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**Project Title:** Microbiome Composition and Environmental pH Modulate the Behavioral Effects of SSRIs in Larval Zebrafish (*Danio rerio*)

**Category:** Behavioral and Social Sciences

## Research Plan

### A. Rationale

Major depressive disorder (MDD) is a prevalent neurological disorder, affecting approximately 300 million worldwide (WHO, 2018). The consequences of MDD are serious, ranging from detrimental effects on schoolwork to suicide, of which there are almost 800,000 per year (WHO, 2018). Selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed class of drugs used to treat MDD, but even so, they do not always work as intended. In fact, of those who are prescribed SSRIs to treat MDD, only about 30-40% go into remission (Viglione et al., 2017). The exact mechanism by which SSRIs work is not completely understood, so the variability in their efficacy also poses questions. Using zebrafish as model organisms, I plan to investigate the role of two factors on the behavioral effects of SSRIs: the microbiome and water pH.

Currently, the gut-brain axis, or the bidirectional interaction between the microbiome and the brain, is a hot topic in the field of neuroscience. Research has suggested that the two systems are able to communicate with each other through this connection, and one study showed that changing the microbiome population was able to affect depressive-like and anxiety-like behavior in mice (Neufeld et al., 2010). In addition, gut bacteria may be responsible for the production of

neurotransmitters, including serotonin (Yano et al., 2015), a key player in MDD and the main target of SSRIs.

Zebrafish are an excellent model organism for studying the microbiome due to being easier to breed and raise as opposed to mammalian models. In order to see what impact the presence or lack of a microbiome has on the effects of SSRIs on zebrafish, I plan on treating both conventional and germ-free zebrafish with various doses of sertraline. By analyzing the data collected from this experiment, more light can be shed on the role that the microbiome plays in the gut-brain axis, as well as how SSRI effects are altered. In addition, I intend to use three different pH levels as another factor to see if it influences the effect of the microbiome on zebrafish behavior. This would provide an interesting look into how individual and environmental factors play a role in their response to sertraline. In addition, the implications of testing pH go beyond neuroscience, as it is a very significant component of the environment, and including it as a third independent variable may allow for further insight into how the negative impact of SSRIs on aquatic ecosystems can be reduced. Using a Zebrabox would enable high-throughput drug screens on these zebrafish to be carried out.

## B. Research Question

What role does the larval zebrafish microbiome play in their behavioral response to sertraline?

### Hypothesis

If zebrafish larvae lack a microbiome, then their swimming activity will increase. Since SSRIs increase the reuptake of serotonin and have been shown to have hypoactive effects, then the absence of microbes that contribute to the production of serotonin may have an opposite, hyperactive effect.

## C. Procedures

*Fish husbandry*

Adult zebrafish from a hybrid strain of Tubigen Longfin/Brian's wildtype will be kept in a zebrafish facility of 28.5°C in a 13/11 hour light/dark cycle. Feeding will consist of pellet food or newly hatched brine shrimp every other day. Five to six mating pairs will be set up in the late afternoon, with each pair separated by a plastic divider. Dividers will be pulled out the next morning, and eggs collected one to two hours later.

*SSRI and pH experiment*

Zebrafish embryo media (0.3 g/L Instant Ocean, 7.5 mg/L $HCO_3^-$, 1 mL/L methylene blue) will be used as the solvent for the exposure solutions. Exposure solutions will then be adjusted to pH 7, 7.5, and 8, using pH 7.0 HEPES, pH 7.5 Tris, and pH 8.0 Tris, respectively. 30 mL of the pH solutions will be transferred into twelve Petri dishes, with four dishes for each distinct pH solution. Stock solutions of the SSRIs sertraline, citalopram, and fluoxetine dissolved in methanol will be diluted with pH adjusted embryo media to create final drug treatment solutions at 1, 10, and 100 µg/L (methanol solvent concentrations were less than 0.1% of total exposure solutions). Embryo media will be used as a control treatment. Each Petri dish will be a unique combination of pH and SSRI dose. Thirty larval zebrafish collected from the morning will then be transferred to each of the Petri dishes and incubated at 28.5°C overnight.

Embryos will be individually transferred into plastic 48-well plates containing 1 mL of their respective drug/pH treatment solutions. Each plate contained 4 wells (technical replicates) of each drug/pH combination. Final sample sizes will range from 14-20 per dose/pH treatment.

80% of the exposure solutions will be renewed daily to prevent pH decrease from larval respiration as well as to remove any waste material and discarded chorions, maintaining a clean environment as well as eliminating any confounding variables. At 6 days post fertilization (dpf), larval swimming behavior will be assessed via the visual motor response.

*Behavior paradigm*

Behavior will be analyzed at 6 dpf using a ZebraBox (ViewPoint, CA). A 50-minute light-light-dark (LLD) paradigm will be used to induce a visual motor response (VMR) in the zebrafish larvae (Fig. 1). VMR is a well-studied, well-stereotyped behavioral response characterized by a sudden increase in behavior when the light is turned off. The paradigm will consist of 20 minutes of acclimation in full light conditions, 15 minutes of spontaneous swimming in full light conditions. Lights will be turned off to stimulate the VMR and be followed by 15 minutes of spontaneous swimming in dark conditions. All plates will be screened between 10 a.m. and 4 p.m. to reduce any differences in behavior caused by circadian rhythm. Empty wells or wells containing dead/malformed fish will be excluded from further analysis.

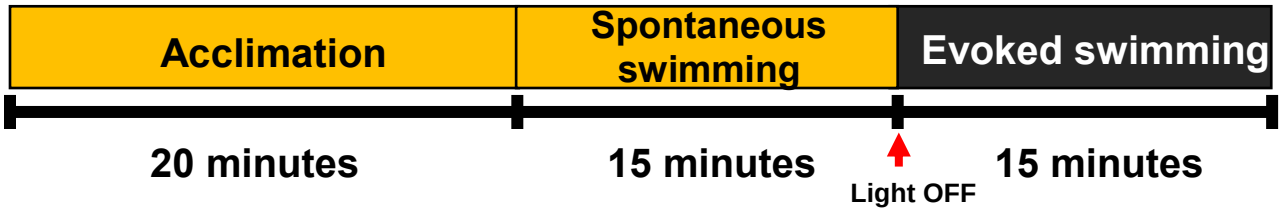


**Figure 1.** The behavior paradigm to be used in this study will incorporate visual motor response (VMR), including a 20-minute acclimation period, 15 minutes of spontaneous swimming, and then 15 minutes of evoked swimming after the visual stimulus of turning off the lights.

*Gnotobiotic zebrafish derivation*

An extensive washing process (Pham et al., 2008) will be used to derive germ-free (GF) zebrafish, in which all microbes are to be eliminated. By making all microorganisms absent, this will allow for gnotobiotic research where all microorganisms in the zebrafish are known.

Zebrafish eggs will be collected via natural breeding as described previously. Large debris will be removed and eggs will be transferred to 15 mL plastic test tubes. Control embryos will be stored in regular embryo media while experimental embryos will be stored in antibiotic gnotobiotic zebrafish media (AB-GZM, which will consist of filter sterilized embryo media with 250 ng/mL amphotericin B, 5 μg/mL kanamycin, and 100 μg/mL ampicillin). Notably, gnotobiotic media does not contain methylene blue. The tubes will be placed in the incubator for 4-6 hours and intermittently washed with fresh AB-GZM. Embryos will be sorted for fertilized embryos before proceeding with the procedure.

The tubes from the experimental group will then be moved to a cell culture hood. Eggs will be washed three times with fresh AB-GMZ followed by a 1 minute 45 second wash with 0.1% povidone-iodine (PVP-I, or betadine). This will be followed by three consecutive washes in sterile GZM. Tubes will then be filled with 0.003% bleach for 20 minutes to 1 hour, before being washed with GZM three times again. Zebrafish eggs will be transferred into 48-well plates containing exposure solutions made from sterile GZM, which will then be wrapped in Parafilm and placed inside a bleached Tupperware before being transferred to the incubator. To control for any confounding variables caused by the washing process, half of the zebrafish eggs that had been washed in the cell culture hood will be conventionalized by being plated in normal nonsterile zebrafish media so that microbes will be reintroduced into the zebrafish.

Sterile treatments will be checked for microbial contamination at the end of the experiment. 5 μL from each well plate will be spotted on LB agar plates, incubated at 28°C for 24 hours and visually inspected for microbial growth.

*Microbial status and pH experiment*

pH solutions (7, 7.5, 8) will be made, and zebrafish will be separated into control, GF, and conventionalized categories. GF and conventionalized zebrafish will be derived according to the process described above. To minimize probability of external contamination of the GF zebrafish, exposure solutions will not be renewed. The VMR assay will be run at 6 dpf following the procedure described above.

*Microbial status and sertraline experiment*

pH will be held constant at 7.0. Zebrafish will be assigned to either control, conventionalized, and GF groups. Sertraline will be the only SSRI used in this experiment, at doses of 1 μg/L, 10 μg/L, and 100 μg/L, as well as controls. To minimize probability of external contamination of the GF zebrafish, exposure solutions will not be renewed. The VMR assay will be run at 6 dpf following the procedure described above.

*Microbial status, sertraline, and pH experiment*

Lastly, an experiment containing all three variables (microbial status, drug dose, and pH) will be run. Only pH 7 and pH 8 solutions will be made, and sertraline will only be administered to 100 μg/L, along with controls. Each distinct condition (pH and sertraline dose) will be separated into its own quadrant on each plate. To minimize probability of external contamination of the GF zebrafish, exposure solutions will not be renewed. The VMR assay will be run at 6 dpf following the procedure described above.

**Risk and Safety**

*HEPES buffer*

- Material may be irritating to the mucous membranes and upper respiratory tract
- May be harmful by inhalation, ingestion, or skin absorption
- May cause eye, skin, or respiratory system irritation

*Tris buffer*

- Harmful if swallowed
- Material may be irritating to the mucous membranes and upper respiratory tract
- May be harmful by inhalation, ingestion, or skin absorption
- May cause eye, skin, or respiratory system irritation

*SSRIs: Sertraline, Citalopram, Fluoxetine*

- Material may be irritating to the mucous membranes and upper respiratory tract
- Harmful if swallowed
- May be harmful by inhalation, ingestion, or skin absorption
- May cause eye, skin, or respiratory system irritation
- Very toxic to aquatic life
- SSRIs used in subclinical concentrations

*Gnotobiotic Zebrafish Media*

- Amphotericin B
    - Causes serious eye irritation
    - Causes skin irritation
    - Material may be irritating to the mucous membranes and upper respiratory tract
    - May be harmful by inhalation, ingestion, or skin absorption
    - May cause respiratory system irritation.
- Kanamycin
    - Material may be irritating to the mucous membranes and upper respiratory tract
    - May be harmful by inhalation, ingestion, or skin absorption
    - May cause eye, skin, or respiratory system irritation

    - May damage fertility or the unborn child.
- Ampicillin
    - Material may be irritating to the mucous membranes and upper respiratory tract
    - May be harmful by inhalation, ingestion, or skin absorption
    - May cause allergy or asthma symptoms or breathing difficulties if inhaled
    - May cause an allergic skin reaction
    - May cause eye, skin, or respiratory system irritation.

*Safety Precautions*

- Personal protective equipment (gloves, goggles, and lab coat) were worn when handling these materials

**Data Analysis**

Data will be binned into 1-minute intervals. For all experiments except the final microbial status, SSRI, and pH experiment, a 3-way factorial, nested sample design with a mixed effects model for repeated measures will be used. For the pH and SSRI experiment, the fixed effects will be drug dose, pH, and behavior paradigm stage (acclimation, pre-stimulation, and post-stimulation). For the microbial status and pH experiment, the fixed effects will be microbial status, pH, and behavior paradigm stage. For the microbial status and SSRI experiment, the fixed effects will be microbial status, drug dose, and behavior paradigm stage. For the final experiment, a 4-way factorial, nested sample design with a mixed effects model for repeated measures will be used, with the fixed effects being microbial status, drug dose, pH and behavior paradigm stage. For all experiments, the fish number and repeated measures will be nested random factors.

For all models, the significance of fixed effects will be determined by ANOVA using Satterthwaite's method for denominator degrees-of-freedom and F-statistic. Additional pairwise comparisons will be made using the least squared means method. A p-value of 0.05 will be used to determine significance. All analyses will be conducted in R (CRAN).

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**Addendum**

# NO ADDENDUMS EXIST