way similar to a predatory attack (Kaatz & Stewart, 2012). Additionally, the varied morphology of the pectoral spine in catfish that produce sound, coupled with the diversity of environments catfish inhabit, suggest there are evolutionary links between sound production, morphology, and habitat (Kaatz et al., 2010).
Body size has also been noted to influence sound characteristics in fish (Ladich et al., 1992; Myberg et al., 1993). For example, it was found that sound pressure level and pulse duration increase while dominant frequency decreases in larger weakfish (Cynoscion regalis) (Connaughton et al., 2000). The relationship between acoustic characteristics and body size is not exclusive to fish and has been documented in many other groups, such as anurans and mammals (Gingras et al., 2013b; Libera et al., 2015). This relationship can partially be explained by the correlation between body size and the size of sound-producing organs (Fletcher, 1992). Additionally, body size has been noted as one of the most important morphological influencers on animal acoustic frequency (Marquet & Taper, 1998).

Morphological features beyond body size also play a large role in sound production. The structures involved in stridulatory catfish vocalizations are the dorsal process of the pectoral spine (Fine et al., 1997), spine locking processes, pectoral girdle (Gainer, 1967), and bony ridges on the pectoral spine (Kaatz & Stewart, 1997; Fabri et al., 2007). The locking mechanism that makes vocalizations possible for catfish is thought to function as a passive predator defense (Alexander, 1981). These structures also help to define the catfish order Siluriformes (Alexander, 1966). Catfish disturbance calls in particular occur when a catfish is physically restrained in a way similar to interspecific attacks (Kaatz, 1999). It has been hypothesized that these sounds are a form of acoustic aposematism (i.e., signaling unfavorability to predators using sound), but this hypothesis has not garnered support (Pfeiffer & Eisenberg, 1965). However, support has been shown for the idea that disturbance calls function in place of chemical signals, given the tradeoff there appears to be between chemical signaling and vocalizations in catfish (Heyd & Pfeiffer, 2000).

Therefore, this study aims to help fill in the literature gap on freshwater fish acoustic descriptions. I accomplished this by comparing the disturbance calls of 4 Ictaluridae species

across 3 genera: Brown Bullhead (Ameiurus nebulosus), Yellow Bullhead (Ameiurus natalis), Stonecat (Noturus flavus), and Channel Catfish (Ictalurus punctatus). Similar methods of recording fish air disturbance have been employed in past studies and have helped to inform this study (Kaatz, 2010; Knight & Ladich, 2014).

First, I ask if there are acoustic feature differences across species. I hypothesize (H1) that acoustic features between Brown Bullheads and Yellow Bullheads will be the most similar, acoustic features between Bullheads and Channel Catfish will be moderately similar, and Stonecats will have the least similar acoustic features in comparison to other species. This is because both bullhead species are from the Ameiurus genus, which have knobs on the shelf of their dorsal process and have hemispheres/convolutions on their pectoral spine (Kaatz et al., 2010). Ictalurus morphological features are similar to Ameriurus but have flat convolutions, which should result in increasingly dissimilar sounds to the bullheads (Kaatz et al., 2010). Finally, Noturus morphology is the most different, with no knobs, hemispheres, or convolutions (Kaatz et al., 2010), which should result in the greatest acoustic differences in comparison to other Ictaluridae species tested here.

Secondly, I explore how acoustic features are correlated with one another. I hypothesize (H2) that some acoustic measurements will be correlated because acoustic features are partially a function of morphology. Thus, acoustic measurements should be correlated with one another since the morphology used to make the sound is the same, including the same constraints and affordances of that morphology.

Thirdly, I ask if body size influences acoustic characteristics. I hypothesize (H3) that acoustic measurements differ between fish of varying lengths. This is because body size has been found to be negatively correlated with fundamental frequency, setting precedence for body size in fish impacting acoustic measurements (Myberg et al., 1993; Knight & Ladich, 2014).

Materials & Methods

Fish Collection

A total of 2 Brown Bullheads (Ameiurus nebulosus), 3 Yellow Bullheads (Ameiurus natalis), 9 Stonecats (Noturus flavus), and 14 Channel Catfish (Ictalurus punctatus) were recorded in this study. Brown Bullheads were collected on November 2020 from the Cornell Experimental Ponds (42.50543521664607, -76.4636035547431), Yellow Bullheads were collected on September 2021 from the Hudson River, Stonecats were collected by electrofishing on October 2021 from Fall Creek (42.45485160008576, -76.44787772736022), and Channel Catfish were purchased on October 2021 from Fish Haven Farm located in Candor, NY. The fishes were housed in Corson-Mudd Hall on the Cornell campus.

Audio Recordings

All the fish from a single tank were placed with a scoop net into a bucket filled with water from their tank. Another bucket filled with water from that tank was placed to the right of the fish-filled bucket. Using water only from their tank helped to ensure that changes in water temp and other related factors did not influence the acoustic measurements of their calls. A Zoom H5 recorder with an attached Zoom H5 microphone and Aquarian H2A hydrophone were used to make audio recordings.

I would take a single fish and hold it in the right-side bucket with a hydrophone placed near the top. I would announce the species being recorded, the fish number based on the order of recording, and the medium (water at this stage) of the recording at this time. After one minute of recording, I would announce the end of the water recording and the start of the air recording. The fish would then be recorded in the air for another minute. The recording would exceed a minute if a call were still going by the end of the minute until the call stopped. I would then announce the end of the air recording and proceed to take a standard length measurement of the fish, which I also announced. Thus, each sound file contained all the sounds produced by all fish in one tank. This would be repeated

for all the fish in the tank, which would all be the same species. This procedure would then be repeated for the rest of the tanks. The order that each tank was recorded rotated over ten trials so that differences in time of day would be less of an influence on their acoustic measurements. At least 24 hours passed before another trial began. Similar methods to obtain acoustic measurements have been successful, where catfishes were held in air/water and audio recorded (Kaatz, 2010; Knight & Ladich, 2014). The fishes are held to illicit disturbance calls and recorded in multiple mediums since disturbance calls have been identified in both, arguably because these calls are aimed at different predators in different mediums (Kaatz, 2010; Knight & Ladich, 2014).

Sound Analyses

Audio recordings were analyzed using Raven Pro 1.6 (https://ravensoundsoftware.com/software/raven-pro/), a bioacoustics analysis software program that allows users to visualize sounds and annotate them. First, I went through each file and boxed out my voice every time I made announcements and recorded the content from those announcements in order to know what fish recording was starting/ending. Next, I went through each air medium file and boxed out all sounds I could reasonably attribute to be fish sounds. Then, I annotated the pulses of each of these sounds to collect measurements on the number of pulses, 90% duration (s), 90% bandwidth (Hz), center frequency (Hz), and peak time (s) for each sound. A random sample of 4 out of 20 files was taken for the Channel Catfish to help bring the overall sound sample size across species closer in number, while all files were annotated for the other species to keep the number of sampled sounds more even across species. While annotating pulses, I filtered out the bottom 1500-2500 Hz depending on the background noise level in the recording due to high levels of background noise and minimal fish sound prevalence in this frequency range (Figure 2). The waveform was on the top of the window, and the spectrogram was on the bottom of the window to more clearly identify pulses. A "sound" was classified as a discrete "sound" as opposed to a pulse in a sound if the distance between the two pulses was greater than 0.1 seconds. I could not reasonably attribute sounds to fish in water medium files; thus, those sound files were not further used or analyzed as the air medium files were.

Statistical Analyses

Statistical analyses were conducted in R 4.1.3 (https://www.r-project.org/). To evaluate if acoustic measurement characteristics were different among species, nested ANOVAs, where individuals were nested within tanks, were conducted for all acoustic measurements (bandwidth 90% (Hz), duration 90% (s), peak time (s), center frequency (Hz), and number of pulses) versus species. For significant nested ANOVAs, Tukey HSD was then conducted to determine which species differed significantly from each other for acoustic measurement(s) found to be significantly different across species. To assess whether there were associations between signal properties, I correlated acoustic metrics with one another using the lmer package in R. I report the marginal and conditional r2 (denoted as r2m and r2c, respectively). In addition, a principal component analysis (PCA) was conducted to visualize the correlations between the broad suite of acoustic measurements. Conducting a PCA helped reduce noise from multi-collinearity, making isolating the relationship between just two variables easier. To evaluate if acoustic characteristics were varied with body size, I correlated standard length and acoustic features using the lmer package in R. I report the marginal and conditional r2.

Results

H1: Acoustic Feature Differences Between Species

Bandwidth 90% (Hz), duration, peak time, and center frequency were not found to be significantly different across species (Table 2). The only acoustic measurement significantly different across species was number of pulses ($p = 0.003$). This difference was seen between Channel Catfish vs. Brown Bullheads ($df = 8.58$, $p = 0.026$) and Stonecats vs. Brown Bullheads ($df = 170.14$, $p = 0.011$) (Table 3 & Figure 3). All other

species pairings were not statistically significant for differences in the number of pulses.

H2: Acoustic Feature Correlations With One Another

Bandwidth was correlated with duration where estimated bandwidth = 4024.05 * duration + 1000 ($p = <0.001$, $r^2m = 0.022$, $r^2c = 0.127$, df = 1164.11). Bandwidth was similarly correlated with center frequency where estimated bandwidth = 0.448 * center frequency + 7410 ($p = <0.001$, $r^2m = 0.180$, $r^2c = 0.233$, df = 1080), and number of pulses where estimated bandwidth = 150.05 * number of pulses + 10108.47 ($p = <0.001$, $r^2m = 0.013$, r^2c = df = 1278.96). Duration was also found to be correlated with peak time where estimated duration = 0.689 * peak time + 0.054 ($p = <0.001$, $r^2m = 0.341$, $r^2c = 0.476$, df = 1283), and number of pulses where estimated duration = 0.029 * number of pulses + 0.041 ($p = <0.001$, r2m = 0.392, $r^2c = 0.630$, df = 1247). Finally, peak time and number of pulses were correlated where estimated peak time = 0.019 * number of pulses + 0.0288 ($p = <0.001$, $r^2m = 0.238$, $r^2c = 0.361$, df = 1274). Pairings that were not correlated were bandwidth and peak time, duration and center frequency, peak time and center frequency, and center frequency and number of pulses.

It was also found that 44.9% of the variance could be explained by PC1, which was predominantly composed of duration, peak time, and number of pulses (Figure 4). 22.7% of the variance could be explained by PC2, which was predominantly composed of center frequency and bandwidth.

H3: Acoustic Feature Correlations with Standard Length

Only number of pulses was found to be significantly related to standard length, where the estimated number of pulses = 0.071 * standard length (cm) + 1.132 ($p = 0.007$, $r^2m = 0.010$, $r^2c = 0.047$, df = 66.56) (Table 5). Notably, the correlative effect is minimal. The vast majority of variation was between sounds and not between different fish or tanks.

Discussion

H1: Acoustic Feature Differences Between Species

Although I had hypothesized that the most morphologically/phylogenetically related species would have the most similar acoustic measurements, this was not the case, as Brown Bullheads were found to be the most dissimilar compared to other Ictaluridae. This is in contrast to the pattern seen in other animal groups where phylogenies reconstructed with acoustic signals are often congruent with phylogenies based on morphological/molecular data (Cocroft & Ryan, 1995; Peters & Tonkin-Leyhausen, 1999; Laiolo & Rolando, 2003; Robillard & Desutter-Grandcolas, 2004). My findings may suggest that ecology, rather than morphology and phylogeny, plays a greater role in Brown Bullhead acoustic patterns. For example, only Brown Bullheads of the four species studied live in thick vegetation (Fish NYS DEC Atlas, 2022). Their larger acoustic niche may be a function of them being much more prevalent across New York waterways than the other species. Thus, a wider range of acoustic measurements would allow communication to be less hindered across the various acoustic niches they might have to compete with across habitats. It should be noted, however, that my Brown Bullhead sample size was only two individuals, so it is possible that the Brown Bullheads I recorded had especially varied calls. However, if this were particularly a problem, I would have expected more frequent outliers in the other species, which I do not see.

Another point of interest is that Brown Bullheads significantly differ from other species in the number of pulses they produce. This is similar to studies on gobies, where pulse rate was determined to be the most prevalent indicator for differentiating closely-related species with acoustics (Malavasi, 2008; Horvatić, 2016). Although pulse rate and the number of pulses are not the same measurement, pulsation-related measurements consistently were reported as the strongest species-differentiating acoustic factor in closely related species. The number of pulses

and pulse rate have also been found to be the predominant acoustic features damselfish used for species-species recognition (Myrberg, 1972; Spanier, 1979). Given the above, more work is needed to understand the importance of acoustic pulsation in species recognition and how these measurements relate to fish phylogenies.

H2: Acoustic Feature Correlations With One Another

I found that acoustic measurements correlated with one another, and PCAs explained the majority of variance through PC1 and PC2. In the PCA, acoustic measurements with hertz units were grouped into PC2, while acoustic measurements measured in seconds or counts were grouped into PC1. Another point of interest in the PCA is that, although Brown Bullheads were the species with the smallest sample size, they occupied the most acoustic niche space, which aligns with the ideas discussed in H1. As for the correlations between acoustic features, this appears rarely documented in fish acoustic studies. Perhaps more data of this nature could provide further insight into sound production mechanisms and how sounds are intertwined with morphology in fishes.

H3: Acoustic Feature Correlations with Standard Length

Body size has often been found to be highly correlative in other clades, such as anurans (Gingras et al., 2013a) and, to an even further extent, cetaceans, where up to 97% of their frequency variation can be attributed to body size (Matthews et al., 1999). However, this high correlation was not seen here with Ictaluridae, as only one acoustic measurement was correlated to standard length, a proxy for body size in this study. Even then, the correlative effect was almost 0. A similar finding was found in the California spiny lobster (*Panulirus interruptus*), where pulse rate was correlated with body size but not typical measurements correlated with body size, such as dominant frequency (Patek et al., 2009). It is somewhat counterintuitive that a stridulatory sound (i.e., sound produced from the friction of body parts) would not display greater correlations between acoustic measurements and body size. This might suggest that the morphological features used to make sound may not be very correlated with the rest of their body size. However, it still begs the question as to why the number of pulses in fish would be the highest correlate to body size. Given the seemingly stand-alone prevalence of number of pulses in both differentiating between species and body size, it appears that acoustic pulsations would be the best acoustic means for communicating information on Ictaluridae.

Conclusion

Insight into acoustic measurements and their relationships is needed to properly conduct conservation bioacoustics methods, which could be useful in combating declining freshwater fish biodiversity. This study tested if acoustic features among ictalurid catfishes corresponded to their morphology/phylogeny, if acoustic features are correlated with one another, and if acoustic features differ between fish of different lengths. It was instead found that acoustic features were not congruent with morphology/phylogeny, unlike in other animal groups. Of the acoustic features studied, only the number of pulses could be used to differentiate species. The number of pulses demonstrated again that it is a comparatively strong acoustic indicator, as it was also the only feature correlated with body size. This is continuously demonstrated by acoustic features measured in seconds and counts (duration, peak time, & number of pulses) primarily accounting for variance, while features measured as rates (center frequency & bandwidth) secondarily accounted for variance. These findings suggest number of pulses to be the most varied and indicative acoustic feature among ictalurid catfishes, which is consistent with other groups of fish. Thus, further research attempting to predict various traits from acoustic features in Ictaluridae and potentially other fish may be well served by focusing on pulsation-related measurements.

Figures and Tables

Table 1: Summary Statistics of Acoustic Measurements and Sample Sizes. For the acoustic measurements, the formatting is mean +/- standard deviation.

	Brown Bullhead	Channel Catfish	Stonecat	Yellow Bullhead
# of Fish	2	14	9	3
# of Sounds	451	699	33	111
Length (cm)	17.06 +/- 0.50	13.85 +/- 1.22	7.73 +/- 2.05	9.14 +/- 1.17
Bandwidth (Hz)	10481.27 +/- 2255.38	10257.44 +/- 2605.21	10453.39 +/- 2745.70	10744.87 +/- 2645.74
Duration (s)	0.119 +/- 0.121	0.080 +/- 0.070	0.073 +/- 0.048	0.089 +/- 0.065
Peak Time (s)	0.082 +/- 0.090	0.054 +/- 0.065	0.054 +/- 0.038	0.056 +/- 0.052
Center Freq (Hz)	6854.72 +/- 2304.56	6256.52 +/- 2262.61	6525.21 +/- 2927.57	6753.28 +/- 2771.85
# of Pulses	2.51 +/- 2.12	1.82 +/- 1.78	1.33 +/- 0.69	2.09 +/- 1.53

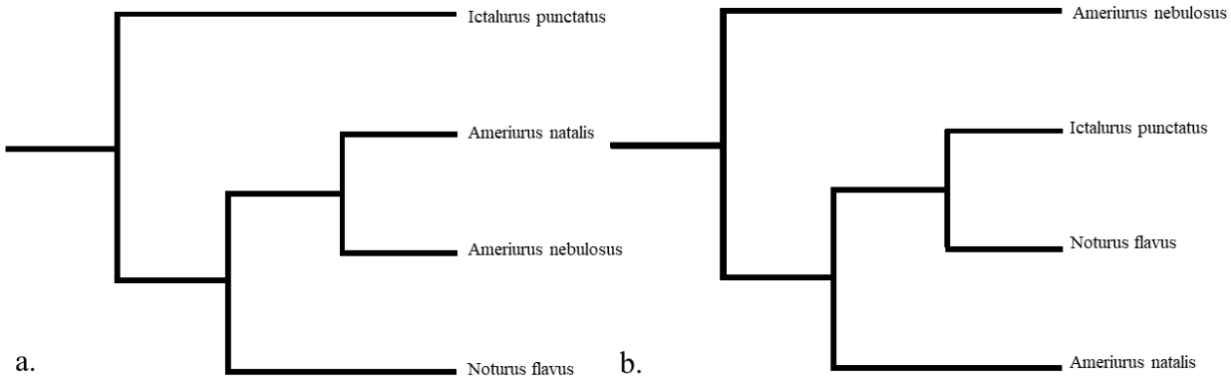

Fig 1. True Phylogenetic Tree of Tested Species (a.) vs. Tree Built from Phenotypic Data of Number of Pulses (b.) a.) Note that *Ameiurus natalis* and *Ameiurus nebulosus* are the most closely related. The Ameiruses are more closely related to *Noturus* than *Ictalurus*. b.) Note that the tree built from the phenotypic data of number of pulses is not congruent with the true phylogenetic tree of tested species.

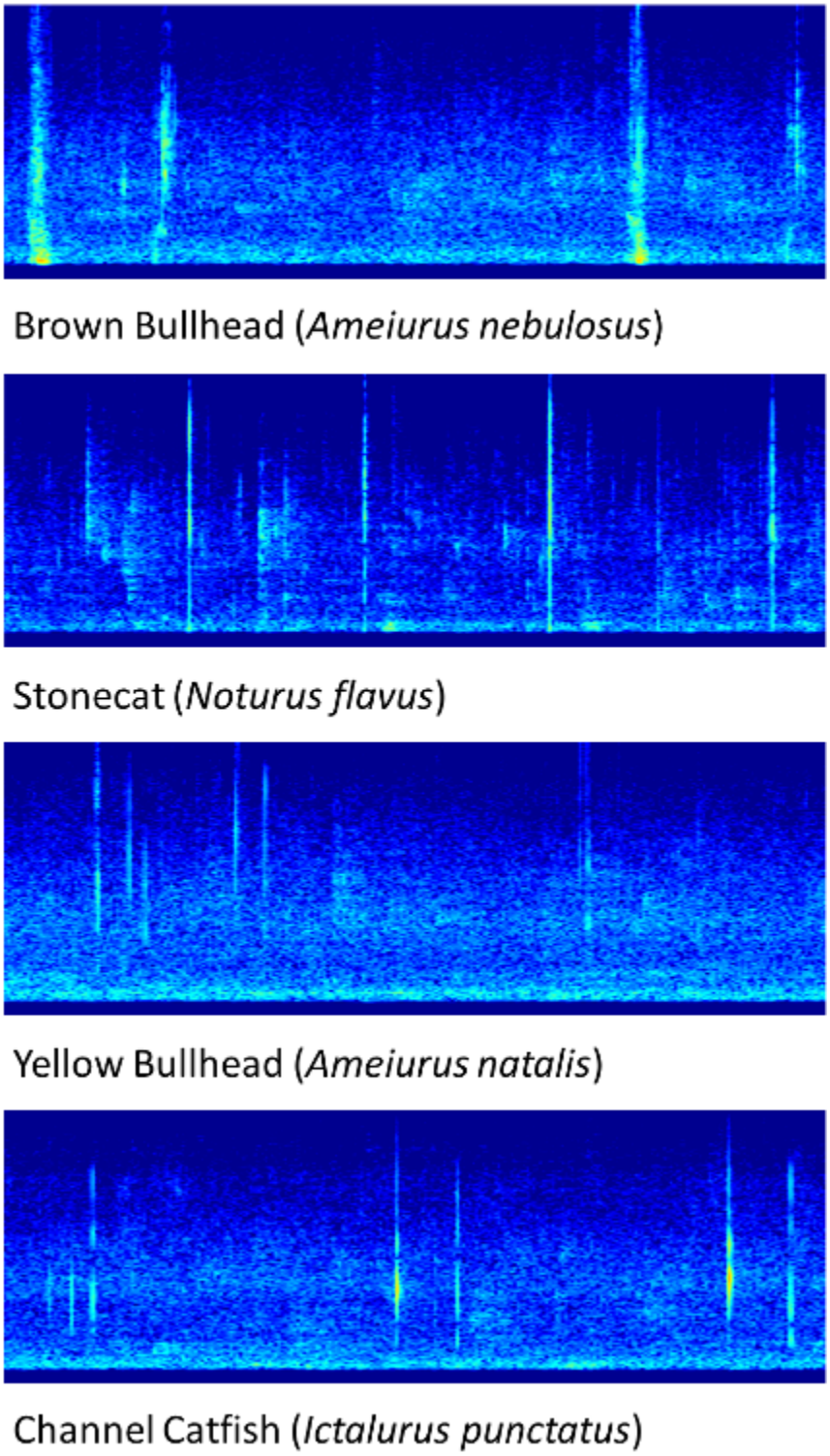

Fig 2. *Example Spectrograms of Disturbance Calls in Ictaluridae*. Y-axis = 22 kHz, X-axis = 1.5 seconds.

H1

Table 2. Nested ANOVA Results P-Value Chart for Acoustic Measurement Differences Across All Species. Note: Italicized text indicates statistically significant.

Acoustic Measurement	p-value
Bandwidth	0.709
Duration	0.413
Peak Time	0.344
Center Frequency	0.880
# of Pulses	*0.003*

Table 3. P-Value & DF Chart for Number of Pulse(s) Differences Between Species. CC = Channel Catfish, SC = Stonecat, YB = Yellow Bullhead, and BB = Brown Bullhead. Note: Note: Italicized text indicates statistically significant.

	df	p-value
CC - BB	8.58	*0.026*
SC - BB	170.14	*0.011*
YB - BB	51.59	0.328
SC - CC	87.06	0.572
YB - CC	22.33	0.702
YB - SC	196.08	0.245

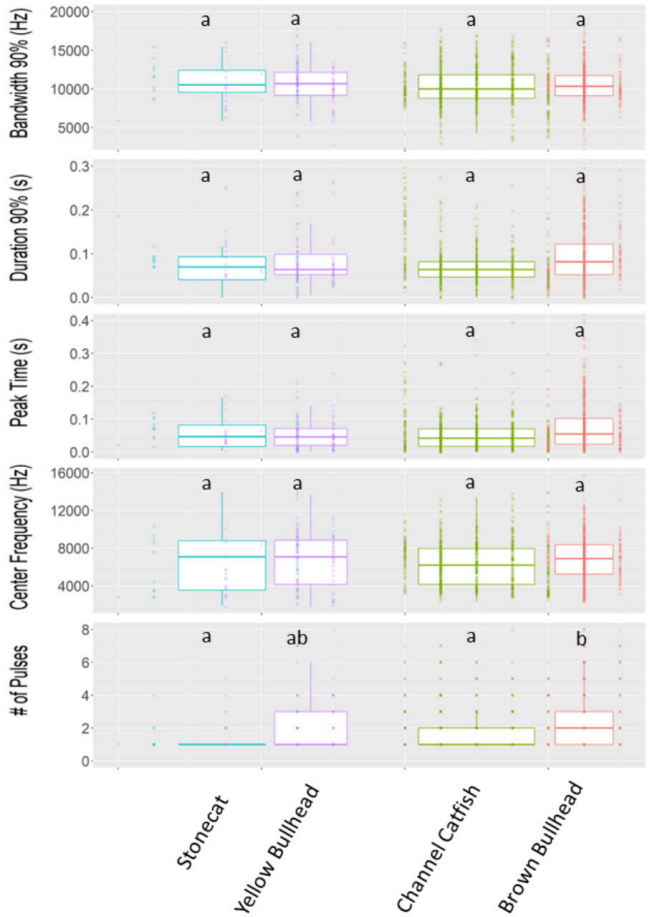

Fig 3. Acoustic Measurement Differences Across Species. The letters above the boxplots represent which species belong to which group for that particular acoustic measurement, denoted on the y-axis. For example, if a boxplot is labeled "a," then it belongs to the same group as species also labeled "a" or "ab". Brown Bullheads (n sounds = 451), 14 channel catfish (n sounds = 699), 9 Stonecats (n sounds = 33), and 3 Yellow Bullheads (n sounds = 111) were used in these analyses.

H2

Table 4. Correlations Between Acoustic Measurements. The first numeric value in the cell represents the p-value, the second is their y = mx + b equation, and the third numeric value represents df. Note: Italicized text indicates statistically significant.

	Bandwidth	Duration	Peak Time	Center Frequency
Duration	*p = <0.001, r^2m = 0.022, r^2c = 0.127, y = 4024.05x + 1000, df = 1164.11*			
Peak Time	p = 0.359, r^2m = 0.001, r^2c = 0.104, y = 879.16x + 10360.53, df = 1284.85	*p = <0.001, r^2m = 0.341, r^2c = 0.476, y = 0.689x + 0.054, df = 1283*		
Center Frequency	*p = <0.001, r^2m = 0.180, r^2c = 0.233, y = 0.448x + 7410, df = 1080*	p = 0.245, r^2m = < 0.001, r^2c = 0.279, y = -1.21e-06 + 0.109, df = 1280	p = 0.806, r^2m = < 0.001, r^2c = 0.140, y = -2.21e-07x + 0.069, df = 1257	
# of Pulses	*p = <0.001, r^2m = 0.013, r^2c = 0.115, y = 150.05x + 10108.47, df = 1278.96*	*p = <0.001, r^2m = 0.392, r^2c = 0.630, y = 0.029x + 0.041, df = 1247*	*p = <0.001, r^2m = 0.238, r^2c = 0.361, y = 0.019x + 0.0288, df = 1274*	p = 0.609, r^2m = < 0.001, r^2c = 0.325, y = 16.27x + 6701.35, df = 1265.26

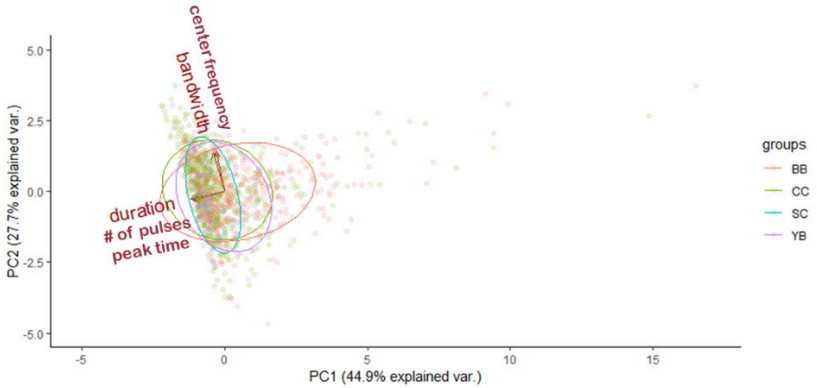

Fig 4. Principal Component Analysis Biplot. 44.9% of variance is explained by peak time, duration, and number of pulses (PC1). 22.7% of variance is explained by center frequency and bandwidth.

H3

Table 5. Acoustic Measurement Differences Between Standard Lengths Results Chart. Note: Italicized text indicates statistically significant.

	Bandwidth	Duration	Peak Time	Center Freq	# of Pulses
p-value	0.917	0.375	0.124	0.849	*0.007*
r^2 marginal	< 0.001	0.003	0.006	< 0.001	*0.010*
r^2 conditional	0.108	0.274	0.134	0.330	*0.047*
df	45.53	52.37	54.84	47.26	*66.56*
y = mx + b	y = -4.508x + 10480.25	y = 0.002x + 0.077	y = 0.002x + 0.403	y = 11.37x + 6691.62	*y = 0.071x + 1.132*
% var by fish	6%	17%	5%	3%	*2%*
% var by tank	5%	10%	8%	30%	*2%*
% var by sound	89%	73%	87%	67%	*96%*

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Serotonin and the Brain: Exploring the 5-HT system's role in Depression

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Abstract

Major depressive disorder (MDD) is a highly prevalent and debilitating illness in the modern world. In the 1960s, the theory that low serotonin (5-HT) was a primary cause of MDD emerged due to the efficacy of 5-HT restoring drugs in treating depression. The 5-HT deficiency hypothesis of depression has since been criticized through studies not being able to directly tie low serotonin to MDD. The discovery of the antidepressant efficacy of the glutamatergic priming ketamine led to a reevaluation of depression pathophysiology. Modern perspectives view depression as an issue of disrupted neurocircuitry resulting from stress induced atrophy of certain limbic and cortical brain regions, such as the hippocampus and PFC, and hypertrophy in the fear evaluating amygdala, the reward evaluating nucleus accumbens, and the orbitofrontal cortex. Depression may be treated by supplementing psychotherapy with potentiating neuroplasticity, helping individuals relearn negative emotional associations and restoring dysfunctional neurocircuitry . 5-HT may be viewed as a vulnerability factor in developing depression due to its involvement in stress, as well as a treatment target which indirectly primes neuroplasticity. Other neurotransmitter systems similarly represent depressive risk factors and antidepressant targets, namely the noradrenergic and dopaminergic systems. Serotonergic antidepressants such as Selective Serotonin Reuptake Inhibitors (SSRIs) see high rates of prescription due to their minimal side effects. They demonstrate slower efficacy than ketamine, whose dissociative side effects and potential for abuse are unideal, demanding further research of its mechanism to find safer and more effective antidepressant targets.

Introduction

Major depressive disorder (MDD) is an impairing and chronic illness. With an estimated lifetime occurrence of 16.2% in the USA while affecting more than 300 million people worldwide, MDD is the second biggest cause of disability today (Albert, 2012; Yohn, 2017). With its potential for lost productivity and the danger of suicide, the prevalence of MDD society has given scientists and medical professionals much reason to find an effective treatment. This has naturally led to research into the biomechanisms behind MDD. One popular explanation is the 5-HT/monoamine deficiency hypothesis of depression, which identifies a deficiency of 5-HT and other monoamine neurotransmitters in the brain as the core pathogenetic factor behind depression (Jacobsen, 2012).

The monoamine hypothesis has had an influential role in the history of psychology (Liu, 2017). Much of the evidence supporting the depletion hypothesis comes from the efficacy of serotonin enhancing drugs, as the majority of clinically available antidepressants upregulate 5-HT or norepinephrine (NE), another monoamine neurotransmitter. A summary of drugs currently used to treat depression is shown in Table 1 below.

Despite early pharmacological evidence, the monoamine hypothesis has since received criticism due to a lack of strong evidence to indicate a connection between low 5-HT and depression (Nautiyal, 2017). This, along with issues surrounding the delayed onset of typical monoamine affecting antidepressants presenting danger in cases of suicidal ideation and a meta-

Table 1: *Currently Approved Antidepressant Drugs (Sheffler, 2022)*

Antidepressant Class	Mechanism	Example(s)
Selective Serotonin Reuptake Inhibitors (SSRIs)	Inhibit the serotonin transporter (SERT), preventing serotonin reuptake into the presynaptic terminal. Currently the first line in treatment of depression due to the relatively minimal side effects.	• Sertraline • Fluvoxamine • Fluoxetine • Paroxetine • Citalopram • Escitalopram
Selective Norepinephrine Reuptake Inhibitors (SNRIs)	Inhibit the norepinephrine transporter (NET), and SERT to a smaller degree, preventing both 5-HT and NE reuptake into the presynaptic terminal.	• Venlafaxine • Desvenlafaxine • Duloxetine • Milnacipran • Levomilnacipran
Atypical Antidepressants	Various effects on the 5-HT, NE, and/or dopamine (DA) systems.	• Bupropion • Mirtazapine • Agomelatine
Serotonin Modulators	Inhibit presynaptic 5-HT reuptake to varying degrees while also acting as 5-HT receptor agonists or antagonists.	• Nefazodone • Trazodone • Vilazodone • Vortioxetine
Tricyclic Antidepressants (TCAs)	Inhibit 5-HT and NE reuptake. Also have an affinity for muscarinic M1 receptors and histamine H1 receptors, causing sedation and anticholinergic side effects.	• Amitriptyline • Clomipramine • Doxepin • Imipramine • Trimipramine • Desipramine • Nortriptyline • Protriptyline • Maprotiline • Amoxapine
Monoamine Oxidase Inhibitors (MAOIs)	Inhibit the monoamine oxidase enzyme, which normally breaks down 5-HT, NE, and DA. Not a first line treatment due to adverse drug-drug interactions.	• Selegiline • Moclobemide • Tranylcypromine • Isocarboxazid • Phenelzine
NMDA Antagonists	Blockades NMDA receptors, which ultimately increases glutamatergic signaling.	• Esketamine • Dextromethorphan/ Bupropion

analysis that demonstrated SSRIs struggle to outperform placebo (Kirsch, 2008), has led to the development of alternate hypotheses hoping to develop more effective antidepressants (Liu, 2017). While there is evidence that 5-HT may play a role in the pathophysiology of depression (Blier, 2013), modern findings suggest the monoamine hypothesis is overly simplistic, and that serotonin should not be viewed as the primary pathogenetic factor behind MDD (Liu, 2017).

The purpose of this review is to critically evaluate the role 5-HT plays in depression based on modern findings, as well as integrate current ideas about depressive pathophysiology into a central hypothesis about the nature of MDD. First, I will explain the role 5-HT plays

in the brain and along with the history of the 5-HT deficiency hypothesis. After providing background, I will elaborate on the role 5-HT may play in stress and MDD, based on modern evidence. Next, I will describe several issues with the 5-HT hypothesis before presenting a description of the neuroplasticity hypothesis, which has been favored in current literature. Finally, I will discuss my findings holistically, presenting directions for future research into antidepressants.

Based on these findings, I hypothesize that serotonin's involvement in self-control predicts individual resistance to stress, and that a chronic failure of the serotonergic system to maintain goal-directed behavior in the face of adversity is one way depression can develop. I would also like to present the idea that in the case of serotonergic antidepressants such as SSRIs, increases in neuroplastic gene transcription resulting from upregulation of serotonergic signaling is the basis of antidepressant treatment, with the quality of the environment predicting whether new emotional associations resulting from the increased plasticity are positive, neutral, or negative. This emphasizes the need to pair antidepressant treatment with psychotherapy and supportive care.

Materials and Methods

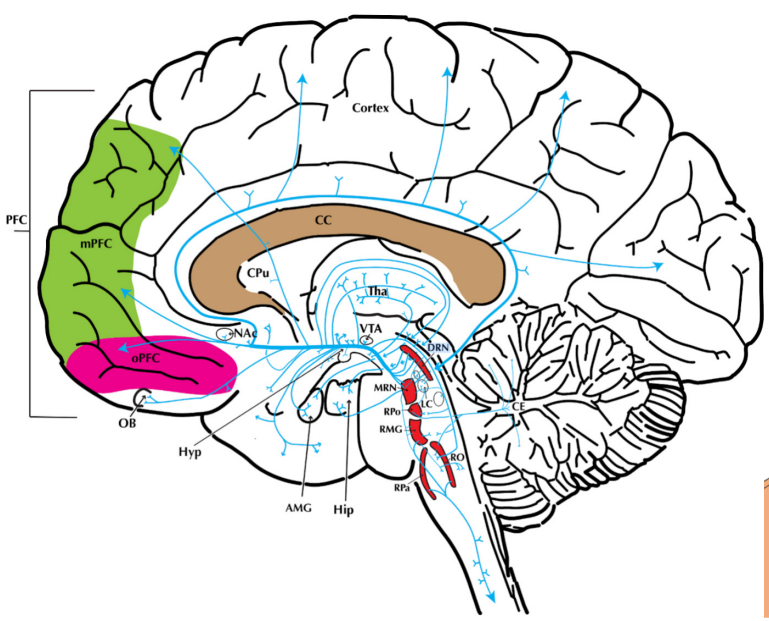
A systematic review of the role of serotonin in depression was conducted, integrating modern perspectives including reviews of stress, neuroplasticity, genetics, and other monoamines, with the focus to better characterize the pathophysiology of depression and examine avenues of future research in antidepressant therapy. The literature search was conducted primarily using PubMed. Papers were found in Pubmed using the website's advanced search function with the keywords, "Serotonin, 5-HT, Depression, Norepinephrine, Dopamine, Neuroplasticity, Stress." Selected papers were qualitatively assessed for their relevance and objectivity before being included. The textbook "Psychopharmacology. Sunderland, MA, U.S.A. Sinauer Associates. (2019)" provided broad information and was used to find additional papers it referenced in text. Additional papers were found through manual search of literature reference lists. This paper is an abridged version of another paper that was submitted as a Cornell Honors Thesis. The original paper can be found at https://ecommons.cornell.edu/handle/1813/111440. Overall, findings of 41 papers were included to conduct the abridged review.

Results

Serotonin's Function in the Brain

Discovered over 60 years ago, 5-HT is a neurotransmitter that modulates a wide range of neural activities and psychological processes (Berger, 2009). 5-HT is mediated by 14 types and subtypes of receptors, that, combined with its role in modulating a large number of physiological and psychological processes, make 5-HT's exact function hard to define (Jans, 2007). Although the cell-bodies of 5-HT-ergic neurons are almost exclusively located in the raphe nuclei of the brain stem, the axons of these neurons spread throughout the entire brain (Jans, 2007). It can be said that every brain cell is close to a serotonergic fiber, with nearly all behaviors and many other brain functions in some way regulated by serotonin (Berger, 2009). The primary raphe nuclei are the dorsal raphe (DR) and median raphe, located in the caudal midbrain and rostral pons (Meyer & Quenzer, 2019). Axons from these regions are not uniformly distributed, innervating areas of high dendritic and synaptic density more than white matter tracts (Meyer & Quenzer, 2019). Figure 1 contains a diagram showing serotonergic fiber distributions in the human brain.

While awake, the dorsal raphe fires tonically at a constant, slow rate. It also can fire in phasic bursts, facilitating motor output while suppressing sensory processing. Phasic firing must be triggered by excitatory inputs to the



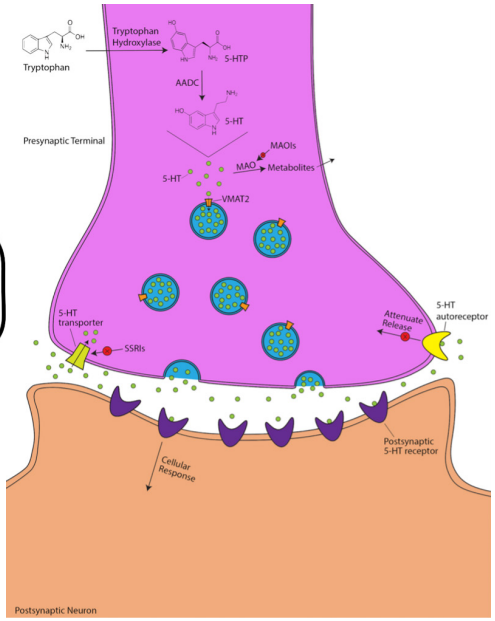


Figure 1 (left): *A sagittal view of human serotonergic neurocircuitry.* The raphe nuclei are shown in red. Blue tracts represent serotonergic projections from the raphe nuclei, with extensive limbic and cortical innervation. Raphe Nuclei: DRN, dorsal raphe nuclei, MRN, median raphe n, RPo, raphe pontis n, RMG, raphe magnus n, RPa, raphe pallidus n, RO, raphe obscurus n; LC, locus coeruleus, Tha, thalamus, Hip, hippocampus, AMG, amygdala, Hyp, hypothalamus, CPu, caudate putamen, VTA, ventral tegmental area, NAc, nucleus accumbens, OB, olfactory bulb, CC, corpus callosum, CE, cerebellum, PFC, prefrontal cortex: mPFC, medial prefrontal c, oPFC, orbitofrontal prefrontal c. Adapted from Charnay, 2010.

Figure 2 (right): *A serotonergic synapse.* 5-HT is synthesized from tryptophan by tryptophan hydroxylase and AADC (Aromatic L-Amino Acid Decarboxylase). It is packaged into vesicles by VMAT2 (Vesicular Monoamine Transporter 2), to be released into the synapse. MAO (Monoamine Oxidase) breaks down 5-HT into metabolites. MAOI antidepressants inhibit this process. The 5-HT transporter (SERT or 5-HTT) mediates reuptake from the synapse and is the primary target for SSRIs. Presynaptic 5-HT autoreceptors maintain the extracellular tone of 5-HT. Postsynaptic 5-HT receptors receive serotonergic signals. Adapted from Meyer & Quenzer, 2019.

DR, such as through glutamatergic pathways from the PFC, lateral habenula, hypothalamus, and various brainstem areas. The DR also receives cholinergic input from the pons, and inhibitory GABA inputs from different areas including the DR itself. 5-HT is also regulated by the monoamine neurotransmitters DA and NE. During slow wave sleep, DR firing becomes slower and irregular, and effectively ceases firing during REM sleep (Meyer & Quenzer, 2019).

5-HT's effects on behavior are varied, modulating a range of processes also affected by depression, such as mood, appetite, sleep, activity, suicide, sexual behavior, and cognition, including learning and memory. In addition, an alteration of 5-HT function has been observed in a plethora of clinical conditions, including anxiety and suicide (Jans, 2007). Serotonergic tracts have been robustly connected to aggression and impulse control. For example, mice prevented from synthesizing 5-HT in the brain demonstrate a large increase in aggression and impulsivity, but decreased anxiety, social communication, and maternal care (Meyer & Quenzer, 2019). For clarity, Figure 2 depicts a serotonergic synapse, including autoreceptors and the serotonin transporter.

Table 2 shows the 14 serotonin receptors and their studied functions. 5-HT1A and 5-HT2A have been widely studied in depression, due to their high expression in limbic and cortical regions respectively (Nichols, 2008). The majority of serotonin receptors have been connected to the modulation of depression (Nautiyal, 2017).

Table 2: *The Functions of the 5-HT Receptors (Meyer and Quenzer, 2019; Nichols, 2008)*

5-HT Receptor	Type	Mechanism	Effect	Notes
5-HT_{1A}	$G_{i/o}$ coupled	Decreases cellular cAMP	Inhibitory	Presynaptically expressed on raphe cells as an autoreceptor, but highly postsynaptically expressed in limbic regions.
5-HT_{1B}	$G_{i/o}$ coupled	Decreases cellular cAMP	Inhibitory	Terminal raphe cell 5-HT autoreceptor.
5-HT_{1D}	$G_{i/o}$ coupled	Decreases cellular cAMP	Inhibitory	Terminal raphe cell 5-HT autoreceptor.
5-HT_{1E}	$G_{i/o}$ coupled	Decreases cellular cAMP	Inhibitory	Expressed cortically, not expressed in mice.
5-HT_{1F}	$G_{i/o}$ coupled	Decreases cellular cAMP	Inhibitory	Expressed primarily in motor regions, antimigraine target.
5-HT_{2A}	$G_{q/11}$ coupled	Increase cellular IP3 and DAG	Excitatory	High cortical expression, primary serotonergic hallucinogenic target (LSD, Psilocin, DOI), activates genes mediating neuroplasticity.
5-HT_{2B}	$G_{q/11}$ coupled	Increase cellular IP3 and DAG	Excitatory	Important for heart and brain development, knockout is lethal, involvement in certain cardiac pathologies.
5-HT_{2C}	$G_{q/11}$ coupled	Increase cellular IP3 and DAG	Excitatory	Inhibits dopaminergic neurotransmission, high expression in amygdala, associated with anxiety when activated.
5-HT_{3}	Cation channel	Depolarize cell	Excitatory	The only ionotropic 5-HT receptor, located on peripheral terminals of vagus nerve, antagonists reduce nausea and are anxiolytic.
5-HT_{4}	G_{s} coupled	Increase cellular cAMP	Excitatory	High expression in hippocampus and motor regions, appear to mediate LTD in hippocampus, high expression in gastric periphery, promotes gut motility.
5-HT_{5A}	$G_{i/o}$ coupled	Decrease Cellular cAMP	Inhibitory	Possibly important in cerebellar functioning.
5-HT_{5B}	$G_{i/o}$ coupled	Decrease Cellular cAMP	Inhibitory	Not expressed in humans.
5-HT_{6}	G_{s} coupled	Increase cellular cAMP	Excitatory	Highly expressed in striatum and cortex, blockade enhances cholinergic neurotransmission and promotes learning/memory.
5-HT_{7}	G_{s} coupled	Increase cellular cAMP	Excitatory	Expressed in the suprachiasmatic nucleus of hypothalamus, appears to regulate circadian processes, high gut and spinal cord expression.

Origins of the 5-HT/ Monoamine Hypothesis

The 5-HT hypothesis of depression can be traced to clinical observations in the 1950s, where Iproniazid, a drug for Tuberculosis, unexpectedly displayed an antidepressant effect in tuberculosis patients. This was attributed to its ability to inhibit monoamine oxidase (MAO) A and B, the enzyme which metabolizes 5-HT and NE (Jacobsen, 2012). These findings served as early evidence in 1965, when Joseph Schildkraut presented the hypothesis that depression could be tied to a deficiency of norepinephrine and other catecholamines, but also emphasized the likely involvement of serotonin. His formulation heavily relied on drug-based evidence, recognizing the theory was likely an oversimplification of a complex biological state (Schildkraut, 1965). In 1967, Alec Coppen identified a connection between 5-HT and MDD, citing pharmacological evidence that depletion of monoamines in the brain could induce depression in a subset of patients receiving reserpine for hypertension, that increasing the effectivity of monoamines with monoamine oxidase inhibitors (MAOI) could alleviate depression, and that there was evidence of disturbances in amine metabolism in MDD. The identification of 5-HT being an important factor was shown by the fact that tryptophan, the amino acid precursor of 5-HT, potentiated the antidepressant action of MAOIs in depressed patients (Coppen, 1967). Today, the theory remains influential in the pharmaceutical industry, and SSRI antidepressants are now among the best-selling drugs in medical practice (Lacasse, 2005).

Role of 5-HT Dysregulation in MDD

As detailed above, much of the basis for the 5-HT hypothesis of depression comes from the efficacy of serotonin-enhancing drugs. Both MAOIs and tricyclics increase synaptic monoamine concentrations and demonstrate antidepressant efficacy. On the other hand, SSRIs are a more pharmacologically specific antidepressant that act by inhibiting 5-HT reuptake into raphe nuclei neurons, leading to increased 5-HT levels throughout the brain after chronic treatment (Yohn, 2017), and serving as a first line treatment for depression (Saavedra, 2021).

In addition to evidence from drug treatments, several interesting observations serve to support the idea that serotonin plays at least some role in MDD. Tryptophan depletion, which effectively lowers brain 5-HT levels, can cause a recurrence of depressive symptoms in recovering MDD patients who were responsive to certain serotonergic antidepressants. Additionally, low 5-HT was found to have a robust correlation with suicide patients, although its correlation to MDD has been inconsistent (Jacobsen, 2012).

While the above findings support the depletion hypothesis, studies into serotonin receptors and genetic polymorphisms served to demonstrate mood disorders modulated by serotonin had more to do with a general dysfunction of serotonergic circuitry rather than a simple reduction in 5-HT. Said mood disorders could result from either hyper- or hypo- function of serotonin pathways, depending on the brain region, stage of neurodevelopment, and receptors involved. For example, increased 5-HT2AR cortical receptors, ante- and post-mortem, have been repeatedly associated with depression and depressive personality traits, with a link to suicidality (Jacobsen, 2012). This receptor is correspondingly seen to be reduced after successful antidepressant treatment, coinciding with the onset of clinical efficacy (Vollenweider, 2010).

Studies into 5-HT2AR have found its signaling to be associated with anxiogenesis, as 5-HT2A KO mice demonstrate reduced anxiety, with restoration of the receptor in the PFC normalizing anxiety-like behavior. This effect may be a result of serotonergic signaling onto the PFC being responsible for modulating downstream signaling in the amygdala (AMG), with serotonin's demonstrated modulation of anxiety being one way it is involved in the intersection of stress and depression. Indeed,

potentiation of this receptor with the hormone CRH, which regulates the stress response, led to increased anxiety-like behavior in mice in response to the 5-HT2A agonist DOI. Finally, frontolimbic receptor density of 5-HT2A in humans is correlated both with anxiety and the ability to cope with stress, with prefrontal 5-HT2A receptors on descending fibers that control the DR being involved in stress responses. In clinical studies, downregulation of these receptors after treatment with various antidepressants was found to coincide with the onset of antidepressant efficacy in patients with MDD (Vollenweider, 2010).

It must be noted that a general upregulation in 5-HT receptors does not occur in depression, as shown by the apparent decrease of hippocampal 5-HT1ARs in chronically depressed patients (Jacobsen, 2012). This corresponds with an observation of postsynaptic 5-HT1ARs mediating anxiolytic effects. It has been theorized that the postsynaptic 5-HT1AR receptor is also involved in impulse control, such as by increasing patience and anti-aggression (Carhart-Harris, 2017). These postsynaptic 5-HT1ARs have differing roles than the presynaptic autoreceptors.

Due to the differing roles of serotonergic receptors, Carhart-Harris et al. (2017) proposed a "bipartite model of depression", where postsynaptic 5-HT1ARs are hypothesized to be involved in resistance to depression through its signaling promoting stress-coping, while 5-HT2ARs are hypothesized to be involved in antidepressant efficacy, shown by their downstream activation of neuroplastic genes in response to psychedelics. This idea is summarized in Figure 3 below.

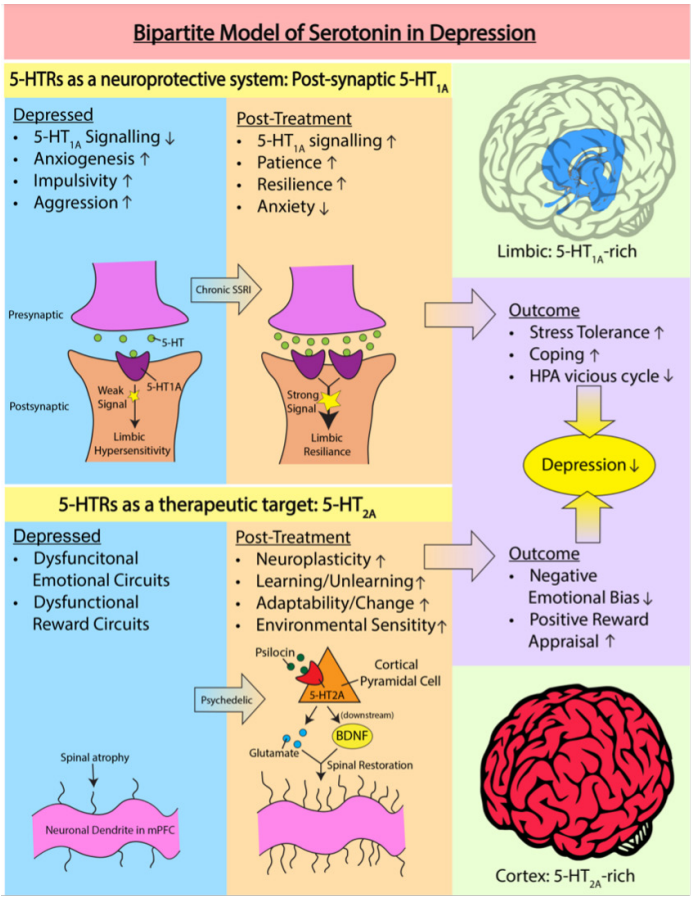


Figure 3: *The proposed bipartite model of serotonin's role in depression.* 5-HT1A signaling corresponds with resistance to stress. 5-HT2A signaling corresponds to neuroplastic changes that may help alleviate depression. Carhart-Harris et al. predicts conventional antidepressants such as SSRIs therefore act by primarily enhancing the 5-HT1A pathway, while serotonergic psychedelics primarily act on pathway 2. Note the findings that serotonergic neuroplasticity could occur independent of 5-HT2A (Hesselgrave, 2021) evidence that this model is an oversimplification. Adapted from Carhart-Harris et al., 2017.

This formulation acknowledges that ignoring the roles of the other serotonin receptors represented an oversimplification, but emphasized the general idea of serotonin mediating both a coping and neuroplastic pathway through separate mechanisms. With results from a study by Hesselgrave (2021) demonstrating psilocybin could mediate an antidepressant and neuroplastic effect in mice despite blockade of 5-HT2A/2CRs with ketanserin, more extensive studies into the roles of other 5-HTRs are warranted to find additional pharmacological targets. For example, blockade of 5-HT7 potentiates antidepressant effects in rats (Yohn, 2017). A summary of data detailing current findings about the roles of varying serotonin receptors in modulating neuroplasticity is shown in Table 3 below (Kraus, 2017).

Table 3: *The roles of various serotonin targets in neuroplasticity (Kraus, 2017).*

Serotonin Target	Downstream Mechanism of Neuroplastic Activation	Modulates
5-HT_{1A}	MAPK, AKT, LTD + LTP via NMDA, s100, BDNF, NF-κB, CREB	Adult neurogenesis, dendritic maturation, neuroprotection, astroglial interaction
5-HT_{1B}	AKT, ERK, LTD	Unknown
5-HT_{2A}	ERK, NMDA, kalirin−7, BDNF	Synaptic plasticity, spine morphology, dendritic morphology
5-HT_{2C}	NMDA, LTP	Synaptic plasticity
5-HT_{3A}	PSA-NCAM, NMDA, LTD	Neuronal migration, synaptic plasticity
5-HT_{4}	ERK, LTP/LTD, BDNF, CREB, AKT	Spine morphology, synaptic plasticity, neurogenesis
5-HT_{6}	ARK, ERK, BDNF	Unknown
5-HT_{7}	MAPK, LTD, TrKB	Neurite length
SERT	BDNF	Spine density
MAO	NMDA, LTP	Neurogenesis

While the examples above serve to support the connection between a general dysfunction in 5-HT modulation and depression, the evidence that the cause of MDD is a particular deficiency of 5-HT is inconclusive at best (Jacobsen, 2012). Nevertheless, the 5-HT system appears to be an important factor in both the pathophysiology (as a risk factor) and treatment of MDD.

Attempts to explain the several weeks before SSRI efficacy begins have identified the serotonin autoreceptors (such as 5-HT1A) as possible culprits mediating the delayed therapeutic onset. This hypothesis identifies the downregulation of 5-HT autoreceptors, which eventually increases synaptic 5-HT, as the key mechanism of SSRI action. As shown in Figure 4, the several days to two weeks these autoreceptors take to desensitize would explain the delayed onset of SSRIs (Liu, 2017).

5-HT May Contribute to Dysregulation of the Stress Axis in Depression

Stress often precedes depressive episodes in humans (Meyer & Quenzer, 2019). Chronic stress induces alterations in dendritic spine densities of various regions, shrinkage of the PFC and hippocampus, and a decrease of hippocampal neurogenesis, all of which is seen to reverse with antidepressant treatment. Interest about the role of stress in depression has spurred research into the dysregulation of the stress activated hypothalamic-pituitary-adrenal (HPA) axis in depressed patients (Mahar, 2013).

Several important observations provide evidence that depression is frequently, although not always (Menke, 2019), associated with HPA dysregulation. Depressed patients often show elevated levels of the stress hormone cortisol, resulting from oversecretion of the stress hormone CRH (Meyer & Quenzer, 2019) along with disrupted negative-feedback mechanisms (Menke, 2019). A vicious cycle of dysregulated cortisol levels may exacerbate hippocampal atrophy, decrease neurogenesis (Krishnan, 2010), and disrupt circadian rhythms (Fig. 5). However clinical trials targeting HPA targets have been largely mixed at best (Menke, 2019).

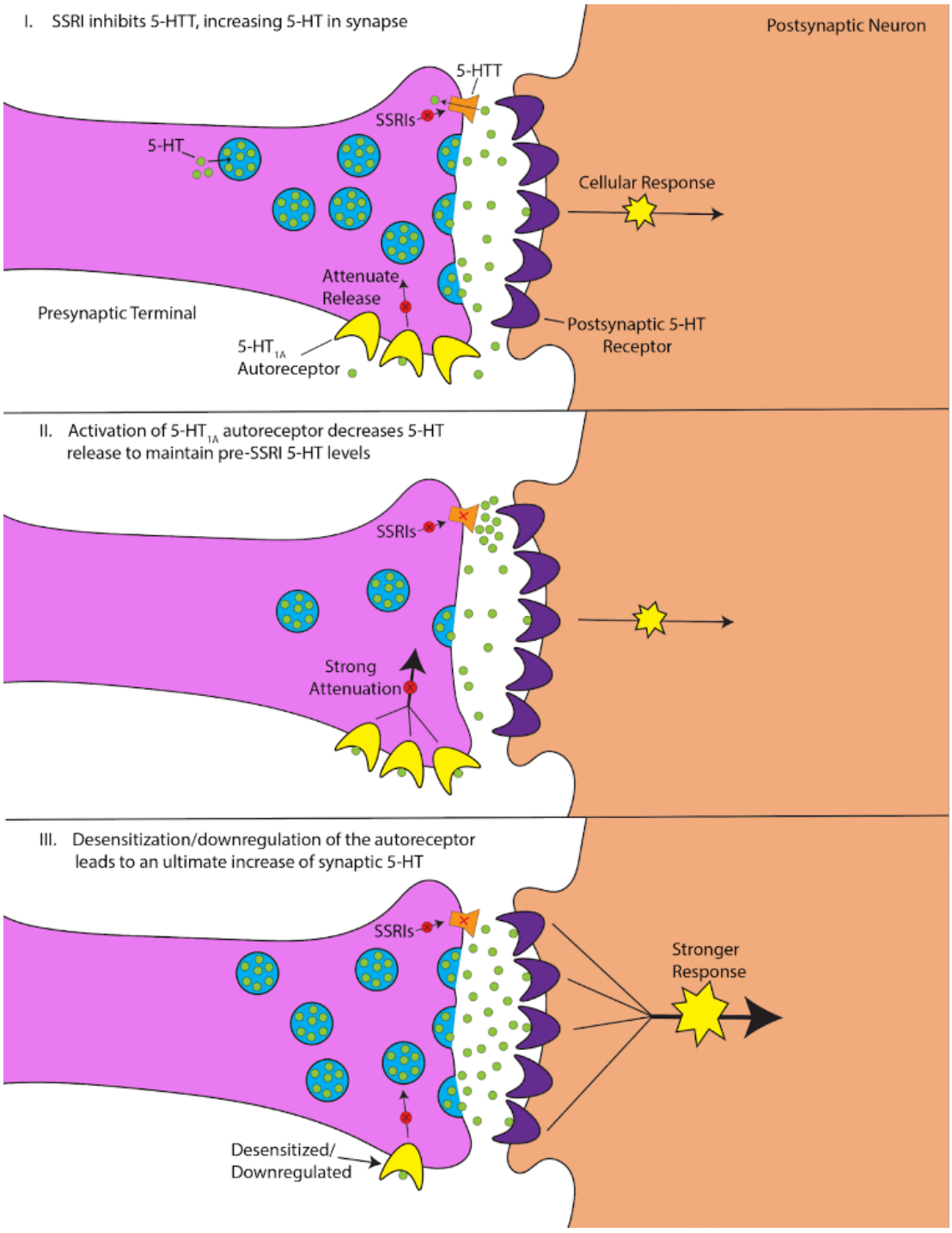


Figure 4: *How 5-HT1A autoreceptor downregulation is necessary for SSRI efficacy.* Serotonin enhancing antidepressants (SSRI, SNRI, TCA) block 5-HTT and initially lead to increased autoreceptor activation which maintains serotonin's extracellular tone. Only after these receptors become desensitized may serotonin release into the synaptic cleft increase above baseline, explaining the ~2 week delayed efficacy of SSRIs. The corresponding increase in postsynaptic serotonin receptor signaling mediates the antidepressant response. Adapted from Celada, 2013.

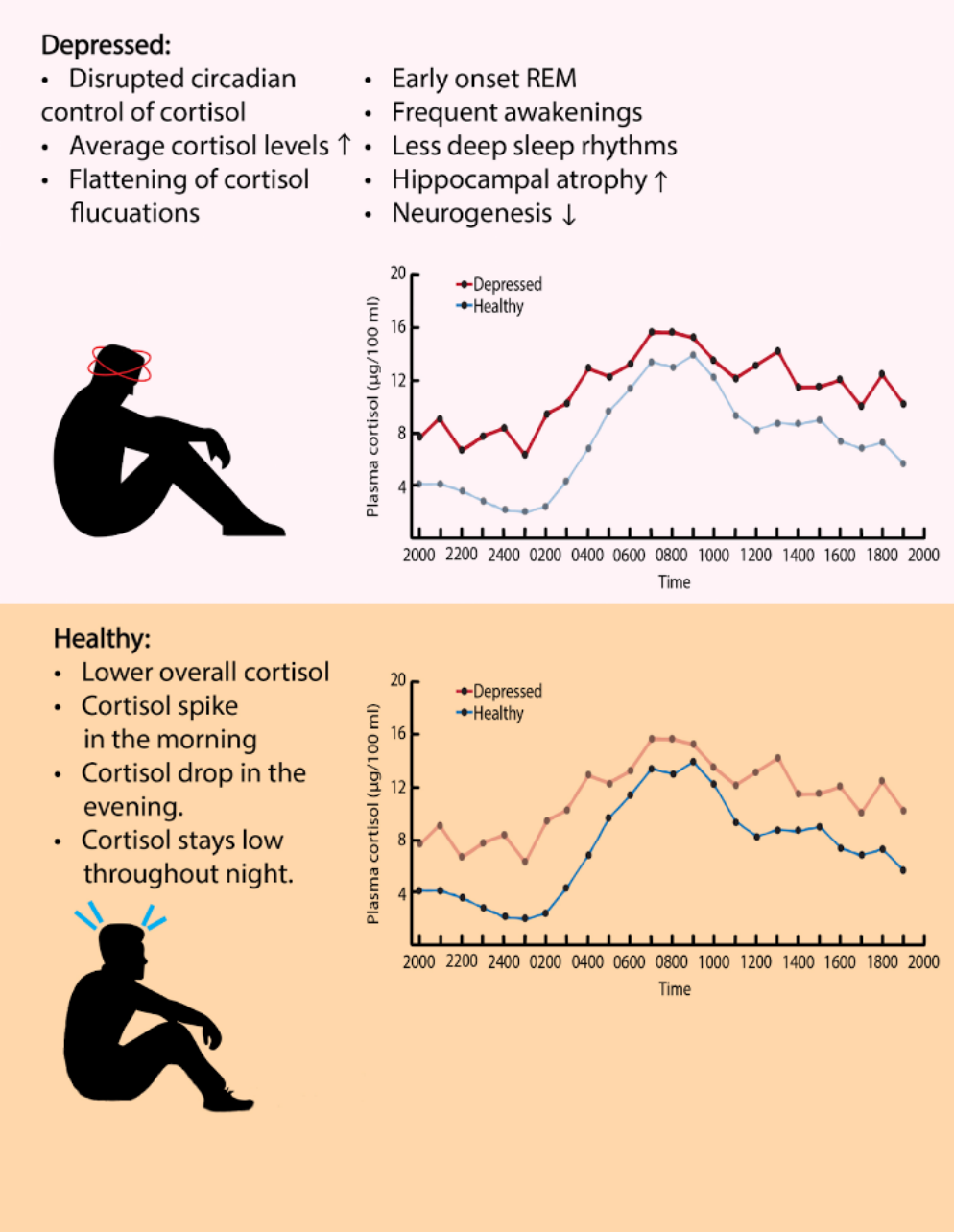


Figure 5: *How depression affects daily cortisol fluctuations.* Healthy individuals show a large decrease in cortisol level corresponding with sleeping hours followed by a large increase in waking hours. Depressed individuals by contrast show a general flattening and overall increase of cortisol levels throughout the day. Adapted from Meyer & Quenzer, 2019.

In a review by Mahar et al. (2013), the role of stress induced serotonergic dysfunction in causing depressive symptoms is discussed at length. 5-HT is observed to modulate the stress response. Under acute stress, extracellular levels of 5-HT in the mPFC are seen to increase with in-vivo microdialysis. This may be in part a result of increased glutamatergic drive from the mPFC onto the dorsal raphe, evidencing the role of the mPFC in interpreting and responding to acute stressors. After chronic unpredictable stress, studies have found decreases in global 5-HT in the brain, a reduction in spontaneous firing of the DR, and a downregulation 5-HT1A autoreceptor receptor function. As it has been hypothesized that 5-HT1A autoreceptor desensitization is necessary for the therapeutic action of SSRIs, it has been proposed that stress induced 5-HT1A downregulation is associated with an opposing behavior profile as downregulation due to SSRI treatment, with some amount of downregulation of this receptor still being necessary for antidepressant efficacy.

Finally, since 5-HT1A downregulation is observed in the mPFC, Mahar et al. (2013) hypothesized that disturbances of the mPFC-DR circuit would lead to impaired cognitive appraisal of stressful situations, and an increase in the negative cognitive distortions seen in depression. A summary of this is shown in Figure 6 below.

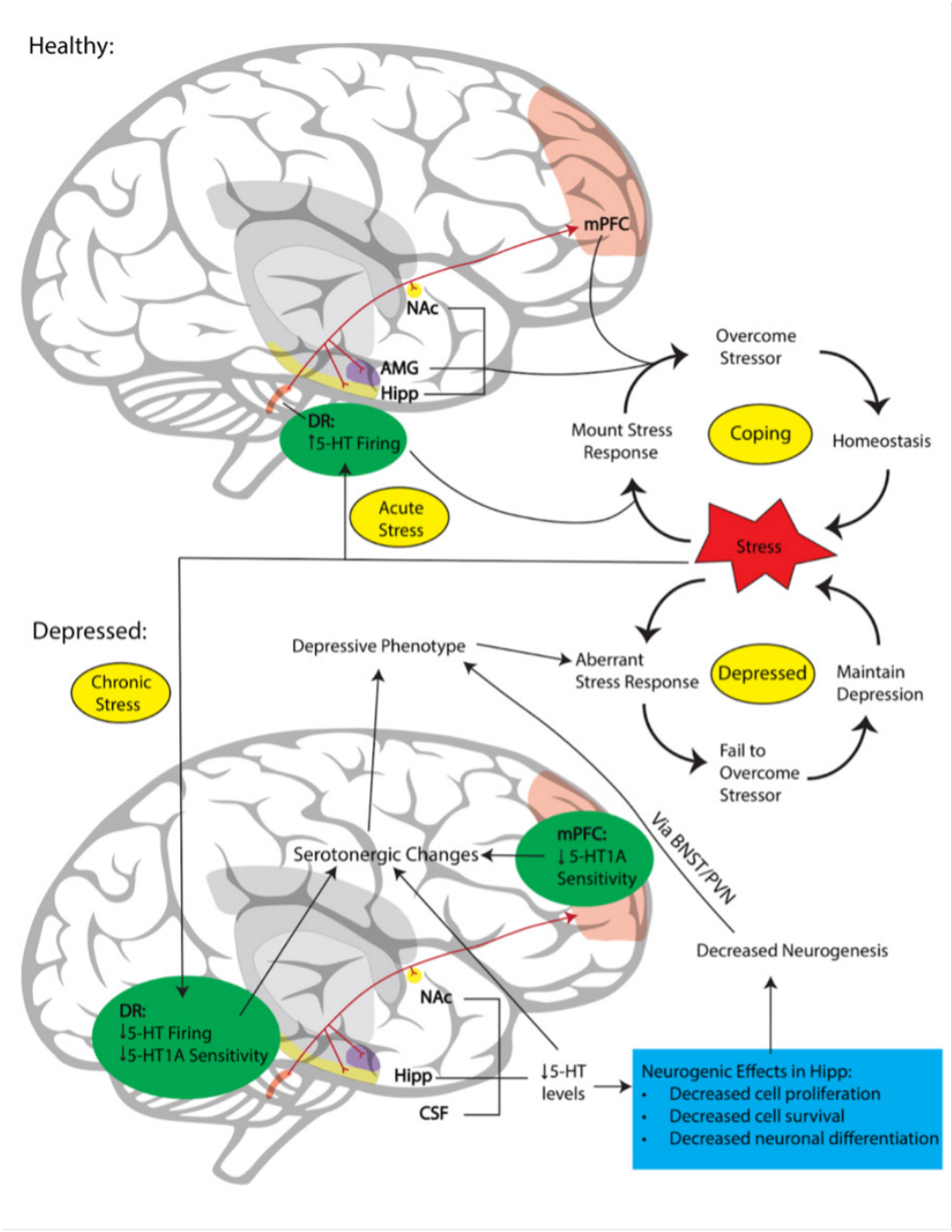


Figure 6: *A model of how chronic stress contributes to serotonergic and neurogenic dysfunction in the brain.* Acute stress increases DR firing onto the mPFC as a way to interpret and respond to stressors. Chronic stress causes changes in the serotonergic circuit, decreasing DR activity, while down regulating pre- and post- synaptic 5-HT1AR sensitivity. It also decreases hippocampal neurogenesis, which may further impair regulation of the HPA axis, as the glucocorticoid receptors regulating negative feedback inhibition of the HPA axis are located in the hippocampus. The resulting dysfunction of the HPA axis contributes to a vicious cycle, where aberrant stress responses maintain serotonergic dysfunctions in the depressed brain. Serotonergic antidepressant treatment modulates hippocampal neurogenesis, restoring HPA regulation there and alleviating depression. DR, Dorsal Raphe; BNST, bed nucleus of the stria terminalis; CSF, cerebrospinal fluid; Hipp, hippocampus; AMG, amygdala; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; PVN, paraventricular nucleus of the hypothalamus. Adapted from Mahar, 2013.

Expanding Beyond the 5-HT Deficiency Hypothesis

Although pharmacological evidence is the basis of the 5-HT deficiency hypothesis, it has also opened up alternative explanations. Both MAOIs and TCAs increase other monoamines in addition to 5-HT, suggesting NE and dopamine (DA) may also play an important role in depression. 5-HT activates excitatory 5-HT2A receptors on GABA neurons that dampen NE neuron activity, and inhibits DA activity in the VTA, potentially explaining the lack of therapeutic benefit of SSRIs in some patients (Blier, 2013), as a lack of dopaminergic signaling is believed to contribute to anhedonia (Grace, 2016). The role of other monoamines in depression is further evidenced by the fact that treatments involving SSRIs combined with drugs that reverse this dampening action (i.e. NE reuptake inhibitors or DA agonists) have led to effective augmentation strategies against MDD in patients resistant to SSRI treatment (Blier, 2013). The involvement of these other monoamine systems makes it hard to narrow down 5-HT as the primary cause of depression.

More of the evidence classically supporting the 5-HT theory of depression has been found to be inconclusive at best under scrutiny. The use of pharmacological evidence citing SSRI efficacy is questionable, as only 50% of patients respond to SSRIs, and effective remission only occurs less than 30% of the time (Albert, 2013). Although controversial, the pooled results of FDA clinical trials of SSRIs showed placebo was able to duplicate the antidepressant response of SSRIs by 80% and demonstrated that SSRIs exactly demonstrate superiority to placebo in severe depression only (Kirsch, 2008). Another review found long-term antidepressant therapy with drugs such as SSRIs to be generally ineffective (Burcusa, 2007). Furthermore, the delayed onset of SSRI efficacy despite its immediate increase of 5-HT in the synaptic cleft shows that simply raising 5-HT levels does not treat MDD (Liu, 2017). Additionally, while tryptophan depletion can cause a recurrence of symptoms in certain recovering MDD patients, such as those responding to the atypical antidepressant mirtazapine, it has been shown to have little to no effect in otherwise healthy controls (Blier, 2009). As a result of these contradictory findings, modern research efforts have sought to expand models of depression beyond 5-HT to better understand the disorder and improve treatment options (Lacasse, 2005).

The Contribution of Neuroplastic and Neurogenic Dysfunctions

A promising way to describe the pathophysiology of MDD is through neuroplasticity. Under this model, the pathology of depression is due to a disruption of the brain's neurocircuitry, rather than a neurotransmitter imbalance and an improvement of neuroplasticity in brain regions altered by MDD serves as the final common pathway of antidepressant efficacy. Antidepressants would therefore act through regulating release of postsynaptic glutamate, enhancing NMDA to AMPA receptor output, improving neuroplasticity through an LTP-like process, and improving hippocampal neurogenesis (Liu, 2017). Central to this hypothesis are alterations in neuronal spines seen in MDD. Spines, which are small protrusions on a dendrite's surface, greatly increase the surface area for synaptic transmission, and therefore enhance functional connectivity. They are highly plastic structures that are enlarged and stabilized when used, but eliminated when inactive (Duman, 2015).

Chronic, but not acute, administration of classical antidepressants that target monoamine systems has been shown to reverse these synaptic deficiencies. The novel antidepressants ketamine, scopolamine, and psilocybin demonstrated a rapid and robust restoration of AMPA mediated neuroplasticity in depressed patients (Duman, 2015). The discovery of the rapid antidepressant effects of ketamine has been crucial to the formulation of the neuroplasticity theory, which Deyama (2020) called "the biggest breakthrough for the treatment of depression in over 60 years."

Although the mechanism is being debated (Kohtala, 2021), ketamine is observed to increase glutamate release and increase AMPA signaling, thus potentiating LTP. One explanation is that inhibiting NMDA receptors on GABAergic interneurons leads to disinhibition of excitatory glutamatergic neurons (Vollenweider, 2010). A meta-analysis of ketamine as an antidepressant showed a single dose after 24 hours produced a response rate of 52.6%, with repeated ketamine infusions associated with an even higher response rate (70.8%) that lasted about 18 days (Liu, 2017), demonstrating drugs acting on the glutamate system exhibit far faster and somewhat stronger antidepressant effects than those acting on 5-HT, whose first line antidepressants likewise produce a remission rate of 60-70% when combined with cognitive behavioral therapy, with an average 2 week delay (Liu, 2017) before drug efficacy representing a danger in cases of suicidal ideation. A similar glutamate burst may also underlie the antidepressant effects of 5-HT2A stimulating hallucinogens such as psilocin or LSD, whose downstream signaling causes a robust increase in glutamatergic synaptic activity in the PFC (Vollenweider, 2010).

The neuroplasticity theory explains the efficacy of 5-HT enhancing drugs both through their indirect regulation of glutamate receptors and the slow, weaker role in neuroplasticity played by monoamines (Liu, 2017). Evidence has shown several serotonin receptors to be regulatory of LTP, hippocampal neurogenesis, and cytoskeletal rearrangement (Kraus, 2017). Additionally, 5-HT has demonstrated a link to levels of Brain Derived Neurotrophic Factor (BDNF), a member of a group of proteins called neurotrophins that signal for increased neurogenesis and plasticity. When coupled with the findings that chronic SSRI treatment restores plasticity in depressed patients and elevates BDNF levels (Kraus, 2017), it is plausible the efficacy of monoaminergic agents for MDD are reliant on these processes. These ideas are displayed in Figure 7 below.

Separate from spine density changes, hippocampal neurogenesis appears important in antidepressant functioning. It must be noted that the exact role of hippocampal neurogenesis in depression is not entirely clear. While evidence of impaired neurogenesis has been found in depression, inhibiting neurogenesis does not affect depressive or anxiety-like behavior in rodents, although it may underlie the cognitive deficits seen in depression. As the PFC and AMG are also key regions involved in depression, it has been proposed that decreased hippocampal neurogenesis is not necessary to trigger depressive behaviors (Kraus, 2017). On the other hand, evidence has shown that restoration of neurogenesis is necessary for antidepressant efficacy (Meyer & Quenzer, 2019), with chronic but not acute SSRI treatment coinciding with an upregulation of hippocampal neurogenesis (Kraus, 2017).

Under this model, all antidepressants function by upregulating glutamatergic signaling in affected brain regions, leading to an increase in neurotrophins, and ultimately restoring the altered neurocircuitry of the depressed brain. The action of BDNF is necessary, as deletion of the BDNF receptor TrkB in progenitor cells blocks both the neurogenic and antidepressant actions of exercise, fluoxetine, and the TCA imipramine (Deyama, 2020). Efforts to find a common chemical pathway underlying neuroplastic antidepressant function have discovered the crucial importance of the second messenger mammalian target of rapamycin (mTOR), which regulates synaptogenesis and BDNF (Ignácio, 2016). An example of this pathway enabling antidepressant therapy is shown below in Figure 8.

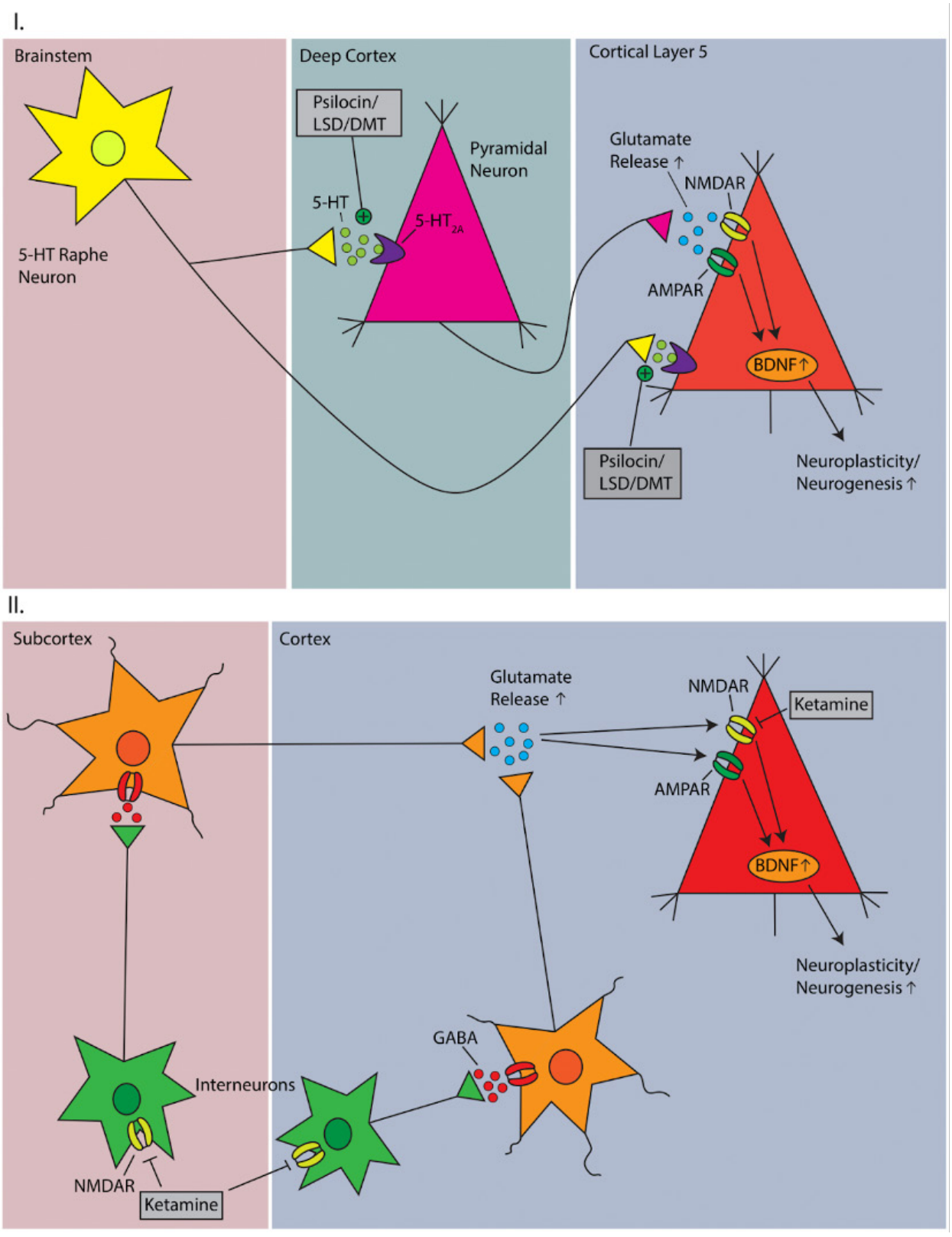


Figure 7: *How hallucinogens may cause a glutamate burst through serotonergic signaling.* I. Hallucinogens such as LSD, psilocin, or DMT, indirectly upregulate glutamate signaling in the PFC by stimulating postsynaptic 5-HT2ARs. The resulting activation of AMPA and NMDA receptors on cortical pyramidal neurons, along with direct 5-HT2AR stimulation, may lead to increased expression of BDNF, mediating neuroplasticity necessary to alleviate depression. II. Ketamine blockades NMDA receptors on GABAergic interneurons in cortical and subcortical regions, upregulating glutamatergic firing and increasing extracellular glutamate in the PFC. This activity increases AMPA signaling, enhancing NMDA throughput through LTP, and activating BDNF to mediate neuroplasticity. Adapted from Vollenweider, 2010.

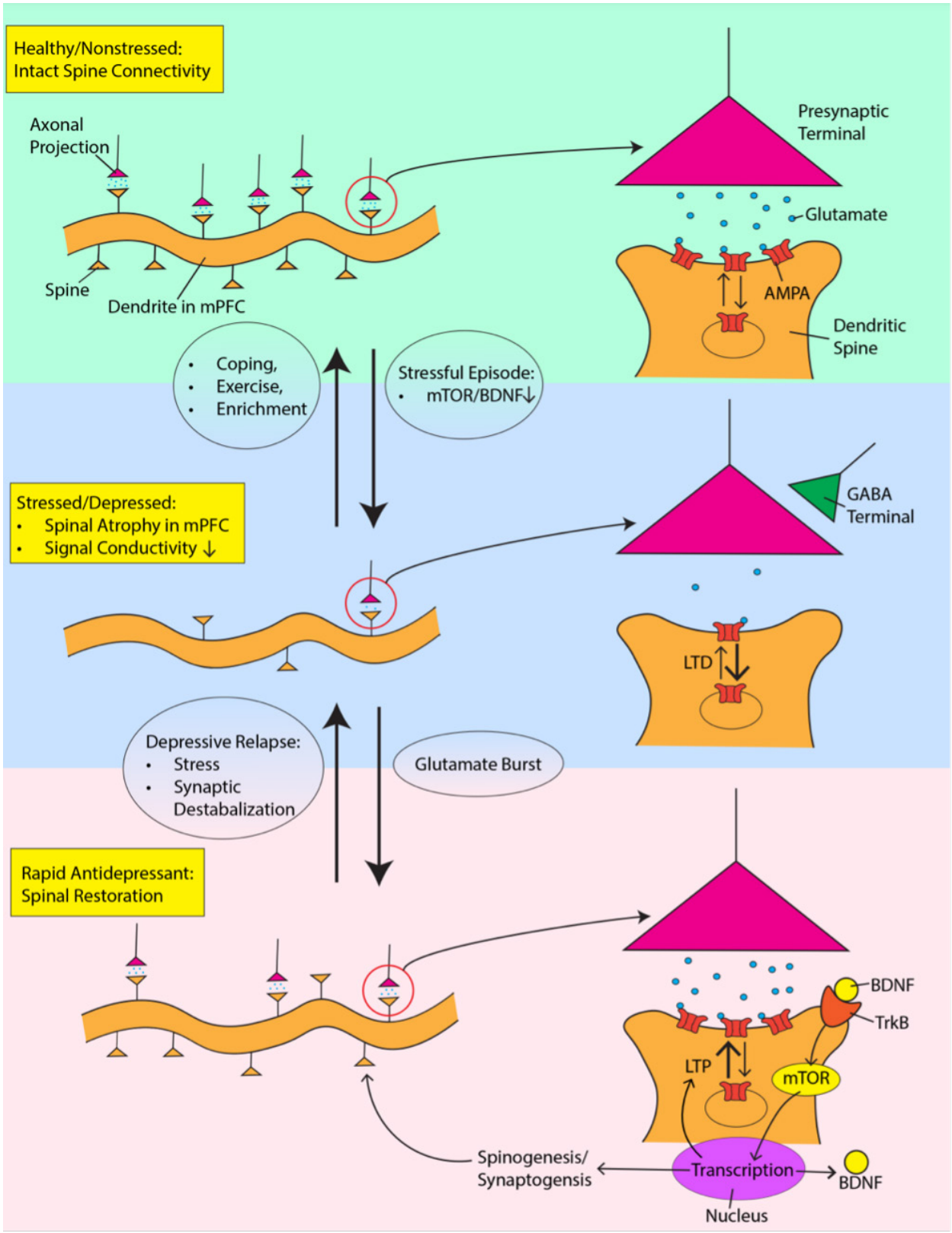


Figure 8: *How mTOR signaling mediates the effects of fast-acting antidepressants.* Under nonstressed conditions, spine synapse connections are normal and contribute to control over mood, emotion, and cognition. Chronic stress and depression decrease BDNF and downstream mTOR signaling, ultimately decreasing spine density in the mPFC. Ketamine reverses spine deficits via a glutamate burst. An increase in AMPA activity leads to release of BDNF and stimulation of mTOR, resulting in a restoration of spine levels. (Duman, 2015).

Discussion

The multitudinous studies that take issue with the 5-HT deficiency hypothesis of depression combined with its lack of solid evidence highlight the need for medical understanding to distance itself from the simple theory that low 5-HT is the primary cause for depression. Modern research concludes 5-HT still likely plays some kind of role in the pathophysiology of depression but looks for alternate explanations of what that role exactly is (Blier, 2013).

MDD is often characterized by its relationship to stress. In general, stressful episodes are frequent in the initial onset of depression (Meyer & Quenzer, 2019). The development of MDD after these episodes depends on an individual's resistance to stress and adversity. As shown by social defeat studies of mice, depression can be considered an aberrant form of an adaptive mechanism to avoid future stress and defeat, where influence of the stress axis realigns neurocircuitry to avoid pursuing reward after an association has been established with punishment. Healthy individuals have neuroprotective mechanisms in place to resist the onset of this pathology, but depressed individuals may become prone to a vicious cycle of stress dysregulation, realigning neurocircuitry to favor negative emotional associations. Stress is not always required for depressive episodes, in particular in cyclical recurrent depression. One proposed explanation is that stressful episodes remain important for initial depressive onset and early recurrent episodes, but that resultant biological changes in the brain cause stressful life events to matter less as a trigger, which could be due to an increased mood liability to once negligible stressors (Burcusa, 2007).

Vulnerability to stress induced depression is an outcome of both genetic and environmental factors. Environmental factors are responsible for establishing the epigenome during prenatal and early childhood development (Saavedra, 2021), explaining the significant association of childhood trauma and adult depression. Besides contributing to depressive risk, epigenetic mechanisms appear to be involved in the physiological onset of depression, as shown by histone methylation of the BDNF gene restricting its transcription after chronic social defeat stress (Meyer & Quenzer, 2019).

Monoamine systems appear to be involved heavily in the stress response. For example, acute stress transiently increases serotonergic drive from the dorsal raphe to the mPFC (Mahar, 2013), likely involved in evaluating stressors and planning the stress response. Serotonergic activation normally promotes patience for reward and inhibits impulsivity (Carhart-Harris, 2017), allowing individuals to maintain goal-directed psychological states necessary to overcome stress and adversity. Noradrenergic fibers from the LC upregulate NE signaling after acute stress (Leonard, 2001), helping the brain coordinate a widespread stress response. Finally, dopaminergic circuits projecting from the VTA in rats upregulate dopamine signaling onto the nucleus accumbens under acute stress (Grace, 2016), likely important for establishing motivation to face stress and challenge.

Effects of chronic stress on monoaminergic systems show how stress-induced disruptions of these symptoms helps maintain depressive pathophysiology. In the case of serotonin, postsynaptic 5-HT1A receptors, which are observed to be anxiolytic and may serve a role in resisting stress (Carhart-Harris, 2017), have been seen in studies to be downregulated as a result of chronic stress (Mahar, 2013). The 5-HT2A receptor, which has been shown to be anxiogenic (Vollenweider, 2010), is seen to be upregulated on the other hand (Jacobsen, 2012). Although an oversimplification, these changes may serve to promote increased anxiety in the response to stressors, translating into an inability to overcome stressful obstacles. LC pathways that innervate the entire brain, including relevant limbic and cortical regions, are disrupted in their noradrenergic signaling after chronic stress (Moret, 2011). Finally, chronic stress downregulates dopaminergic signaling, which

disrupts the ability to form reward associations and may be largely responsible for symptoms of amotivation and anhedonia (Grace, 2016).

Carhart-Harris's (2017) bipartite theory is incomplete, but may aptly characterize 5-HT's specific role in depression as both a risk factor and therapeutic target through separate mechanisms. Hesselgrave's (2021) study demonstrates that prior theories of how exactly serotonin modulates neuroplasticity, for example via 5-HT2A signaling, do not yet fully characterize its mechanism. Further research in how other serotonin receptors combine neuroplastic action and antidepressant potential is warranted.

Individual variation in how monoaminergic circuitry regulates the stress response describes how these symptoms can serve as risk factors for development of depression. Furthermore, individuals more resistant to depression likely have protective pathways that prevent maladaptive changes to monoamine systems after chronic stress, as shown by stress resilient mice being able to upregulate K+ channels in the VTA after social defeat to prevent hyperactivation of the nucleus accumbens, a likely contributor of depressive pathophysiology (Grace, 2016). When considering serotonin's role in patience for reward and impulsivity, I would like to propose that the maintenance of these pathways is crucial to maintain goal-directed behavior in the face of stress. The changes in serotonergic activity observed in MDD reduce this capacity by increasing anxious and impulsive behaviors, leading to a disinclination or even inability to face future stressors and contributing to feelings of low self-worth, amotivation, and reduced concentration consistent with depressive pathology.

Depression is presently best explained by changes in the functional connectivity of different brain regions. Dendritic spine hypotrophy and volume loss in the mPFC (Duman, 2015) may explain the loss of self-control, suicidal ideation, and negative ruminations seen in depression. Spine hypotrophy and reduced neurogenesis in the hippocampus meanwhile may contribute to cognitive deficits (Kraus, 2017) and a bias favoring negative labeling of external stimuli. Spine hypertrophy in the amygdala and nucleus accumbens may be responsible for negative reward associations (Grace, 2016) and fear (Duman, 2015) of normally rewarding stimuli. Finally, hypertrophy of spines in the orbitofrontal cortex may also contribute to reward-association disruption (Edmund, 2020).

The actions of neurotrophins such as BDNF appear necessary to mediate the gene transcription of neuroplastic factors in neurons (Deyama, 2020). In the case of ketamine, BDNF transcription has been connected to the actions of the mTOR pathway after a glutamate burst (Ignácio, 2016), raising the question of whether mTOR may be a common factor in all antidepressant efficacy, for example mediating similar glutamate bursts downstream of serotonin signaling (Vollenweider, 2010).

Under the lens of the neuroplasticity hypothesis, depression is better described as a dysfunction of the neurocircuitry of the brain, especially in areas associated with emotional regulation. Through the serotonergic innervation of limbic circuitry, especially the amygdala, hippocampus, and mPFC, 5-HT dysfunction may change how the brain appraises emotionally laden information. This viewpoint connects 5-HT to the negatively biased emotional responses seen in depressed patients and sees 5-HT not as directly improving mood. Instead, the antidepressant role of 5-HT is viewed as a secondary effect of positive shifts in emotional responses. The use of SSRIs may indirectly potentiate synaptic plasticity and help the brain relearn emotional associations. Over time, this would lead to a positive biasing of emotional experience (Cowen, 2015).

Branchi et al. (2013) substantiates this hypothesis with evidence that SSRI effectiveness in mice is largely dependent on the environment during ongoing antidepressant administration,

with fluoxetine actually potentiating depression in negative environments. Studies on the role of the environment in antidepressant efficacy in humans are few, but they have shown that living conditions modulate patient response to antidepressants. This may explain why low-income groups are less responsive than higher income groups. It therefore appears SSRIs prime the brain for increased plasticity to be shaped by environmental factors, whether positive or negative. Although the possibility of antidepressant-induced exacerbation of depression has not been directly tested in humans for ethical reasons, it would explain some findings demonstrating paradoxical worsening of mood disorders after antidepressant treatment (Fava, 2003).

The above findings emphasize the crucial role of the environment during antidepressive treatment, and underscore why combination treatment with psychotherapy and antidepressants are shown to be significantly more effective than either antidepressant or psychotherapy alone (Heim, 2008). A failure to properly control for environmental factors calls into question the meta-analysis that demonstrated antidepressants such as SSRIs struggle to outperform placebo (Kirsch, 2008), as one might expect depressed patients to be dealing with a higher degree of stress compared to healthy individuals.

It appears the biological culprits responsible for the onset of MDD differs between individuals. For example, individuals with adult depression as a result of childhood adversity likely develop it as a result of epigenetic alterations laid down during critical periods of childhood neural development, that are not present in cases of adult depression without prior childhood trauma (Heim, 2008). Similarly, thyroid disorders may lead to HPA hyperactivation, and have been associated with a higher risk of depression (Hage, 2012). Resultantly, whether the mechanism of depressive onset changes which treatment options are most effective is a question worth considering.

A recent study published in Nature (Taliaz, 2021). substantiated this hypothesis, demonstrating that antidepressant prescription done by an algorithm fed an assortment of patient demographic, clinical, and genetic data was able to predict a patient's response between different antidepressants with an accuracy of 70.1% compared to the 46.8% initial antidepressant response rate of the participants. This holds clinical relevance, as providing initial test screenings for genetic, demographic, and symptomatic markers of patients could lead to more effective initial response rates to antidepressants. This is especially important in patients suffering suicidal ideation. Moving away from always initially prescribing SSRIs, when superior treatments may exist, may help decrease suicide rates.

Conclusion and Future Directions

The discovery of the antidepressant efficacy of serotonin enhancing drugs was central to the development of the theory that low 5-HT was the primary factor in MDD. Over time, the theory began to receive scrutiny due to a growing body of counterevidence, coupled with a lack of solid evidence to directly tie low 5-HT to depression. Despite this, 5-HT still demonstrates a connection to depression, suggesting it may still play a role, even while not being a direct cause of MDD. In a more modern understanding, 5-HT abnormalities are considered as a potential risk factor for MDD. New theories about the pathophysiology of depression consider how 5-HT interacts with the complex systems of the brain, such as its involvement with other monoamine neurotransmitters. The 5-HT neurotransmitter itself has become less of the central focus as evidence instead connects its numerous receptors to the alleviation of depression with SSRIs. This can be seen as a side effect of increased neuroplasticity, rather than simply a result of a correction of 5-HT levels. In the future, studies into alternative treatments of depression are warranted,

stressed by the prevalence of misleading SSRI advertisement campaigns that cloud public perception about potential antidepressants (Lacasse, 2005). This is especially important in instances where an alternative treatment may be superior. While SSRIs are among the bestselling drugs, expanding beyond serotonin regulating treatments may help discover more successful treatments against MDD.

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China in Africa: Soft Power and the Development of Neocolonial States

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Abstract

Through investment, loans, political influence, and migration, China is utilizing an inventive form of neocolonialism on African nations that continues the cycle of underdevelopment. It is a colonialism that does not mirror the tactics and strategies of the Europeans before, but instead creates the façade of partnership and trust for the extraction of natural African wealth through soft power. This study is meant to examine how Sino-African investment is affecting African economies, resources, labor, environment, and diplomacy and argue that it fulfills the requirements of neocolonialism. The methodology for this study is mixed methods, including analytical, applied, and exploratory research. Using secondary sources, such as books, journals, and data sites, the research consists of collecting data and displaying it under different circumstances to support the argument and raise new questions. An example of this is with the section on labor, where the absence of labor rights is attractive or unattractive depending on the context or point of view of the scenario. Although this creates complexities in the research process, it also opens new inquiries and arguments. Through each section, the attributes of China's soft power through a continued interest in African resources will show its equivalence as a neocolonial force that keeps African nations dependent on Chinese investment.

Introduction

Africa is abundant with various resources that China seeks for its economic growth. To invest in those resources, China trades construction projects, through its advanced and heavily invested engineering, in the form of loans to build African infrastructure. This trade-off gives China its missing resources and Africa its basic infrastructure. The root of this exchange runs deep, and it not only heavily favors China's development as a world power but also contributes to the underdevelopment of the African nations accepting the loans, investments, and diplomatic conditions.

European colonization of Africa founded our understanding of underdevelopment. Through decades of brutal regime over African nations, colonialism destroyed their ability to economically profit from their land and globally invest. Walter Rodney, author of *How Europe Underdeveloped Africa*, defined underdevelopment as the product of imperial and colonial exploitation from the developed world. The wealth of colonized nations is extracted through the exploitation of colonized labor, with the profits proceeding only to the colonizer. Using the term underdeveloped over "developing" is an important distinction, according to Rodney (1972), as it indicates the exploitation of that community by another and breaks the conception that those nations are "escaping from a state of economic backwardness relative to the industrial nations of the world" (p. 14-15). This underdevelopment made it difficult for African nations to depend on their own economies to thrive and participate in the global markets.

Defining "Neocolonialism" and "Soft Power"

The difficulty of defining neocolonialism is not

with the word itself but with defining its root word: colonialism. Colonialism will be defined as "the projects and practices of control marshaled in interactions between societies linked in asymmetrical relations of power and the processes of social and cultural transformation resulting from those practices" (Dietler, 2010, p. 30). This broad definition allows for comparative analysis of the multitude of colonial strategies and practices (Dietler, 2010, p. 30). Neocolonialism is, literally, "new" colonialism. It is a form of colonialism that has persisted after the independence movements of newly sovereign nations. Kwame Nkrumah, the first Prime Minister and then first President of Ghana, was the first to coin the term neocolonialism. Nkrumah (1965) stated that "The essence of neo-colonialism is that the State which is subject to it is, in theory, independent and has all the outward trappings of international sovereignty. Its economic system and thus its political policy is directed from outside" (p. ix). This refers to an indirect control of a political entity over another, where one nation profits from the resources and services of another without direct force or command. Nkrumah is exposing how, despite the independence of once colonial nations, foreign capital continued to find a way to guide policy.

Soft power, especially when it comes to China, is the key factor in neocolonialism. It is "defined by a policy of attraction rather than coercion." There are rules that establish soft power strategies: they come from a feeling of value and respect, they are based on intangible assets, such as loans and investments, and they have variations depending on the situation (Kelly, 2017). Soft power as a tactic is essential because it differentiates from the colonial history of Western powers in Africa for a system that comes off as diplomatic. The Chinese Foreign Minister, when touring Kenya, stated that China "will not take the old path of Western colonists, and [it] absolutely will not sacrifice Africa's ecological environment and long-term interests" (Manero, 2022). What essentially appears to be diplomatic favors and resources in exchange for investment and infrastructure, soft power is a hidden, patient tactic for political and economic control over resource-rich nations.

Why is China Interested in Africa?

China is a rapidly growing nation. Their economy averaged an annual growth rate of 10% from 1990-2010 (Albert, 2017) and is currently growing after a drop from the pandemic at around 4% ("China: GDP Growth Rate", 2022). It is the world's largest energy consumer and producer, with a heavy reliance on coal and oil consumption to keep up with those needs ("China: GDP Growth Rate", 2022). Although China exports its own oil, the nation became a net importer in 1993 and recently surpassed the United States as the largest oil importer (Albert, 2017). China, with the goal of finding access to cheaper crude imports, turned to Africa for help. China receives 1.4 million barrels per day, and the nation receives imports from Angola, the Republic of the Congo, and South Sudan (Albert, 2017). Chinese interests extend beyond just oil; there is also substantial interest in using African resources to sustain its electronic industries. China sees Africa as a method of shifting the structure of its economy. It moves away from labor-intensive and pollutant industries, as labor costs continue to increase domestically, and relocates those industries to Africa (Hanauer & Morris, 2014). This is to combat the declining competitive advantage of China's hold on the manufacturing industry, as relocating these industries to regions with a cheaper labor cost would bring back China's competitive advantage. This is shown as "a 5% shift of Chinese export-related investments in the industry could translate into $5.4 billion in additional exports— a 233% increase" (Altenburg, 2019).

Africa is an abundantly rich continent full of natural resources that attract nations for trade. In 2019, the continent produced 1 billion tons of minerals totaling $406 billion, making up 5.5% of all the world's minerals extracted ("Mapping

Africa's", 2022). Africa is the center of 30% of all mineral reserves, 12% of all oil reserves, 8% of natural gas reserves, 40% of the world's gold, and up to 90% of its chromium and platinum ("Mapping Africa's", 2022). The continent is estimated to hold a third of the earth's minerals ("Mapping Africa's", 2022). Most of this is consolidated within five countries that hold two-thirds of the continent's mineral wealth: South Africa ($125bn), Nigeria ($53bn), Algeria ($39bn), Angola ($32bn), and Libya ($27bn) ("Mapping Africa's", 2022). Petroleum is the most abundant resource in 22 out of Africa's 54 countries, and it is close to the top in countries where it is not the first ("Mapping Africa's", 2022). Since 2019, Nigeria has produced most of the continent's petroleum, at 25%, followed by Angola at 17% and Algeria at 16% ("Mapping Africa's", 2022). The Democratic Republic of the Congo alone produced 63% of the world's cobalt in 2019, and with Rwanda, they produce half of the world's tantalum ("Mapping Africa's", 2022). Africa also produces about half of the world's stock of manganese, and significant amounts of coltan, which is used in electronics (Shepard, 2022). Africa is also predicted to house 85% of the global supply of platinum, 30% titanium, and 75% of phosphates (Mohan et al., 2014, p. 57). China wants control of these resources, and so it is heavily investing in these sectors.

China wants to raise its political influence and legitimacy. Strengthening relationships in Africa is believed to raise not just China's political influence, but also its trust as an international investor. An example of this is seen in the fact that most African governments would support Chinese endeavors, such as their "One China" policy that denounces the recognition of Taiwan and their investments (Hanauer & Morris, 2014). This political influence is a crucial step for China to initiate business with Africa. Many of the conditions for receiving Chinese investments are political favors, and this greatly leans in China's favor over the African nations.

Aid or Investment?

There is continuous confusion with the vernacular around Chinese "aid" to Africa. The reference to "aid" usually suggests an altruistic action or charity from one entity to another without the basis of an exchange for something in return. Examples of this are shipping medical supplies overseas for a nation that experiences a drastic hurricane, sending food and water to locations suffering from a drought, or sending volunteers to rebuild infrastructure. These actions described are at the expense of a country other than the one affected.

When discussing Chinese aid to Africa, the focus is on systemic aid: aid payments from governments to other governments (Moyo, 2010, p. 7). Within systemic aid from China to Africa, the categories are complete projects, goods and materials, technical cooperation, human resource development cooperation, medical assistance, emergency humanitarian aid, volunteer programs, and debt relief (Moyo, 2010, p. 7). The aid categories include issues of education, transportation, energy, communications, and health (Moyo, 2010, p. 7). Most of these aid categories are within financial aid, encompassing infrastructure, and China's own policy actively contributes to the confusion within its loans and aid. The CCP encourages its entities to "closely mix and combine foreign aid, direct investment, service contracts, labor cooperation, foreign trade and export" (Sun, 2016). This dishonestly combines attributes defined as aid with those that are loans to be repaid. A theory with that distinction is that the Chinese government "pays for the difference between the interest rates of concessional loans provided to Africa and comparable commercial loans" (Sun, 2016). This difference being paid would be quantified as Chinese aid, and China markets that capacity to its fullest, bringing about the idea that they bring aid to Africa.

Although Chinese FDI and loans in Africa are seen as "aiding" African nations, they should not be recognized as aid since what is happening

involves an exchange of goods and services. The distinctions of aid are constantly unsuccessful "to separate Chinese foreign direct investment (FDI) in Africa from its aid projects, commercial deals, and implementa¬tion of contracts for African governments and other organizations," which leads to these activities being intermingled (Shinn, 2016, p. 27). Although CCP analysts silently acknowledge the blur between aid and financing (Sun, 2016), there is yet to be a public distinction with the type of aid constantly implemented and stated by China. The category of systemic aid fills the gaps to acknowledge the finance as a form of "aiding" African nations while indicating that there is an exchange in return for the aid. Note that systemic aid is used synonymously with Chinese FDI and loans.

Chinese Investment

In the 1960s, Maoist China made an investment with Tanzania and Zambia. They would build a railway line from the port city of Dar es Salaam in Tanzania to Kapiri Mposhi in Zambia — the heart of the copper belt. The project took almost 50,000 Chinese managers and project leaders and 100,000 workers from Zambia and Tanzania, performed the majority of the hard labor (Poplack, 2016). For Tanzanian and Zambian revolutionaries, this railway was a crucial development as it allowed the nations to export their resources and reap the profits. This railroad was fully paid for by the Chinese with an interest-free loan to be paid back over 30 years (Poplack, 2016). This started the initial developments of a "win-win" relationship with China and African nations as it allowed China to push its way to have a seat at the UN (Poplack, 2016). On top of creating strong political allies, China was able to expand its rule to encompass new areas full of resources. Seeing the benefits of the copper mine, this deal was the beginning of a new era of Chinese interest in African affairs.

Investment from China into Africa has rapidly increased from $210 million in 2000 to $3.17 billion in 2011 (Sun, 2016). In 2018, that number rose to $5.4 billion (Fu, 2021). Between those years, China promised up to $67 billion in FDI, loan packages, and infrastructure spending (Kelly, 2017). This funding has gone into 50 of the 54 African countries, including the construction of entire cities in Angola, railroad systems across Tanzania, and highways across all nations (Kelly, 2017). There are five main sectors for Chinese investment: construction, mining, manufacturing, financial services, leasing, and business services (Fu, 2021). Construction and mining make up the bulk of that investment.

Infrastructure is China's main area for Chinese investment. Construction capital from China is categorized into two forms: concessional loans from Chinese policy banks and Chinese-owned contracts implementing the designs (Lee, 2017, p. 47). Concessional loans, the emphasis in this essay, are attractive to African nations. This is because they focus on construction rather than human capacity building, and the loans are prioritized by the African governments' interests in infrastructure, which skip criteria such as social or environmental benefits. The main, and most recognizable, reason for the attractiveness of Chinese concessional loans is that China and African nations have a shared definition and understanding of development. China recognized the urgency for infrastructure (Lee, 2017, p. 48) and this is likely due to China's own development through the building stages of China's economy after WWII.

Chinese loans and economic growth in Africa are linked in a positive relationship that helps both entities (Mlambo, 2022, p. 18). It is reliant, however, upon the African nations' ability to control the openness of their trade and their import levels. Boosting international trade is essential, and it is shown to be most efficient with raw material exports and manufactured material imports, which is exactly what China provides. FDI, however, is shown to require additional investment in human capital, such as skills and apprenticeships for jobs, for it to validate economic growth (Mlambo, 2022, p. 19).

Examples of investments include China giving out more than $60 billion in construction plans that have been used to build "stadiums, highways, airports, schools, hospitals and, in Angola, an entire city that still stands empty" in 2014 (Poplack, 2016). China was there from the beginning with Sudanese oil fields and invested early in its development (Shinn, 2016, p. 48). China invested $12 billion on the Coastal Railway in Nigeria, $4.5 billion for the Addis Ababa-Djibouti Railway, and $11 billion on a megaport in Bagamoyo (Shepard, 2022). China also invested in energy schemes, which included financing $6.7 billion in hydropower projects to assist with Africa's power crisis (Ofosu & Sarpong, 2022, p. 145). China also assisted Tanzania by funding a $1.2 billion gas field development project (Ofosu & Sarpong, 2022, p. 146). Through these investments, Chinese companies have begun to build a reputation for "good quality and timely work," which has increased their popularity (Ofosu & Sarpong, 2022, p. 146). China has established itself in Africa as a sophisticated business entity that markets its ambition for partnership with African nations. This partnership, however, comes with provisions.

Political Influence

Political favors are a common tactic utilized by China to maintain its share of resources and diplomatic achievements. There is a strong correlation between the amount of aid that China gives to a nation and that nation's support for China's foreign policy (Manero, 2022). The most prominent one is isolating Taiwan. It is the most consistent demand of China for any nation to which it grants loans or systemic aid. Chinese officials forced a deal with Zambia stating that for the nation to continue to receive investment for the copper mines, it must denounce its ties with Taiwan (Lee, 2017, p. 35). Kenya was forced into a similar situation in 2016, when the nation deported 50 Taiwanese nationals to China (Manero, 2022). Sao Tome and Principe broke off its relations with Taiwan to re-establish them with China (Manero, 2022). China has also enacted an approval rating scheme, where every 10% increase in United Nations voting support leads to increased aid averaging 86% (Manero, 2022). These schemes are very attractive to the underdeveloped nations of Africa, but in the end, they take away the autonomy of those nations from developing on their own and making proper diplomatic ties to benefit their citizens.

The alternative to these deals is with the EU: the same nations that colonized them decades prior. The EU announced in 2021 that they "pledged to mobilize up to $340 billion by 2027 to support a green and digital transition around the world" to combat China's investment strategies (Farand, 2021). It would include "€2.4 billion ($2.7bn) in grants for sub-Saharan Africa and €1.08bn ($1.2bn) for North Africa to support the roll out of renewable energy" (Farand, 2021). The problem with these alternatives is that the EU is not trusted across Africa when compared to China because they do not consult African nations. Ovigwe Eguegu, a Nigerian policy adviser, stated that the EU does not listen to African demands and that "You can't expect any enthusiasm because there is just ignorance of what these promises will mean and how they will translate on the ground" (Farand, 2021). In contrast to China, where they continuously hold meetings and consult with their African partners, partnerships with Europe feel rushed and inconsiderate of African issues (Farand, 2021).

The presence of Chinese activities has garnered overall strong support from African people. African officials "overwhelmingly view China's role in Africa positively, welcoming China's heavy emphasis on government-to-government contracts with few, if any, strings attached" (Hanauer & Morris, 2014) which indicates the strength of these relationships. Much of this comes from the contributions of Chinese infrastructure, which improves economic activity in these countries and sets up viable telecommunications and transportation networks that

benefit citizens. Statements made by African leaders indicate strong favoritism for partnership with China. Former Kenyan President Uhuru Kenyatta stated that he believes "it has huge potential for a win-win situation for all who are involved, and that is why we are very keen as a country, and I believe also as a continent, to partner strongly with China" (Mlambo, 2022, p. 9). Former Ugandan President Yoweri Museveni strongly defended Uganda's partnership with China, taking on loans, and he pushed back against critics who stated Uganda was afraid of opposing China Mlambo, 2022, p. 9). With strong support from African leaders, it is persuasive that Chinese investment is positive for the nation and its people.

Outside of political leaders, China's presence in Africa has generally good reviews from locals. There are generally favorable views of China in Africa: 90% of those living in the Ivory Coast and Mali, 81% from Senegal and Kenya, 75% of Ghanaians and Nigerians, and 78% of Tanzanians (Moyo, 2010, p. 109). With that, most Africans view China's presence as more beneficial than the United States' presence (Moyo, 2010, p. 109). When it came down to those who state Chinese business is bad, a survey done with the 25% of Nigerian and Ghanaian locals who stated unfavorable views of China in Africa showed that the reasons were 35% taking natural resources, 25% flooding markets, and 16% taking jobs (Mohan et al., 2014, p. 8). Overall, Africans have a positive view of Chinese influence.

Labor

Chinese enterprises are heavily criticized by labor unions for poor labor conditions, unsustainable environmental procedures, and job displacement from Chinese enterprises (Hanauer & Morris, 2014). Zambia had a significant incident of abusing African laborers in 2017 when over thirty Chinese nationals were arrested for illegal mining practices that included child labor and unlicensed smelting (Kelly, 2017). Within 48 hours of the arrest, all the miners were sent back to China, escaping any form of legal action from the Zambian government (Kelly, 2017). The Chinese government claimed that the evidence was not substantial, and they used their political power, such as their billion-dollar investments in the nation, to protect their citizens (Kelly, 2017). This is an early indicator of neocolonialist tendencies that mirror those of European colonization. There was no accountability system for the colonial supervisors who brutalized the colonized, and the imperial powers would not hold trials for the crimes done by their citizens. Problems within Zambia go back further to 2013, when the nation was forced to drop murder charges against two Chinese nationals (Kelly, 2017). The crime? Shooting 13 African miners. Under pressure from the Chinese government, Zambia was forced to let the men go (Kelly, 2017).

A similar situation occurred in Ghana. In 2013, 168 Chinese nationals were arrested for illegal gold mining (Kaiman & Hirsch, 2013). Their activities created various problems for water pollution through toxins such as pesticides, mercury, and cyanide (Kaiman & Hirsch, 2013). The arrests were made after numerous pit collapses killed dozens of Ghanaian miners (Kaiman & Hirsch, 2013). The cost of cleaning that water is expensive, and the Chinese are not investing in Ghanaian water foundations to remedy the issue. On top of that, China sent diplomats to the country to secure the release of the convicted Chinese nationalists (Kaiman & Hirsch, 2013). These are consistent activities that show China's dominance, which is taking over the justice systems of the African nations. There is no guarantee that those Chinese nationals were held accountable for their actions.

Regarding job displacement, the reality is not as negative. Around 89% of workers in these Chinese companies were African, with about two-thirds of those companies providing skill or apprenticeship training (Jayaram et al., 2020). There is a substantial emphasis on opening jobs and opportunities for African workers as a part

of China's plan. This contributes to a common theme in Sino-African trade: migration. The CCP had set up websites, one of which was called "Go to Africa," to help interested citizens find the resources they needed to travel abroad. The establishment of banks has also encouraged Chinese citizens, as it would help transfer funds (Mohan et al., 2014, p. 46). When it comes to Chinese corporations, the hierarchy of labor is skewed favorably toward the Chinese. Most managers and people in high-ranking positions at job sites are Chinese (Ofosu & Sarpong, 2022, p. 149). This is shown across Angola, Tanzania, Zambia, Rwanda, etc., and it can be concluded that most of these companies did not employ local African workers to fill high-ranking positions (Ofosu & Sarpong, 2022, p. 149). It is likely that more than 80% of the workers will be locals, but the highly skilled or upper-management positions will be occupied by Chinese nationals (Ofosu & Sarpong, 2022, p. 150). This indicates an issue: Chinese officials actively favor Chinese nationals over Africans to fill higher ranking positions. This takes away the autonomy of the local workers, as they are not able to bring back higher wages and skills to their communities. Instead, that money goes to the Chinese nationals, and therefore, the profits are more likely to recirculate back to China through the banking system.

Environmental Impacts

China has been repeatedly criticized for environmental degradation through its investment in Africa. The main issue has to do with China's relocation of its most polluting industries. China has been making strides to decrease its output of pollution within its national boundaries, but that has also come at the cost of moving those high-polluting industries overseas. Africa is one of those places, and China has moved many of its steel, glass, leather, and cement industries to African nations for a cleaner environmental footprint and cheaper labor (Shinn, 2016, p. 40). This reduces all the pollutants in China while also meeting the demands of African nations to invest in job-creating industries (Shinn, 2016, p. 40).

An example of this is Hebei Iron and Steel, which is China's largest steel producer. Originally in the Hebei Province, the company built a plant that could make five million tons of steel every year in South Africa (Shinn, 2016, p. 40). This will continue to increase in the future, with hopes to encapsulate offshore productions of steel and cement into Africa by 2023 (Shinn, 2016, p. 41). With these projects being expanded, the amount of pollution will continue to drastically increase.

Alongside these natural pollutants from certain industries, China has utilized poor waste cleaning methods. Somalian residents near the Jeronimo Group Industries & Trading PLC, a glove-making firm, complained that "the company has been dumping waste into the river, causing harm to the livestock industry" (Shinn, 2016, p. 41). Instead of acting against it, the Somalian government refused to interfere as they felt it would discourage additional foreign investments (Shinn, 2016, p. 41).

As previously stated, Chinese loans are attractive as they do not contain criteria for proper environmental practices or labor rights, which create costs. It seems that those requirements should have been taken into consideration or even handled by the governments of African nations themselves to ensure those regulations are put in place. Ethiopia is an example of this, as in 1997, their government "enacted a wide range of legal, policy, and institutional frameworks regarding the environment, water, forests, climate change, and biodiversity," which takes environmentalism more seriously than most other African nations (Shinn, 2016, p. 44). China actively adapts its guidelines to improve upon the criticisms of its negative environmental effects. These improvements are slow, and they recognize a lack of care for the well-being of African laborers.

Neo-Colonialism through Soft Power

China has consistently argued against claims about its neocolonial structure in Africa. In 1996, the Chinese Ministry of Foreign Affairs stated five public foundations for Sino-African trade: "To foster a sincere friendship between the two sides and become each other's reliable 'all-weather friends,' To treat each other as equals and respect each other's sovereignty and refrain from interfering in each other's internal affairs, To seek common development on the basis of mutual benefit, To enhance consultation and cooperation in international affairs, To look into the future and create a more splendid world" (Kelly, 2017). These statements hide the reality of China's practices and goals of exploiting the African continent for cheap labor, resources, political influence, and settler expansionism.

The soft power concessional loan system brings the strongest criticism for Chinese neocolonialism. Considered "predatory loans," they seem attractive for economic and infrastructural development, but these loans do not guarantee timely investment (Mlambo, 2022, p. 12). As loans pile up when nations continue to accept them, these nations owe China billions of dollars through the extraction of their natural resources. This lack of transparency when it comes to debt points in the favor of the Chinese economy. Alongside transparency, China has been criticized for the creation of vanity projects. These are projects that do not reinvest profit back into the African nations: airports with few passengers, bridges that lead nowhere (Mlambo, 2022, p. 11), or empty cities that are left deserted (Poplack, 2016).

Angola, for example, received a loan of $2 billion in infrastructure, which included the construction of the city of Nova Cidade de Kilamba, in exchange for the nation's oil (Kelly, 2017). As oil prices drop, Angola's debt has skyrocketed to $25 billion, and they have fallen behind on paying back their loan (Kelly, 2017). Because of this, China gained more control over Angola's petroleum reserves. Chinese imports comprise 35% of Angola's economy, so Angola has the choice of either giving up control of its mines or abandoning its deals with China altogether. If the first option is chosen, the nation is at risk of China refusing to purchase Angolan imports, which would sink Angola's economy. If the second option is chosen, Angola is stuck in a continuing debt situation. This suggests Angola as a neo-colony (Kelly, 2017); it's economically dependent on an outside nation.

These are the workings of debt-traps where China is "saddling Africa with unsustainable debt and seeks to use indebtedness to further its geopolitical control over the continent" (Mlambo, 2022, p. 13). Since China is the nation providing the loans, and these countries have built up a system where they depend on exports to China, China can have a strong influence on diplomatic and economic concessions outside of the loan and investment agreements (Mlambo, 2022, p. 13). The term "debt-trap" was coined by Brahma Chellaney (2017), who showed that China exchanges by "requiring countries to award it contracts for additional projects, thereby making their debt crises interminable". To get out of these debt-traps, nations are told to hand over their project leadership or management to Chinese state-owned firms, sell majority ownership of investments, or even sign new contracts for additional projects that accumulate the debt (Chellaney, 2017).

Although there is criticism of the idea of Chinese debt-traps, these studies are misguided. China consistently calls the debt-traps a tactic of the US to discredit China's investments abroad (Bartlett, 2022) and that it is "motivated more by frantic Sinophobia than any genuine concern for the economic and fiscal health of African countries" (Singh, 2020, p. 242). In August 2022, China announced that 23 interest-free loans across 17 African countries had been forgiven (Savage et al., 2022). Specifically, it was a cancellation of the outstanding balance from the loans (Bartlett, 2022). With the interest-

free loans, from 2000-2019, China canceled at least $3.4 billion of such debt in Africa and also restructured or refinanced $15 billion (Savage et al., 2022). However, as pointed out by Reuters, the interest-free loans that China has forgiven make up only a small portion of China's lending, which has been called "low hanging fruit" (Savage et al., 2022). These debt cancellations do not apply to interest-bearing commercial loans (Bartlett, 2022) which make up the majority of lending and debt in African nations (Savage et al., 2022).

Another argument against the debt-trap idea comes from author Ajit Singh. He states that debt-trap diplomacy is not accurate in its depiction of China's overall international lending practices (Singh, 2020, p. 241). With examples in Africa, Singh states that "the vast majority, over two-thirds, of the external debt owed by African governments is to multilateral institutions such as the World Bank and non-Chinese private creditor" (Singh, 2020, p. 242). While this indicates that most of the debt in African nations is not with China, this statistic is independent of China setting up debt-traps. Having the World Bank and non-Chinese private creditors make up most of the debt is separate from China's debt traps and should be seen as two separate issues. The argument that Singh presents makes a stronger case to explain why African nations decide to trade with China rather than the nonexistence of a Chinese debt-trap in Africa.

Conclusion

An ongoing trend with Sino-African investment is the absence of investments in budget support, health, or education. Aside from investing in human capital skills, China has not invested in any sort of economic area for the health of African people. This lack of investment, as well as consistent labor rights abuses, indicates the façade of partnership between the nations. With China's marketing of its supposed "aid" in Africa, it would be normal to expect investments in health and education. For an underdeveloped country, those are the main investments to make for social mobility. Tied to the vanity projects, it is evident that China's loans lack transparency to allow African governments to decide where their funding is headed.

The cities, railroads, mines, airports, high-rises, highways, and everything else built under the concessional loans from China do not belong to the nations of Africa. They belong to China. Until those debts are entirely paid off, the concessional loan system will continue to pile on as debts climb. China can then formulate new agreements that sway heavily in their favor. This is neocolonialism through soft power: a nation's attraction to intangible loans and investments that then consolidate a dependent relationship to an authoritative power that regulates diplomacy, migrates its citizens, and receives raw exports from the said nation. African nations must recognize this theory of underdevelopment and take a stronger stance toward self-sustaining their own economies without having to rely on one predatory and colonial entity.

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The Influence of Virtual Out-of-Body Experiences on Fear of Death

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Abstract

Virtual reality (VR) has a demonstrated capacity to embody participants in a virtual avatar and induce body ownership illusions. Previous research has leveraged avatar embodiment to create virtual out-of-body experiences (OBEs) for participants. These induced experiences have subsequently been linked to influencing participants' fear of death (FOD). Because perceptions of mortality have important clinical implications for palliative and hospice care, there has been growing research interest in the efficacy of utilizing VR technologies to influence FOD. This exploratory study extends this line of research by examining the impact of VR OBEs on FOD in a between-groups experiment with three conditions: (1) a control condition where participants remained in control of the avatar body; (2) an out-of-body (OBE) experimental condition in which participants drifted out of the avatar body and lost visuotactile contact with their avatar; and (3) a "drifting body" (DBE) experimental condition in which participants drifted out of the avatar body but retained visuotactile contact with their avatar. Preliminary data analysis revealed non-significant reductions in FOD in the OBE and DBE experimental conditions. Furthermore, qualitative measures indicated that participants were more disturbed by the OBE condition than by the DBE condition. We provide a discussion of these results, as well as study limitations and future continuation of research under ideal conditions.

Chapter 1: Introduction

1.1 Out-of-Body Experiences

Out-of-body experiences (OBEs) are one of the key phenomenological characteristics of near-death experiences (NDEs), or altered states of consciousness that typically occur during clinical death (Parnia, Waller, Yeates, & Fenwick, 2001). Survivors of NDEs have described OBEs as "experience[s] in which a person seems to be awake and see his body and the world from a location outside the physical body" (Blanke, Landis, Spinelli, & Seeck, 2004). Because patients experiencing OBEs have also reported reduced fear of death (FOD) and enhanced belief in life after death, research has focused on investigating the correlation between OBEs and perceptions of mortality (Van Lommel, Van Wees, Meyers, & Elfferich, 2001).

The relationship between OBEs and the idea of survival after death has various accounts in literature, with previous reports linking naturally occurring OBEs with enhanced belief in life after death (Metzinger, 2005). Those undergoing an OBE retain sensory perception, which illustrates the possibility of the existence of consciousness outside a physical body. Because of this implicit evidence that survival beyond the body is possible, research has linked OBEs to a reduction in FOD (Tassell-Matamua & Lindsay, 2016). Recent advances in virtual reality have now raised the possibility of virtually replicating OBEs in a controlled environment. Therefore, this study induced OBEs in a research setting to examine whether an experience in which the center of perception was located outside a participant's body might influence a participant's FOD.

1.2 Virtual Reality

The recent proliferation of immersive virtual reality (VR) tools has presented the opportunity to study OBEs within a controlled environment.

VR creates a perceptual illusion of "presence" in a virtual environment by utilizing a head-tracked stereo head-mounted display, real-time motion capture, and multisensory stimulation. A study by Bourdin, Barberia, Oliva, and Slater (2017) successfully simulated OBEs within virtual environments, and found that the experimental group (i.e., participants experiencing OBEs) had lower FOD than of the control group. Similarly, a 2018 exploratory study in Cornell University's Virtual Embodiment Lab utilized consumer VR technology to examine the implications of exploiting virtual embodiment to improve end-of-life clinical care; interestingly, the study linked greater feelings of embodiment over the virtual body with greater FOD in an OBE condition (Chan, Hwang, Sun, Birckhead, & Won, 2020). The contradictory findings and non-uniform methodology across these studies were particular points of emphasis when preparing the study procedure (1.4 *Research Overview*).

Utilizing the methodology from Bourdin et al. (2017), a perceptual illusion of ownership over the virtual body will be induced via visuotactile and visuomotor synchrony. These techniques have been well documented to reproduce body ownership illusion within VR (Kilteni, Maselli, Kording, & Slater, 2015). Visuomotor synchrony is induced as real-time motion capture from the Oculus Quest virtual reality headset allows the virtual body to move synchronously with the real body. Visuotactile stimulation is a technique borrowed from the rubber-hand illusion in which participants see a virtual brush stroking their virtual hand as the researcher synchronously brushes the participant's hand (Botvinick & Cohen, 1998). Past studies have confirmed that participant self-location in VR is systematically biased to where a visual–tactile event is seen (Lenggenhager, Mouthon, & Blanke, 2009).

1.3 Clinical VR Therapy

There is increasing interest in incorporating VR within clinical settings. Early clinical VR studies leveraged the technology in exposure therapy (Rothbaum, Hodges, Watson, Kessler, & Opdyke, 1996) where VR helped patients gain a sense of control by allowing them to feel like they were present in simulated fear-provoking environments. Since then, VR has also been used in other various clinical contexts, such as treatment for posttraumatic stress disorder, schizophrenia, and complex regional pain syndrome (Cukor, Spitalnick, Difede, Rizzo, & Rothbaum, 2009; Rus-Calafell, Garety, Sason, Craig, & Valmaggia, 2018). The potential of VR for therapy has also led several medical centers to develop VR therapy teams (Delshad, Almario, Fuller, Luong, & Spiegel, 2018).

Simulating OBEs within VR holds an important clinical implication for palliative and hospice care. In addition to mitigating physical symptoms, palliative and hospice care also encompass a psychosocial and spiritual component where clinicians help patients achieve a sense of meaning and control while preparing for death (Rome, Luminais, Bourgeois, & Blais, 2011). During these discussions, patients typically undergo a therapeutic life review involving the meaning of life, resolving lingering conflicts, legacies, as well as perceptions of future life (Keall, Clayton, & Butow, 2015).

Within these contexts, VR OBEs offer an end-of-life intervention that may influence patient perception of mortality and death. Because experiencing OBEs has been linked to a reduced FOD and enhanced belief in life after death, VR provides a mechanism to artificially create these OBE experiences for patients (Van Lommel et al., 2001). Additionally, current therapeutic life review interventions are not widely practiced, partially because of their time-consuming nature; VR OBEs may aid in streamlining the standard discussions in palliative and hospice care.

Despite recent advances in leveraging VR treatment as a non-pharmacological therapy, there is still limited research exploring the clinical capabilities of this technology. A major hindrance to the advancement of clinical VR has

been the commercially available technology; a majority of the randomized, controlled studies described in literature have utilized computer-tethered VR headsets, such as the Oculus Rift and HTC Vive, or even more immersive systems that involve full-body tracking (Bourdin et al., 2017; Dascal et al., 2017). While studies showed promising results, these expensive, high-end VR systems will be difficult to utilize in health care settings widely. Ultimately, the progression of VR into widespread clinical viability will be dependent upon advancement in both VR scientific literature and availability of consumer technology.

1.4 Research Overview

Bourdin et al. (2017) demonstrated a viable methodology of replicating OBEs within a VR environment. The study utilized two study conditions: (1) an out-of-body (OBE) condition, in which participants drifted out of the avatar body and lost visuotactile contact with their avatar, and (2) a "drifting body" condition (DBE), in which participants drifted out of the avatar body but *retained* visuotactile contact with their avatar. Results found that the experimental OBE group had lowered FOD compared to the control DBE group. Notably, whereas the Bourdin et al. (2017) study design intended for the DBE condition to serve as the control for the study, participants assigned to the DBE condition arguably still underwent an out-of-body experience.

With this in mind, Chan et al. (2020) replicated Bourdin et al. (2017) with two study conditions: (1) an OBE condition, with a similar implementation to the Bourdin et al. (2017) OBE condition, and (2) a control condition, in which participants remained in the avatar body and retained visuotactile contact with their avatar. Additionally, Chan et al. (2020) sought to leverage consumer VR technology; the study's VR simulation was developed with the Oculus Go — a standalone headset that sacrifices graphical resolution and processing power for a portable, lighter, and non-tethered VR experience. This study ultimately revealed an indirect effect of perceived virtual embodiment *increasing* FOD through a heightened degree of reported OBE in the experimental condition.

This study extends the line of research examining the impact of VR OBEs on FOD. Our contributions are as follows:

- We examine the pairwise comparison of each of the conditions mentioned above (OBE, DBE, and control).
- We report the results of a qualitative interview with participants.
- Building off Chan et al. (2020), we assess the clinical viability of the Oculus Quest — a successor to the Oculus Go with superior performance hardware, no requirement for thether to a PC, inside-out tracking, 6 degrees of freedom movement, and hand tracking.

Chapter 2: Methods

2.1 Participants

A total of thirteen participants (six men, seven women) were recruited from a large northeastern university and received cash or course credit points for participating. The original study was set up as a single factor between-groups design with a minimum of 16 participants in each of the three study groups, in other words, a minimum of 48 total participants. However, because of university closure due to the global COVID-19 pandemic, only a total of thirteen participants were successfully run. Participants' ages ranged from 19-23 years (mean = 20.38), including one American Indian/Alaskan Native, 4 Asian Americans, 3 Asians/Pacific Islanders, and 6 European/European Americans. All participants consented, and the IRB approved all procedures.

2.2 Virtual Environment

The virtual environment was designed via Unity3D and delivered in an Oculus Quest head-mounted display (HMD). The virtual bodies used were a male and a female character. To ensure participants feel connected to their

avatars, the characters were programmed to customize skin tones from a scale of 1 (*very light*) to 10 (*very dark*).

The built-in sensors in the Oculus Quest HMD and Touch controllers tracked movement data in order for the virtual avatar to mirror the participant's real life head and hand movements (Appendix A.1). Additionally, an inverse kinematics script was applied in order to determine the joint parameters that provide the desired positions of the avatar's other limbs.

2.3 Procedure

Participants were first asked to complete an online questionnaire a few days prior to their arrival at the lab, at which time they also provided informed consent. This pre-study survey measured participants' baseline FOD and demographic data.

During the experiment day, participants came to the lab for the experimental session in VR. Upon arrival, they were instructed about the experimental procedures and again provided consent. Afterward, they were randomly assigned to one of the three conditions: control, OBE, or DBE. Participants were assisted in putting on the VR headset and shown how to hold the hand controller. A researcher stood next to the participant to give instructions for the duration of the experiment.

Drawing from Bourdin et al. (2017), the virtual reality portion of this study was divided into two phases.

In the first phase, all participants experienced the control condition consisting of an in-the-body phase. The overall strategy was to induce the participant's initial embodiment of the avatar. Each experimental session began with the researcher describing the virtual environment to help the participant become familiarized with it. Participants saw a reflection of their avatar body in a virtual mirror seated on a comfortable chair in a virtual room. Because participants were seated, they observed their avatar in approximately the same posture and position as their real body. Participants were also asked to look around and test their wrist movements in order to observe the synchronous visuomotor correlation between real and virtual body movements (Figure 2.1).

Figure 2.1: Control condition. Participant is seated on a chair and view a reflection of their avatar body in a virtual mirror.

To further induce avatar embodiment, participants were asked to use their virtual hand to stroke a cloth placed in front of their avatar in the virtual environment. Simultaneously, the researcher placed a piece of cloth in front of the participant's hand so that visuotactile synchrony could be achieved. Participants felt the cloth touching their hand as they moved their hand, and they could also see these movements represented by their avatar. The virtual model of the cloth was animated so that it moved when contacted by the hand of the avatar. Participants were asked to stroke the cloth for a minute in order to achieve avatar embodiment.

To evaluate if we successfully embodied participants in their avatars, we utilized methodology adapted from Lenggenhager et al. (2009), where a mental ball-dropping exercise was shown reflect the participant' vestibular system and time perception during OBEs. In this exercise, participants were asked to imagine how long it would take for an imaginary ball dropped from their hand to reach the floor. Participants were asked to hold a stopwatch (Figure A.2 in Appendix A) and click a button on the stopwatch, first when releasing the imaginary ball and second when they estimated that the ball would reach the floor. All participants were asked to complete this task five times at the start of the in-the-body phase. These values were recorded, with the average drop times reported as *Drop1* in the results section.

After completing the mental ball dropping exercise, participants entered the second phase of the experiment. This phase lasted for two minutes. During the first minute, participants once again experienced the in-the-body phase. During the second minute, VR experiences differed depending on the assigned condition.

Participants in the control condition remained in the first-person perspective of the avatar. They continued to stroke the cloth and see their avatar hands move in accordance with their movements for another minute.

Participants in the OBE condition had their perspective lifted out of their virtual body to the ceiling room; participants also lost control over their avatars, with their physical movements no longer mapping to their avatars virtual movements. At the same time, the researcher moved the cloth away from the participants' hand to end visuotactile synchrony. Even if participants continued to move their wrists, they could no longer see their avatar's wrist movements nor feel the touch from the cloth. The ultimate final result was that participants viewed their avatar from above and behind the avatar's unmoving body (Figure 2.2).

In the DBE condition, the viewpoints of the participants were also lifted out of their virtual body to the ceiling room. Participants viewed themselves above and behind their avatar body as in the OBE condition, but in this case, their physical body movement continued to map onto their virtual body. The participants continued to move their wrists while the researcher continued to hold the cloth in front of their hands. This condition split perception between a visual point of view behind the avatar and the continuing tactile sensation on the hand (Figure 2.2).

At the end of this second phase, the participant's avatar embodiment was evaluated again using the mental ball-dropping exercise. All participants were asked to carry out the task five times. These values were then recorded, with the average of the drop times reported as *Drop2* in the results section.

At the conclusion of the experiment, participants were assisted in removing the VR equipment. All participants completed a post-study survey with the measures described in the following measures section. Finally, the researcher conducted a short qualitative interview with those assigned to the experimental OBE and DBE conditions.

Figure 2.2: OBE and DBE conditions. Participant has their perspective lifted out of their virtual body to the ceiling room.

Chapter 3: Measures

3.1 Embodiment

At the conclusion of the VR portion of the study, participants were asked to complete a post-experimental survey. The first questions were adapted from the standard questionnaire proposed by Gonzalez-Franco and Peck (2018) to measure avatar embodiment. A subset of these questions were nearly identical to those in the original Bourdin et al. (2017) study; these are noted in italics.

At the conclusion of the study, participants completed a post-experimental survey evaluating three components (Appendix F):

- *Avatar embodiment:* adapted from Gonzalez-Franco and Peck (2018) and Bourdin et al. (2017).
- *Out of body experience:* adapted from Bourdin et al. (2017).
- *Fear of Death:* adapted from the Collett-Lester Fear of Death scale, which has demonstrated reliability and factorial congruence (Lester, 1990; Lester & Abdel-Khalek, 2003). The pre-study survey took these same FOD measures, alongside demographic data to establish the participant's FOD baseline.

In order to control for extraneous variables, we collected data on participants' self-esteem and religious convictions, with the intention of comparing responses compared between groups (Appendix D & E). Unfortunately, a sufficient analysis of the impact of these variables was unable to be conducted due to the small sample size (Limitations).

After completing all the surveys, the researcher had a short conversation with the participant covering the participant's lived experience, etc. These qualitative measures were analyzed for any noticeable trends.

- Did you feel in ownership of the virtual body during this study? If yes, why so?
- Briefly describe the experience of leaving your body. What was going through your mind?
- Did this experience make you think about death? How so?

Chapter 4: Results

The statistical software R was used to determine all descriptive statistics and analyses for this study.

Due to the small sample size of this study (Limitations), many considerations were made in the statistical testing and analysis portion of this study. Firstly, we were unable to assume normal distributions and therefore used nonparametric testing. Additionally, a majority of the statistical comparisons returned non-significant results; instead, a power analysis has been conducted to inform future research of the smallest sample size necessary to detect significance. Discussion of sample sizes for future research is also provided.

4.1 Mental Ball Dropping Task

To establish that the experimental methodology is valid, we utilize the ball drop test to show that the participants successfully underwent an out-of-body experience.

As described in the methodology, participants were asked to conduct a mental ball dropping task twice throughout the experiment, with each task consisting of five trials. *Drop1* designates the mean of the first five trials, which were recorded at the beginning of the experiment. *Drop2* designates the mean of the last five trials, which were recorded at the end of the experiment. *Drop1* was used as the baseline comparison for *Drop2*.

The Kruskal-Wallis *H* test was used to determine if there were statistically significant differences between *Drop1* and *Drop2* (Figure 4.1). There was no significant difference in *Drop1* and *Drop2* of the control condition (H= 0.08, p= .77) and OBE condition (H= 2.08, p= .15). The DBE condition returned a significant difference (H= 6.82, p= .009); a Dunn's Multiple Comparison posthoc test affirmed these significant differences (z= -2.61, p= .0045).

These results support the idea the participants in the DBE condition may have had their sense of location shifted above and out of the body. Although the differences between pre- and post- trials for the OBE condition were not significantly different, the data visually displayed in the box plot (Figure 4.1) suggests differences in perceptions of embodied location for this condition. To inform further research, a power analysis was conducted with power (1 - β) set at 0.80 and α = 0.05, proposing a minimum of 14 participants will be needed to reach a statistically significant, medium effect size (*E.S.*= 0.5) for this measure.

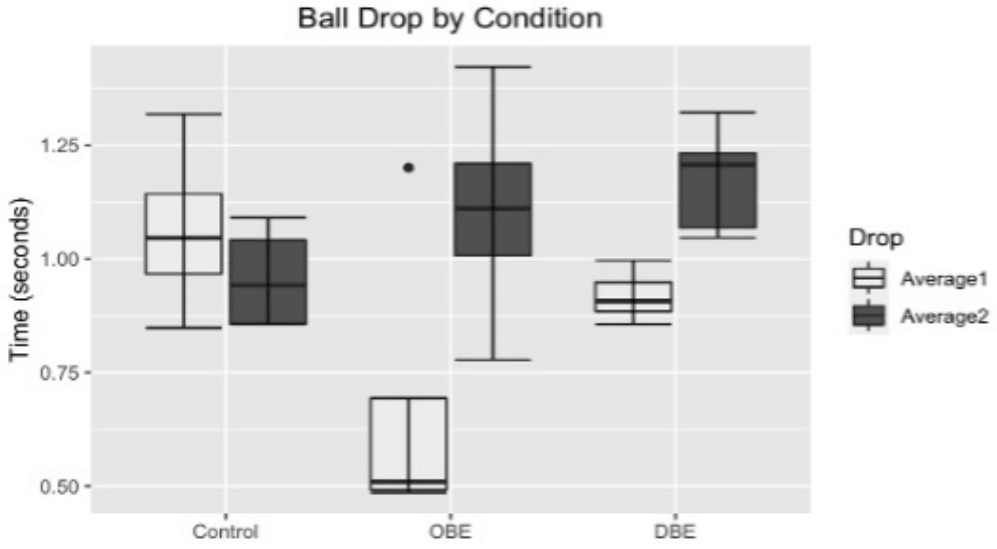

Figure 4.1: Box plots for *Drop1* and *Drop2* by condition.

4.2 Fear of Death

The pre and post-experimental surveys contained eight questions relating to the perception of death adapted from the Collett-Lester Fear of Death (Lester, 1990; Lester & Abdel-Khalek, 2003). An aggregate score was calculated for each participant by adding up participant responses for each of the eight questions. In the following figures, *FOD1* refers to the aggregate pre-experimental FOD score, and *FOD2* refers to the aggregate post-experimental FOD score.

Kruskal-Wallis *H* tests were used to compare the *FOD1* and *FOD2* scores within each study condition (Figure 4.2). No significant differences were found between the *FOD1* and *FOD2* scores in either the control (H= 0.02, p= .88), OBE (H= 1.71, p= .19), or DBE (H= 0.54, p= .46) conditions. Although there were non-significant differences between pre- and post scores for all three conditions, on visual inspection the trends are in the expected direction.

The recorded data suggest that a larger sample size could yield significant differences between reported FOD for the OBE and DBE conditions in the future. A power analysis was conducted with power (1 - β) set at 0.80 and α = 0.05. This revealed that in future research, the study will need a minimum sample size of 7 participants to identify differences between pre and post scores in the OBE condition, or 44 participants in the DBE condition.

The changes in FOD score within each condition, defined as (*FOD1 - FOD2*), were calculated for further analysis. Kruskal-Wallis *H* tests were used for pairwise comparisons of FOD differences between the three conditions (Figure 4.3). No significant differences were found between the changes in FOD between control and OBE (H= 2.55, p= .11), control and DBE (H= 2.67, p= .10), and OBE and DBE (H= 1.86, p= .17).

Although there were non-significant results in the FOD differences, on visual inspection these results might indicate a gradual divergence between the three conditions. As discussed before, future research could investigate whether these differences are significant with larger sample sizes.

4.3 Qualitative Measures

At the conclusion of the survey, the researcher conducted a short interview with the participants in the OBE and DBE conditions. Generally, all participants reported feeling "connected" to the avatar body, which reflected successful embodiment in the in-the-body phase. Participants commonly agreed that their

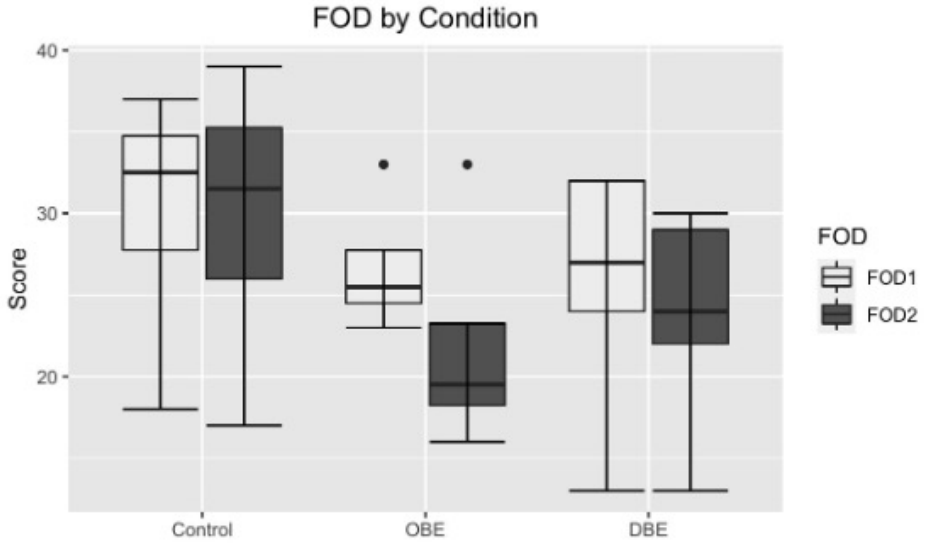

Figure 4.2: Box plots showing the total FOD score by condition.

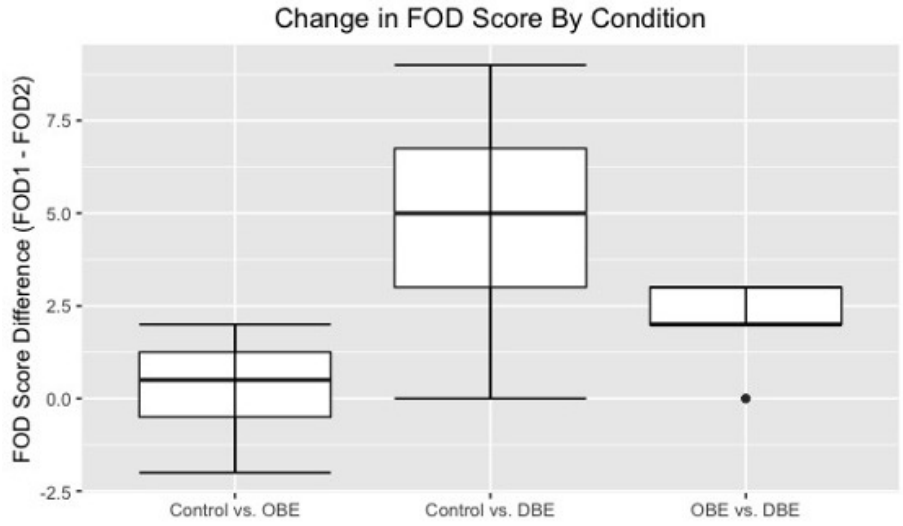

Figure 4.3: Box plots showing differences in FOD score by condition. Greater differences in FOD score indicate a greater decrease in FOD.

real movements mapping onto the avatar, as well as touching the piece of cloth, elicited an increased sense of embodiment in the avatar. The following enumerate selected quotes from participants reflecting perceived embodiment in the first phase of the study.

- *Participant 2:* When I had the controllers, the body moving around in virtual reality was important. And when I touched the cloth, I felt how it actually touched me in real life which was immersive.
- *Participant 4:* When [the researcher] told me to touch the cloth, I felt that my gestures were controlling that. When I moved my head with the VR headset, I also felt in control of my actions in the environment.

Responses from participants for the second phase of the experiment varied between the conditions. Generally, those in the DBE condition felt some initial discomfort, but quickly adapted to the third-person point of view. In contrast, those in the OBE condition were more disturbed by their experience, describing it as intimidating. Once again, we list participants' feedback on the experimental stimulus below.

- *Participant 1 (OBE Condition):* I'm pretty scared of heights ... and heights are something I associate with fear ... so there was something there.
- *Participant 7 (OBE Condition):* I thought I was on a higher point of view... [death] might be like that a little bit, just looking down on yourself like that.
- *Participant 3 (DBE Condition):* [The experience] didn't exactly feel I left my body. It felt more like I was just looking at a birds-eye view of my body ... I still felt control over my body, it was just from a different [view] point.
- *Participant 6 (DBE Condition):* [Moving out of the body] was a weird feeling, but I think after moving and realizing I could still control myself, I felt more normal.

Chapter 5: Discussion

5.1 Implications

This study primarily sought to further lines of research regarding VR OBEs in studies such as Bourdin et al. (2017) and Chan et al. (2020). In Bourdin et al. (2017), researchers utilized a similar methodology presented in this study and found that overall FOD was lower in the OBE condition than in the DBE condition. In Chan et al. (2020), researchers once again utilized a similar methodology presented in this study and found that participants with a greater embodiment of their avatars experienced greater FOD in the OBE condition than the

control condition. Although this study returned insignificant results, insights from the small sample data may be drawn.

The Bourdin et al. (2017) and Chan et al. (2020) studies each used two conditions: OBE/DBE and OBE/control, respectively. This study incorporated all three conditions (OBE/DBE/control) into the study methodology, and preliminary results appear to support the findings from Bourdin et al. (2017). Both the OBE and DBE conditions were correlated with a reduction in FOD, with OBE having a medium effect size and DBE having a small effect size.

These differences in effect size may be attributed to differences in sensory arousal and experience between the OBE and DBE conditions. Although participants in both conditions drifted out of their body, the DBE condition allowed for participants to continue receiving external sensory information through the cloth and retain control over their virtual avatar. In the post-experimental qualitative interview, many participants expressed quickly adapting to the condition, referring to it as essentially a "third person point-of-view." Conversely, the OBE condition removed all sensory information and avatar control, which may have more realistically simulated an out-of-body experience.

In evaluating the clinical viability of the Oculus Quest, this study lent support to the HMD's ability to create portable and immersive OBE experiences. The qualitative interview revealed that participants perceived embodiment in their virtual avatars, with those in the experimental conditions reporting varying levels of disturbance once they shifted out of their body. Nevertheless, in order to ascertain these effects, further research with the Oculus Quest must be conducted in clinical contexts and with palliative and hospice patients.

5.2 Limitations

Limitations of this research primarily lie in the methodology used to complete the study. Most notably, this study contains a small sample size; the researcher was regretfully unable to recruit and run a sufficient amount of participants because of Cornell University's closure due to the global COVID-19 pandemic.

Additionally, because this study was conducted amidst the global COVID-19 pandemic, it is important to note that fear of death may have been particularly salient among participants. These unusual circumstances may have served as a confounding factor in this study.

The methodology could also be adjusted to obtain clearer results from participants. During research sessions, it was never measured whether participants had experience in virtual reality or other immersive technologies. Because the experience level of the participants may have affected avatar embodiment, it may have also served as a confounding factor in this study.

It is important to note that while this study adds to the discussion of the potential of out-of-body experiences for reducing fear of death, it does not, and was not intended to, directly replicate the study conducted by Bourdin et al. (2017). As elaborated above, there are several fundamental differences between the two studies. Instead, it was meant to extend this line of research. We also acknowledge the limitations in using portable devices to create an out-of-body experience.

Furthermore, while this study refers to possible future use in clinical contexts, the participants were not meant to be representative of a clinical population. As noted above, the participants were healthy college undergraduate students from Cornell University with a mean age of 20.38 years; this is in contrast to the more advanced age populations who have a terminal diagnosis and would be under palliative or hospice care. As alluded to before, the Cornell student population may have more experience with immersive technologies; additionally, older populations contrast with Cornell students in terms of life experiences, age-associated physical and mental ailments (i.e., Alzheimer's,

dementia, visual impairments), and other factors. Each of these components may serve as a moderating or mediating factor. Ultimately, although this study supports existing literature on inducing embodied experiences in portable VR devices, further investigation within a clinical setting will be needed to determine what aspects of an embodiment experience affect FOD.

5.3 Future Research

There are several opportunities to further investigate out-of-body experiences in VR and their effect on individuals' fear of death.

Future work replicating OBEs in VR may allow more time to establishing the participant's embodiment and attachment to their virtual avatar. Additionally, it would be beneficial to add contextual cues such that participants are more aware that they are experiencing the death of their character. These changes would help in addressing feedback from participants which indicated minimal embodiment and confusion as to whether they were dying. A methodology to model future studies is described in Barberia, Oliva, Bourdin, and Slater (2018), where the researchers embody participants in a body on a beautiful island with two companions; participants are then allowed to explore the island and carry out tasks together over the course of a longitudinal study.

Researchers can compare active and passive tactile feedback while they attempt to achieve visuotactile synchrony and embody participants during the in-the-body phase. In this study, participants actively moved their avatar hand and brushed against the cloth that the researcher held. This is qualitatively different from a passive-touch experience, where the participant would passively hold their hand still as the researcher holds the hand controller and use it to animate an object that touches the participant's hand in the real world.

Additionally, it would be interesting to examine the relationship between participants' out-of-body experience and their FOD responses when there are additional sensory cues for the leaving-the-body phase of the stimulus. It would also be intriguing to assess how different environmental factors (e.g. higher vs. low sensory arousal) could impact sensory perception and affect the VR out-of-body experience.

Regarding quantitative measurement, future work might seek advice from clinicians or experts to extend the FOD questionnaire in capturing more dimensions of participant FOD. We can examine whether and when an experimental stimulus itself may induce fear or anxiety. Post-stimulus survey questions should also capture the degree to which participants attend to cues of body ownership as well as visuomotor contradiction.

Regarding qualitative measurements, future work might incorporate interviews with participants from the control condition rather than solely from the experimental OBE and DBE conditions.

5.4 Conclusion

In this study, we created an out-of-body experience in the Oculus Quest loosely based on previous studies examining VR OBEs. The study examined the effect of OBEs on participant FOD in three conditions: control, OBE, and DBE., Although there were observable differences between the pre and post-experimental FOD scores of the OBE and DBE conditions, these divergences were non-significant. A power analysis was provided to inform future studies on the number of participants needed to return significant results for this study. Future research is also advised to focus on factors that lead to different degrees of sensory cues, visuomotor contradiction, and stimulus arousal to comprehend the best practices for creating VR OBEs using the simplest possible paradigm.

Appendix A

Materials

Figure A.1: Oculus Quest HMD and Touch Controllers.

Figure A.2: Stopwatch Timer

Appendix B

Research Setting

Trials for this study were conducted in a designated room in the Virtual Embodiment Lab at Cornell University. This room was a 380cm X 450cm room with a ceiling height of 321cm. During each trial, at least one researcher was always in the room to run the study and for safety reasons.

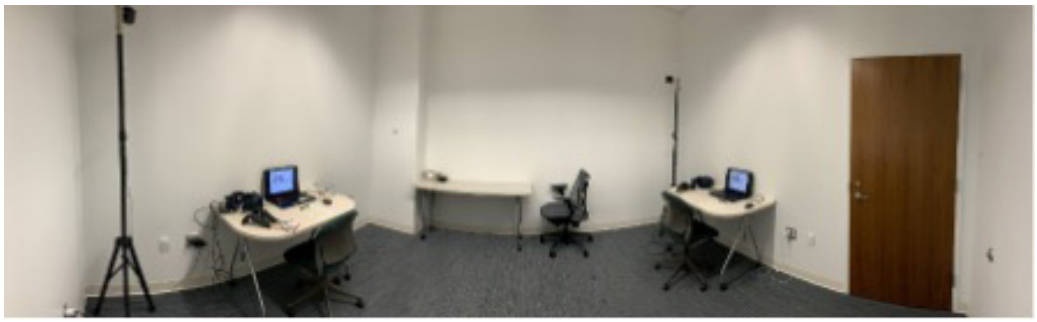

Figure B.1: Research Setting

Appendix C

Demographics

Participant demographics were taken in the pre-study survey.

1. How old are you? Please specify: _____

2. What is your gender?
   - Male
   - Female
   - Other gender identity, Please specify: _____

3. How do you typically describe yourself?
   - American Indian or Alaskan Native
   - African-American, of African descent, African, or Black
   - Asian or Pacific Islander
   - Asian American
   - Hispanic, Latino, or Latina
   - Native Hawaiian or other Pacific Islander
   - European-American, of European descent, or European (but not of
   - Hispanic descent or Hispanic)
   - Biracial/bi-ethnic or multiracial/ multiethnic
   - Other (specify)

4. What is your year in school?
   - 1st year undergraduate
   - 2nd year undergraduate
   - 3rd year undergraduate
   - 4th year undergraduate
   - 5th year undergraduate or more
   - Other; please specify: _____

Appendix D

Self-Esteem

Self-esteem was identified as an extraneous variable in this study. This study's measure of self-esteem was adapted from the Rosenberg Self Esteem Scale (Rosenberg, 1965). Participants were asked to rate on a Guttman scale of 1 (*Strongly agree*) to 5 (*Strongly disagree*) when responding to the following statements:

- On the whole, I am satisfied with myself.
- At times I think I am no good at all.
- I feel that I have a number of good qualities.
- I am able to do things as well as most other people.
- I feel do not have much to be proud of.
- I certainly feel useless at times.
- I feel that I'm a person of worth.
- I wish I could have more respect for myself.
- All in all, I am inclined to think that I am a failure.
- I take a positive attitude toward myself.

Appendix E

Religious Conviction

Religious conviction was identified as an extraneous variable in this study. Drawing from the methodology in Bourdin et al. (2017), participants were asked to respond to the question *What is your religious preference?* and given the following options to select:

- Believer and practicing
- Believer non-practicing
- Agnostic
- Atheist
- Other

Appendix F

Avatar Embodiment

- *I felt as if the virtual body I saw when I looked down was my body.*
- *It felt as if the virtual body I saw was someone else.*
- It seemed as if I might have more than one body.
- *I felt as if the virtual body I saw when looking in the mirror was my own body.*
- *I felt as if the virtual body I saw when looking at myself in the mirror was another person.*
- It felt like I could control the virtual body as if it was my own body.
- The movements of the virtual body were caused by my movements.
- I felt as if the movements of the virtual body were influencing my own movements.
- I felt as if the virtual body was moving by itself.
- It seemed as if I felt the touch of the cloth in the location where I saw the right hand of the avatar touched.
- It seemed as if the touch I felt was located somewhere between my physical body and the virtual body.
- It seemed as if the touch I felt was caused by the virtual cloth touching the virtual hand.
- It seemed as if my hand was touching the virtual cloth.
- I felt as if my body was located where I saw the virtual body.
- I felt as if my (real) body were drifting out of the virtual body or as if the virtual body were drifting out of my (real) body.

Out of Body

Participants were asked to rate on a Likert scale of 1 (*I did not feel that at all*) to 7 (*I felt it to the maximum intensity*) when responding to the following statements:

- I felt as if the body I was seeing was my own body.
- I felt as if the body I was seeing belonged to someone else.
- I felt as if I was floating in air.
- I felt as if I was in an elevated position in the room.
- I felt a connection with the body, as if I was looking down at myself.
- I felt as if I had an invisible body.
- I felt out of my body.

Fear of Death

Participants were asked to rate on a Likert scale of 1 (*Not at all*) to 5 (*Very much*) on how disturbed or made anxious they felt by the following aspects of death:

- The total isolation of death
- The shortness of life
- Missing out on so much after you die
- Dying young
- How it will feel to be dead
- Never thinking or experiencing anything again
- The possibility of pain and punishment during life-after-death
- The disintegration of your body after you die

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Authors' Biographies

Narda Bondah holds a B.Sc. in Biology and Society with Distinction in Research from CALS. She is from Kumasi, Ghana. Her research interests lie in immunology, especially in issues related to maternal and fetal health. In her freetime, she volunteers to teach children about Africa.

Jan-Paul Ramos-Dávila is a sophomore in the College of Arts & Sciences studying mathematics, computer science, and philosophy. His main research interests involve developing practical tools for software verification using both formal and lightweight methods. At Cornell, he worked under Dr. Adrian Sampson in the CAPRA research group – whereas currently – he works with researchers at Carnegie Mellon University on exploring the application of gradual verification techniques to smart contract technology. His hobbies involve regularly visiting the Cornell Cinema and playing Minesweeper pseudo-professionally.

Alice Lou graduated from Cornell University in May 2022 with a major in biological sciences and minors in business, nutrition, and health. She is currently obtaining a second bachelor's and a master's in nursing at the University of Pennsylvania. Alice's research interests are primarily in Microbiology. At Cornell, she was involved in the Filiatrault Lab, a plant microbiology lab where she completed her Honors thesis studying the response of plant pathogens to a salt compound. Currently, she is a part of the ARES Lab at the University of Pennsylvania and is involved in several projects investigating S. aureus in hospitals. Alice is from the San Francisco Bay Area, and while she misses the food from the Bay, she enjoys trying new restaurants wherever she is.

Jessica Lecorchick graduated from Cornell University in 2022. There, she triple majored in biological sciences, information science, and communication with concentrations in neurobiology and behavior, behavioral science, and technology, respectively. Throughout her undergraduate career, she was heavily involved with the K. Lisa Yang Center for Conservation Bioacoustics, where she explored topics such as fish acoustics, effects of smoke on apes, and forest elephant conservation. Other organizations she has worked with include the Organization for Tropical Studies, where she researched ungulate-vegetation interactions, and the Price Lab of The University of Chicago, where she assisted with bird banding and helped to design an exploratory study using earth mound presence to help inform reserve forest land purchases. She is also a Princeton Ecology and Evolutionary Biology Scholar, CALS Global Fellow, and Hunter Rawlings III Presidential Research Scholar. These days, Jessica is a project manager for Epic Systems, a leading Electronic Health Records software, where she manages the implementation of a population health module with care coordination and data analytics functionality across healthcare facilities. She hopes to continue her passion for behavior in her future endeavors, whether that be in zoology, health, tech or beyond.

Liam McLaughlin graduated from Cornell in May 2022 from the College of Arts and Sciences. While there, he majored in Biological Sciences with a concentration in Neurobiology, and minored in creative writing. In undergrad, he did research in a computational neurobiology lab and he currently works at the Washington University School of Medicine studying cancer biology. Besides academics, Liam volunteers as an ESL tutor for refugees and as a patient representative at Barnes-Jewish hospital in St. Louis. In his free time, he enjoys writing creative fiction and is working on publishing a recently drafted novel. He also loves to ski and play a good game of chess.

Naveen Sharma is a junior at the ILR School minoring in Philosophy, Africana Studies, and Law and Society. He intends to go to law school to study international law and also intends to pursue a Ph.D. in Philosophy or Africana Studies. Naveen's area of interest combines ethical philosophy with international affairs to continue his work research on global development and anti-trust policy. He will continue doing research on developing economies with a focus on breaking down mining monopolies. Outside of his academic work, Naveen is the founder and Chair of the Student Assembly Office of Ethics, the Vice President of MiXed at Cornell, and the Treasurer for the Scheinman Conflict Resolution Club. For fun, Naveen can be found practicing various martial arts, cooking new recipes, and participating in new campus events.

Joshua Zhu is a recent graduate and majored in Information Science. He is currently a medical student at the Renaissance School of Medicine at Stony Brook University. At Cornell, he was a researcher in the Virtual Embodiment Lab where he worked under the mentorship of Andrea Stevenson Won, PhD. His research interests include digital health, personalized medicine, and healthcare informatics.

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