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

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Abstract

Major depressive disorder (MDD) is one of the most prevalent neurological disorders, affecting approximately 300 million people worldwide and having the potential to cause severe debilitation or even suicide. Selective serotonin reuptake inhibitors (SSRIs) are one of the most commonly prescribed classes of drugs used to treat MDD. However, despite their wide use, the exact effects of SSRI on the brain are unknown and treatment efficacy varies widely between individuals. This variability in effect has been speculated to be related to variations in individual traits such as genetics or microbiome composition, or differences in environmental factors. Recent studies have only begun to highlight how the gut microbiome affects mental health, and it has been linked to various mental illnesses including MDD. The objective here was to study how factors including microbiome composition and pH influence the effect of SSRIs on larval zebrafish behavior. Zebrafish embryos were exposed to different doses of SSRI, their microbiome and environmental pH were altered, and their combined impacts on larval swimming behavior in a light/dark spontaneous swimming paradigm were observed. Sertraline consistently caused a hypoactive effect, which was further enhanced by increasing pH. Fish without a complete microbiome tended to be hyperactive relative to their conventional counterparts, which counteracted the hypoactive effects of SSRIs. The additive effects on locomotor behavior resulting from the interaction between increasing pH and high SSRI dose counteracted the effect of microbiome reduction. The nature of how pH and the microbiome modulate SSRI effects on larval zebrafish were somewhat variable and highly context dependent. The results show that SSRI effectiveness may rely partially on the condition of an organism's microbiome as well as environmental factors, which provides more insight into their mechanism of action.

1. Introduction

Depression is a prevalent neurological disorder that affects approximately 300 million people worldwide (WHO, 2018). Besides causing debilitating problems in daily life and contributing to almost 800,000 annual suicides (WHO, 2018), depression has been shown to be correlated with reduced educational advancement (Breslau et al., 2008), lower financial and job security (Marcotte & Wilcox-Gök, 2001), higher instances of teenage pregnancy (Kessler et al., 1997), as well as certain lethal and sublethal diseases, including cancer (Gross et al., 2009), diabetes (Carnethon, 2003), and coronary heart disease (Kooy et al., 2007). Selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed drug used to treat depression (Mayo Clinic, 2019). Even so, only about 30-40% of patients go on to remission (Viglione et al., 2017), and the exact mechanism of action of SSRIs is not known either.

Research has shown that the gut microbiome may play a major role in cross-communication with the brain through the gut-brain axis, with possible implications for the treatment of neurological disorders (Cryan & Dinan, 2012). Changing the microbiome population has been shown to affect anxiety-related and depression-like behaviors in mice (Neufeld et al., 2010), as well as mood in human subjects (Benton et al., 2006). Gut bacteria may also be capable of contributing to the production of neurotransmitters such as serotonin (Yano et al., 2015; Lyte, 2011), which strongly suggests a connection to how SSRIs mitigate depression. In addition, approximately 95% of the body's serotonin is produced in the intestines, which supports the case for a relationship between depression, SSRIs, and the gut microbiome. In humans, microbiome composition is known to be diverse (Gilbert, 2015), even during infant stages (Raveh-Sadka et al., 2015). While zebrafish possibly have a core enteric microbiome (Roeselers et al., 2011), it is known that their microbe populations also vary among individuals (Stephens et al., 2015). These individual distinctions can be pronounced by artificially altering the microbiome to determine the role of gut bacteria in depression treatment.

While a multitude of genes and pathways may be associated with depression, it is important to note that environmental factors may also play a role in the efficacy of SSRIs (Kovacs et al., 2014). Studies have indicated that SSRI treatment is correlated with altered levels of brain-derived neurotrophic factor (BDNF), the most commonly expressed neurotrophin in the brain (Mostert et al., 2008). In addition, BDNF plays a major role in brain plasticity (Bramham

& Messaoudi, 2005), and studies have concluded that SSRIs may increase neuroplasticity, thereby allowing organisms to become more susceptible to external stimuli (Andrade & Rao, 2010). As an environmental stressor, water pH is relevant to zebrafish physiology. Therefore, pH was chosen as the environmental variable. Acidic and basic pH levels have been shown to induce stress responses within zebrafish. For example, due to pH variations, changes in oxygen uptake and hatching rate abnormalities were observed (Zahangir et al., 2015).

Zebrafish as model organisms enable low-cost and large-scale testing to be performed (Lieschke & Currie, 2007), making it feasible to even conduct high throughput drug screens for various diseases (Spomer et al., 2012; Kithcart & Macrae, 2017). In this study, zebrafish larvae were used as behavior models to test how individual differences (microbiome) and environmental factors (pH) would affect the impact of SSRIs on behavior. Much of the neurochemistry and brain morphology of the zebrafish brain is highly evolutionarily conserved with the human brain (Kalueff et al., 2014). For example, the habenula, which is a group of nuclei found in the epithalamus that is involved in the release of neurotransmitters including serotonin, is evolutionarily conserved (Mathuru & Jesuthasan, 2013), making these zebrafish studies relevant to understanding human depression.

In this study, the visual motor response (VMR) assay was used as a way to observe the interplay between the three different factors of pH, sertraline dosage, and microbiome composition in contributing to the behavior of the zebrafish when exposed to a visual stress stimulus (Emran et al., 2008). Using assays such as VMR allows neurobehavioral responses to external variables to be directly observed, rather than relying on results from tissue that do not necessarily induce behavioral changes. Since SSRIs are meant to reverse the behavioral symptoms that are caused by depression and have been shown to alter zebrafish behavior (Theodoridi et al., 2017), even in progeny (Vera-Chang et al., 2018), monitoring behavior was an appropriate choice for the dependent variable. The well-established 50 minute light-light-dark (LLD) paradigm, which incorporates a sudden, drastic change in illumination to prompt a startle response, was utilized to elicit behavioral responses. Swimming behavior of zebrafish was measured firstly under different pH levels and SSRI doses. The microbiome was altered in later experiments, which was combined with pH or SSRI dose, or both. Thus, this study investigated

the hypothesis that the microbiome status and pH would affect the behavioral manifestations of SSRI uptake in larval zebrafish.

2. Materials and Methods

2.1. Fish husbandry

All maintenance and experimentation of zebrafish larvae were approved by the Institutional Care and Animal Use Committee (IACUC) of Stony Brook University. Adult zebrafish from a hybrid strain of Tubigen Longfin/Brian's wildtype were kept in a zebrafish facility of 28.5°C in a 13/11 hour light/dark cycle. Feeding consisted of pellet food or newly hatched brine shrimp every other day. Five to six mating pairs were set up in the late afternoon, with each pair separated by a plastic divider. Dividers were pulled out the next morning, and eggs were collected one to two hours later.

2.2. SSRI and pH experiment

Zebrafish embryo media (0.3 g/L Instant Ocean, 7.5 mg/L HCO_3^-, 1 mL/L methylene blue) was used as the solvent for the exposure solutions. Exposure solutions were 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 were 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 were 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 was used as a control treatment. Each Petri dish was a unique combination of pH and SSRI dose. Thirty larval zebrafish collected from the morning were then transferred to each of the Petri dishes and incubated at 28.5°C overnight.

Embryos were 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 ranged from 14-20 per dose/pH treatment.

80% of the exposure solutions were 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 was assessed via the visual motor response.

2.3. Behavior paradigm

Behavior was analyzed at 6 dpf using a ZebraBox (ViewPoint, CA). A 50 minute light-light-dark (LLD) paradigm was 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 consists of 20 minutes of acclimation in full light conditions, 15 minutes of spontaneous swimming in full light conditions. Lights were turned off to stimulate the VMR and was followed by 15 minutes of spontaneous swimming in dark conditions. All plates were 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 were excluded from further analysis.

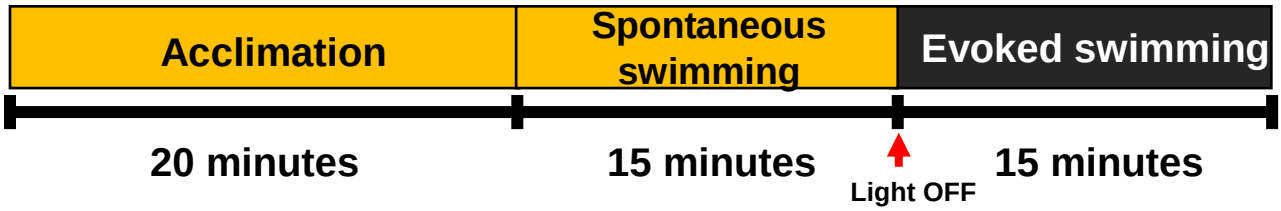


Figure 1. The behavior paradigm used in this study, incorporated visual motor response (VMR), including a 20-minute acclimation period, 15 minutes of spontaneous swimming, and then 15 minutes of evoked swimming after the visual stimulus of turning off the lights.

2.4. Gnotobiotic zebrafish derivation

An extensive washing process (Pham et al., 2008) was used to derive germ-free (GF) zebrafish, in which all microbes were to be eliminated. By making all microorganisms absent, this allowed for gnotobiotic research where all microorganisms in the zebrafish were known. Zebrafish eggs were collected via natural breeding as described in section 2.1. Large debris was removed and eggs were transferred to 15 mL plastic test tubes. Control embryos were stored in regular embryo media while experimental embryos were stored in antibiotic gnotobiotic zebrafish media (AB-GZM, which consisted 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 were placed in the incubator for 4-6 hours and

intermittently washed with fresh AB-GZM. Embryos were sorted for fertilized embryos before proceeding with the procedure.

The tubes from the experimental group were then moved to a cell culture hood. Eggs were 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 was followed by three consecutive washes in sterile GZM. Tubes were then filled with 0.003% bleach for 20 minutes to 1 hour, before being washed with GZM three times again. Zebrafish eggs were transferred into 48-well plates containing exposure solutions made from sterile GZM, which were then 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 were conventionalized by being plated in normal nonsterile zebrafish media so that microbes would be reintroduced into the zebrafish.

Sterile treatments were checked for microbial contamination at the end of the experiment. 5 µL from each well plate were spotted on LB agar plates and incubated at 28°C for 24 hours and visually inspected for microbial growth. In the first set of experiments requiring gnotobiotic zebrafish derivation, which also varied pH, only about 15-20% of the wells clearly indicated contamination. The second set of experiments which altered the microbiome and sertraline doses had a contamination rate 10-15%. In the last experiment combining the microbiome, pH, and sertraline dose, the agar plates showed the least growth, with a contamination rate of approximately 3%. While these percentages are low, and are expected due to the extreme sensitivity of maintaining a sterile environment, there is a probability that the microbes may have spread to other zebrafish in the plate. Therefore, the zebrafish in the experimental group were designated “microbiome-reduced” instead of GF.

2.5. Microbial status and pH experiment

pH solutions (7, 7.5, 8) were made, and zebrafish were separated into control, GF, and conventionalized categories. GF and conventionalized zebrafish were derived according to the process described in section 2.4. To minimize probability of external contamination of the GF zebrafish, exposure solutions were not renewed. The VMR assay was ran at 6 dpf following the procedure described in section 2.3 above.

2.6 Microbial status and sertraline experiment

pH was held constant at 7.0. Zebrafish were assigned to either control, conventionalized, and GF groups. Sertraline was 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 were not renewed. The VMR assay was ran at 6 dpf following the procedure described in section 2.3 above.

2.7. Microbial status, sertraline, and pH experiment

Lastly, an experiment containing all three variables (microbial status, drug dose, and pH) was run. Only pH 7 and pH 8 solutions were made, and sertraline was only administered to 100 μg/L, along with controls. There were enough zebrafish for two 48-well plates of each microbial condition. Each distinct condition (pH and sertraline dose) was separated into its own quadrant on each plate. To minimize probability of external contamination of the GF zebrafish, exposure solutions were not renewed. The VMR assay was ran at 6 dpf following the procedure described in section 2.3 above.

2.8. Statistical Analyses

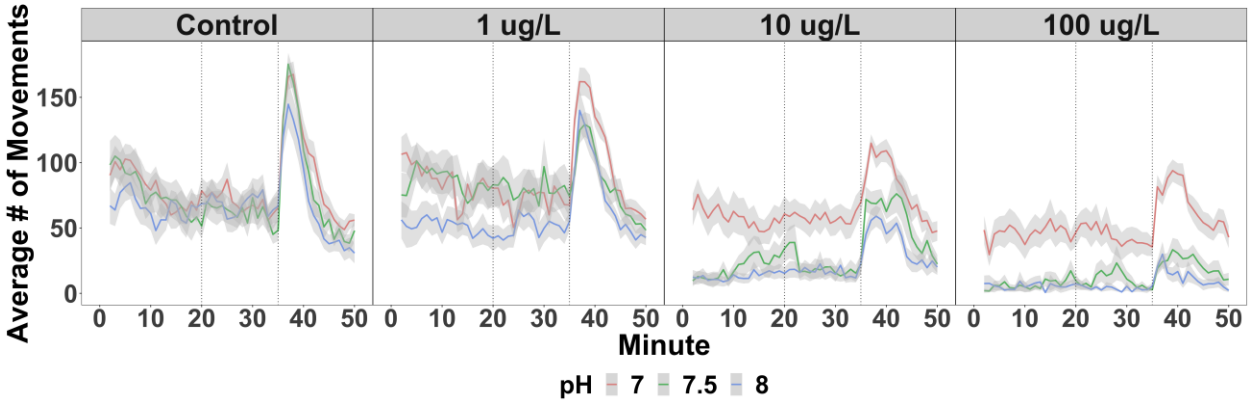
Data was binned into 1-minute intervals. For all experiments except the final microbial status, SSRI, and pH experiment, a 3-way factorial, nested sample design with a mixed effects model for repeated measures was used. For the pH and SSRI experiment, the fixed effects were drug dose, pH, and behavior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

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

3. Results

3.1. Relationship between effects of SSRI exposure and pH on larval zebrafish movement

A



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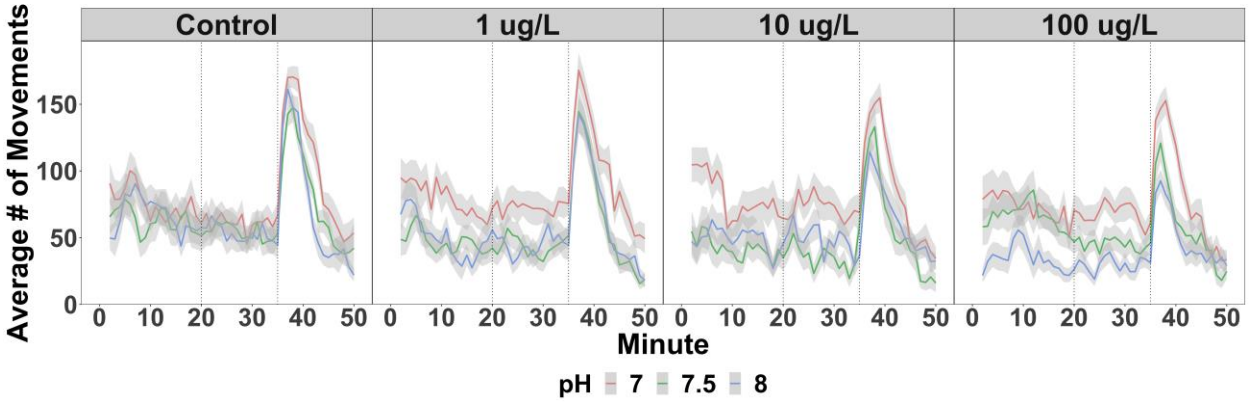

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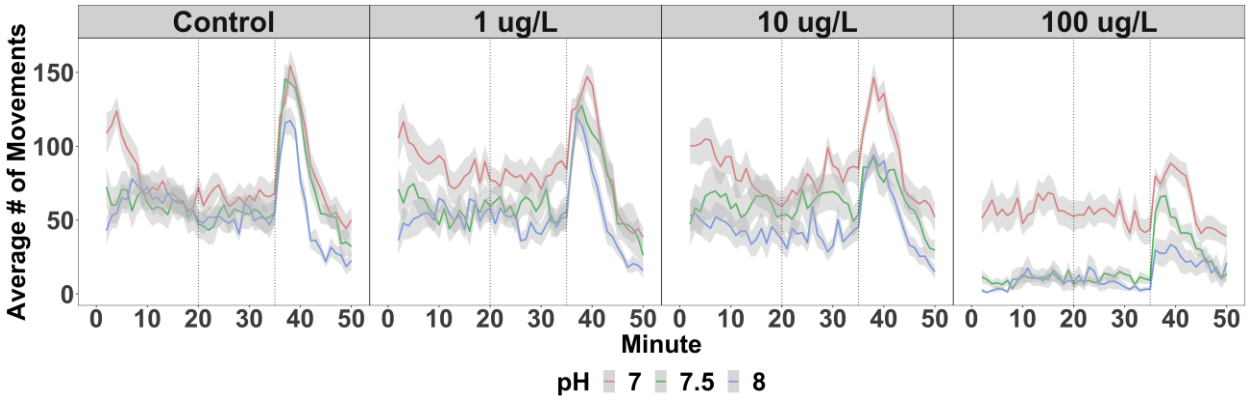

Figure 2. Average number of movements made per minute and standard error intervals in different SSRI doses and pH levels. A represents sertraline, B represents citalopram, and C represents fluoxetine. The

dotted lines separate the different stages of the behavior paradigm. Standard error is represented by the shaded areas. There was a significant effect of pH ($p = 6.377e-10$, $p = 0.025834$, $p = 0.000816$, respectively) in all three experiments on zebrafish movement. Sertraline and citalopram exposure had an overall significant effect ($p < 2.2e-16$, $p = 0.086975$, respectively) on the number of movements, while fluoxetine did not ($p = 0.237605$). A significant synergistic interaction was found between pH and sertraline dose ($p = 0.02874$), while interactions between pH and citalopram or fluoxetine were almost significant ($p = 0.069354$ and $p = 0.109214$, respectively). N = 16-20

In the sertraline (Fig. 2A) experiment, increasing pH had a significant hypoactive effect on swimming behavior. Compared to pH 7, pH 7.5 caused a 30% decrease and pH 8 caused a 46% decrease. Higher doses of sertraline caused significant hypoactivity as well, reducing movement by 68% from controls at the 100 µg/L. There was a significant positive interaction between sertraline dose and pH level, where the combination of high pH and high dose decreased swimming behavior more than anticipated. Pairwise comparisons that fixed sertraline dose but varied pH showed that there no significant differences in the number of movements at 0 µg/L and 1 µg/L. However, when sertraline dose was increased to 10 µg/L, there was a significant difference during the acclimation and post-stimulation stages between pH 7 and pH 8. Furthermore, at 100 µg/L, there were significant differences were between pH 7 and pH 7.5 as well as between pH 7 and pH 8 during the post-stimulation period.

In the citalopram experiment (Fig. 2B), increasing pH also had a significant hypoactive effect on swimming behavior. There was a 31% and 34% reduction in movement for pH 7.5 and pH 8 compared to pH 7, respectively. Increasing citalopram dose showed a near-significant hypoactive trend, exhibited by a 19% decrease in movement at a dose of 100 µg/L compared to the control . In addition, the interaction between these two factors was almost significant and is likely still biologically important despite not making the 0.05 p-value cutoff. Pairwise comparisons showed that while changing pH alone did not cause any significant changes in behavior, it did when combined with citalopram exposure. At 1 µg/L, a near-significant ($p = 0.050234$) difference was determined during the post-stimulation stage between pH 7 and pH 7.5. When the dose was increased to 10 µg/L, there was a significant difference ($p = 0.041966$) during the pre-stimulation stage between pH 7 and pH 7.5. At 100 µg/L, there were significant differences between pH 7 and pH 8 during the acclimation ($p = 0.038388$) and pre-stimulation stages ($p = 0.021933$). The movement reduction of 16% caused by 100 µg/L of citalopram

exposure during the post-stimulation stage was also near-significant ($p = 0.094686$), which may indicate that the stressful environment induced by the sudden light change can increase the hypoactive effect of sertraline.

In the fluoxetine experiment (Fig. 2C), pH again had a significant effect on swimming behavior, reducing movement by 32% at pH 7.5 and 45% at pH 8 compared to pH 7. Overall, while there was a 56% difference in movement between the control and 100 µg/L, the effect of fluoxetine on swimming behavior was not significant, and neither was the interaction between fluoxetine dose and pH. However, several pairwise comparisons indicated otherwise. For example, some significant differences in number of movements include: between 0 µg/L and 100 µg/L during the acclimation stage at pH 7.5 ($p = 0.000279$); between 10 µg/L and 100 µg/L during the acclimation stage at pH 7.5 ($p = 0.000789$); between 10 µg/L and 100 µg/L during the pre-stimulation stage at pH 7.5 ($p = 0.000413$); and between 0 µg/L and 100 µg/L during the post-stimulation stage at pH 7.5 ($p = 0.000393$). Moreover, as fluoxetine dose increased, more pairwise comparisons between varying pH levels showed significant differences in number of movements. At 0 µg/L, there was only one instance that was significant, which was between pH 7 and pH 8 during the post-stimulation stage ($p = 0.022694$). At 1 µg/L, there was also only one instance, but when fluoxetine dose increased to 10 µg/L and 100 µg/L, the number of pairwise comparisons that were significant increased from three and five, respectively.

3.2. Relationship between effects of microbiome status and pH on larval zebrafish movement

As shown in Fig. 3, the microbiome status had a significant effect on swimming behavior. Compared to the control group, microbiome-reduced zebrafish were hyperactive by 17%. Conventionalized fish did not exhibit any significant differences from the controls, with only a 4% increase in movement. Once again, increasing pH also had a significant hypoactive effect, with a 21% and 25% decrease in movement for pH 7.5 and pH 8, respectively, when compared to pH 7 . There was no significant interaction the two factors. However, both the microbiome status and pH had a significant relationship with the paradigm stage ($p = 0.0001081$ and $p = 0.0429257$, respectively), indicating that the effects of each variable on locomotion may be magnified in less stressful situations, since the difference was more significant in the acclimation and pre-stimulation stages.

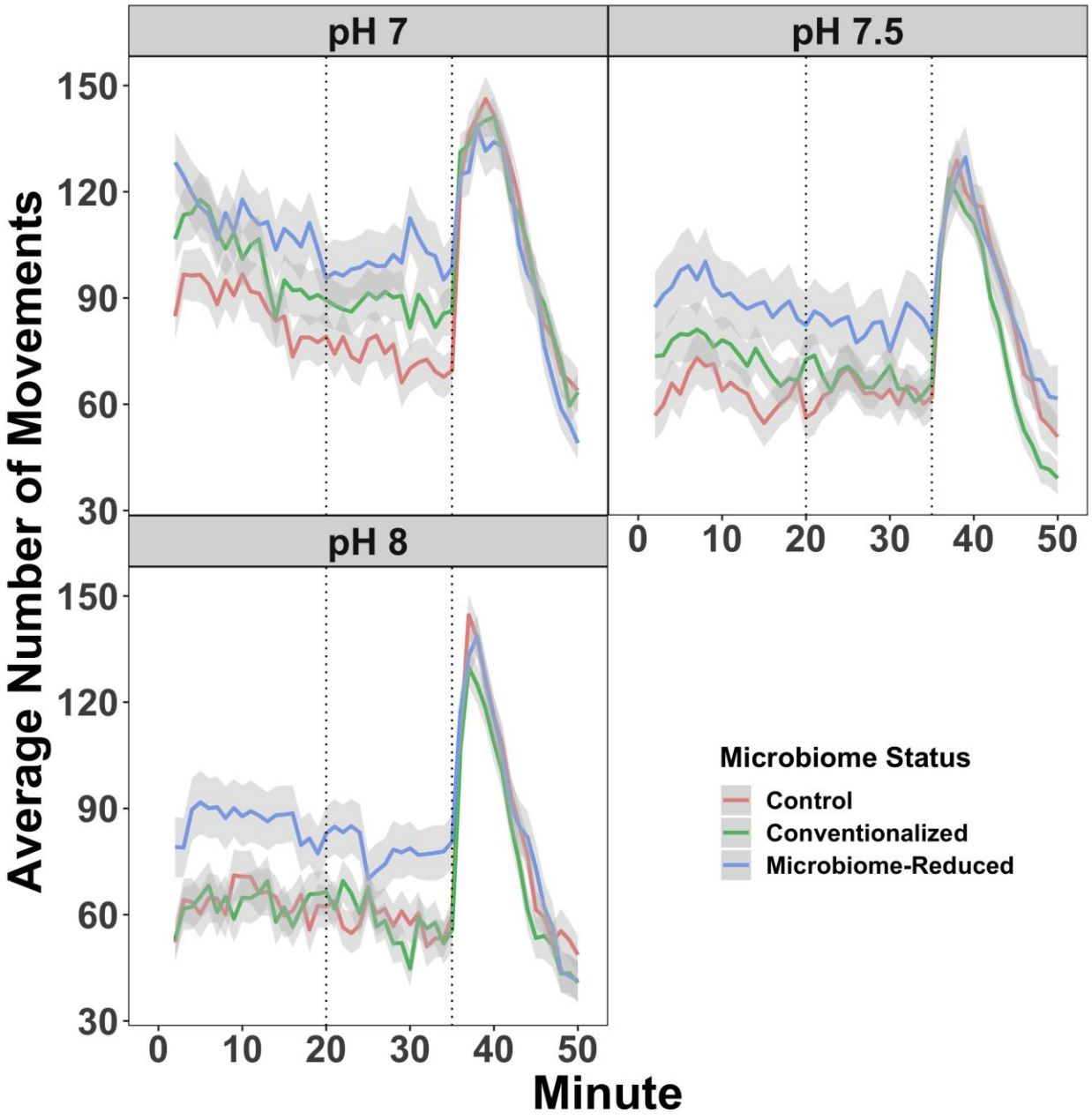

Figure 3. Average number of movements made per minute in different microbiome statuses and pH levels. The dotted lines separate the different stages of the behavior paradigm. Standard error is represented by the shaded areas. There was a significant effect of microbiome status ($p = 0.0019416$) and pH ($p = 5.512e-10$) on the number of movements, but no significant interaction was found between microbiome status and pH. N = 28 - 64

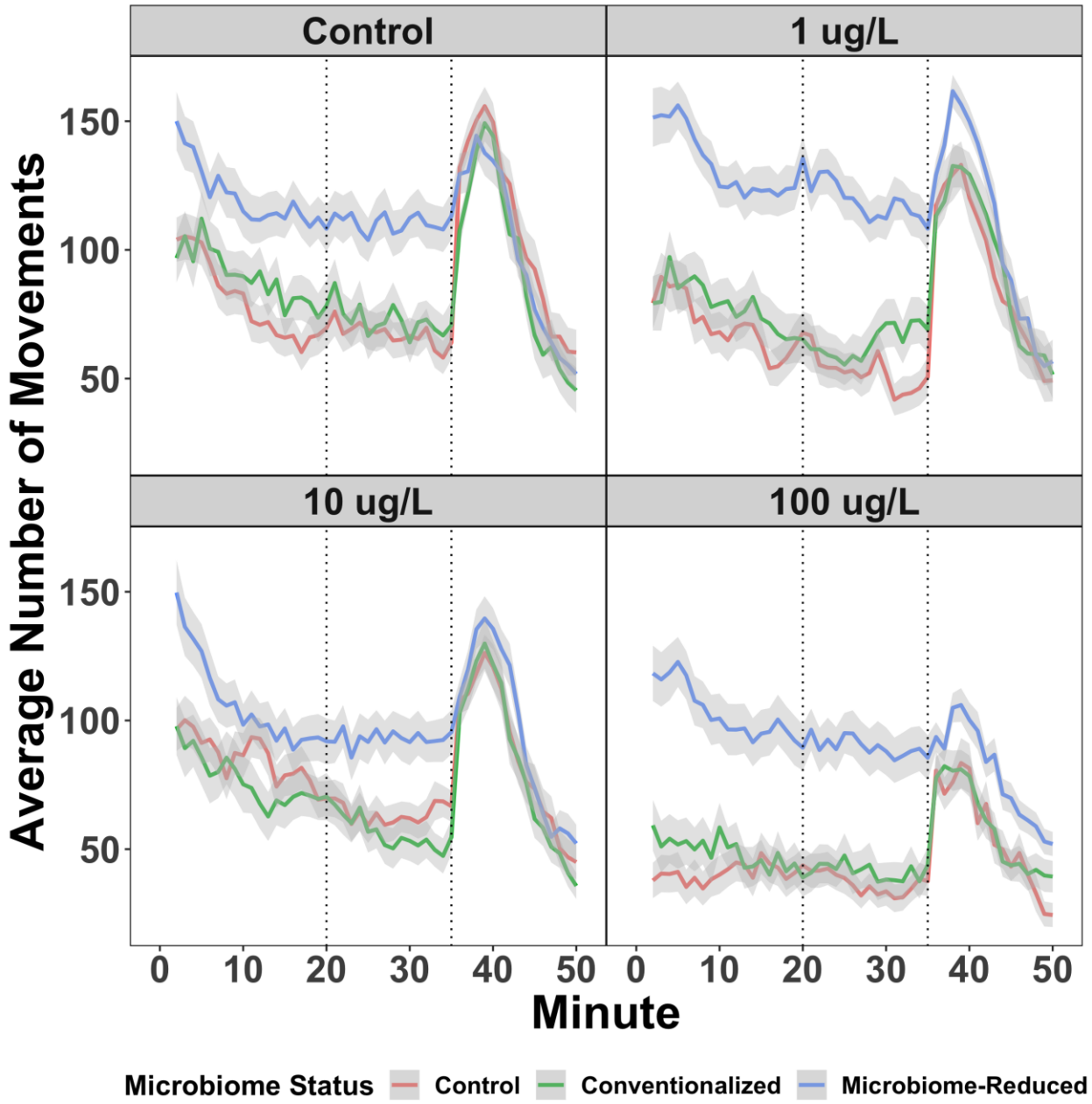

Figure 4. Average number of movements made per minute in different microbiome conditions and sertraline doses. The dotted lines separate the different stages of the behavior paradigm. Standard error is represented by the shaded areas. There was a very significant effect of microbiome status ($p < 2.2e-16$) and sertraline exposure ($p < 2.2e-16$) on the number of movements. Significant interactions were found between microbiome status and sertraline exposure ($p < 2.2e-16$). N = 29 - 43

3.3. Relationship between effects of microbiome status and sertraline exposure on larval zebrafish movement

Based on Fig. 4, both the microbiome status and sertraline dose had extremely significant, although opposing, effects on swimming behavior (Fig 4). Microbiome-reduced zebrafish were hyperactive compared to control and conventionalized zebrafish, with a 34% increase from the control. In contrast, higher sertraline dose caused hypoactivity, as shown by a 33% decrease in movement from control at a 100 µg/L dose. A significant interaction was found between these two variables, meaning that they were either magnifying or diminishing each other's effects. When looked at with the addition of paradigm stage, another significant interaction was determined ($p = 0.001023$). This implied that the extent to which the behavioral effects of the microbiome and sertraline dose were synergistic or antagonistic depended on the paradigm stage. From the data, it seemed that as the sertraline dose increased, the differences in the number of movements among the different microbiome conditions is magnified more so during the acclimation and pre-stimulation stages than during the post-stimulation stage.

Looking at pairwise comparisons, the effect of the microbiome is significant during the acclimation ($p = 5.19e\text{-}5$ at 0 µg/L; $p = 6.49e\text{-}13$ at 1 µg/L; $p = 6.17e\text{-}13$) and pre-stimulation ($p = 3.76e\text{-}6$ at 0 µg/L; $p = 2.62e\text{-}13$ at 1 µg/L; $p = 2.03e\text{-}10$ at 100 µg/L) stages in all sertraline doses except 10 µg/L. However, in the post-stimulation stage, this effect is only significant at 100 µg/L ($p = 0.008401$). From 1 µg/L to 10 µg/L, there was a greater reduction in movement in the GF zebrafish compared to the control and conventionalized groups, which contributed to the lack of a significant difference between the distinct microbiome statuses at 10 µg/L. This difference was significant again at 100 µg/L due to the depression in movement seen in the control and conventionalized groups from 10 µg/L to 100 µg/L, while GF zebrafish movement remained relatively constant between these two doses.

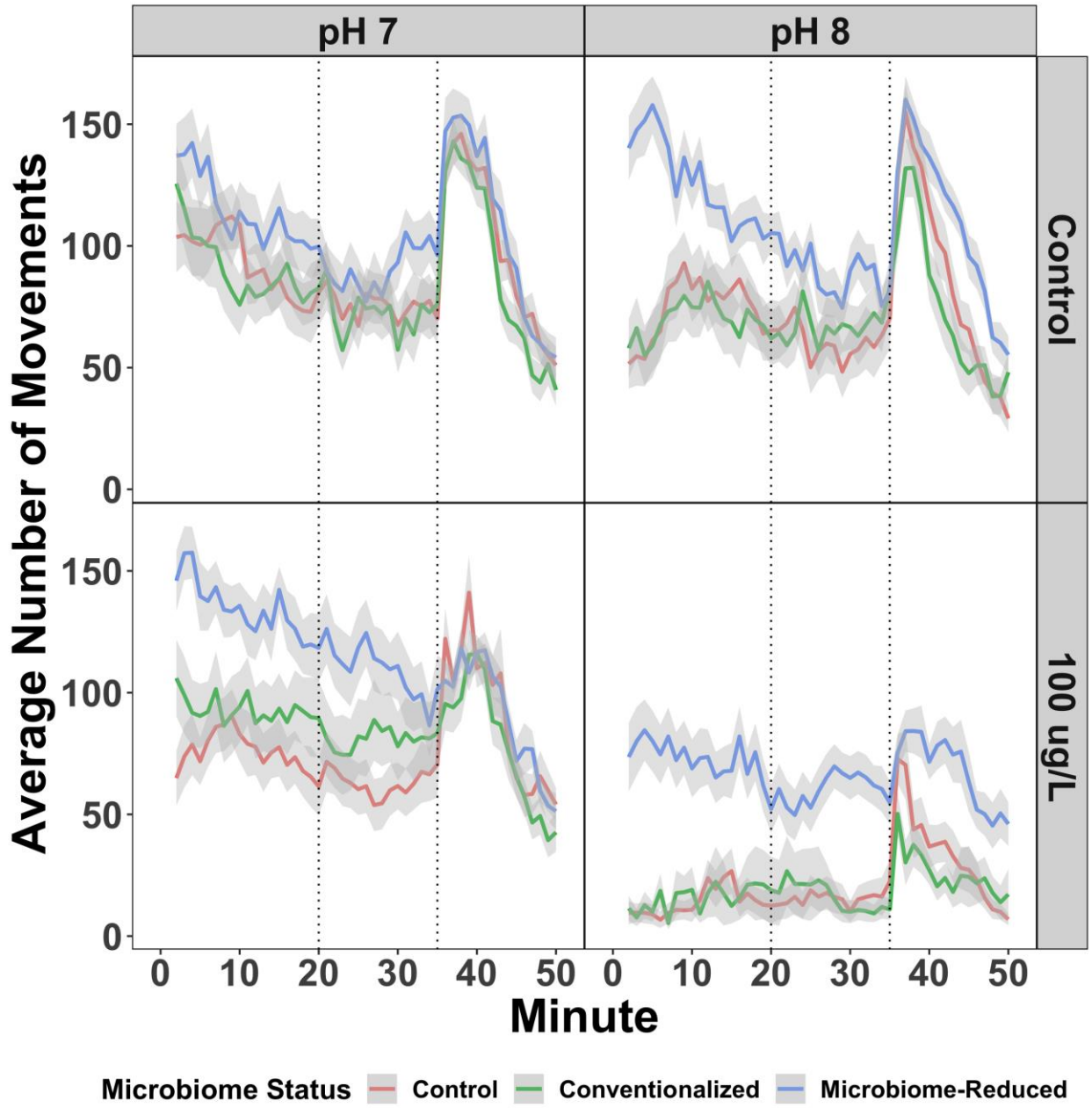

Figure 5. Average number of movements made per minute in different microbiome statuses, sertraline doses, and pH levels. The dotted lines separate the different stages of the behavior paradigm. Standard error is represented by the shaded areas. There was a significant effect of sertraline exposure ($p = 4.994e-8$) and a near-significant effect of pH ($p = 0.05172$) on zebrafish movement, but no significant effect of microbiome status ($p = 0.58902$). There was a significant interaction between sertraline exposure and pH ($p = 4.730e-9$. N = 22 - 24

3.4. Relationship between effects of microbiome status, pH, and sertraline exposure on larval zebrafish movement

According to Fig. 5, increasing sertraline dose once again had a significant hypoactive effect, with a 28% increase in activity at 100 µg/L compared to the control. Contrary to prior results, while there was a similar 34% increase in activity in the microbiome-reduced zebrafish compared to the control, the difference was not significant in this experiment. In addition, while close, pH also did not have a significant effect, even though pH 8 caused 35% hypoactivity compared to pH 7. Especially at 100 µg/L, it does seem that higher pH causes hypoactivity. This is supported by the significant relationship found between pH and sertraline exposure, which was also noted in the first set of experiments involving only SSRI exposure and pH. In addition, this relationship depended on the paradigm stage ($p = 0.04471$), as differences in number of movements were greater during the acclimation stage than during the pre-stimulation and post-stimulation stages.

4. Discussion

4.1. Hypoactive effect between sertraline exposure and high pH level is magnified bidirectionally

The first set of experiments (Figure 2) involving different SSRI doses and pH levels showed a significant interaction between the two factors. Statistical analyses showed that there was a synergistic hypoactive effect on behavior when SSRI dose increased and pH increased. In fact, while fluoxetine was the only SSRI tested whose dose did not have an overall significant effect on the number of movements (Figure 2C), a number of pairwise comparisons showed that behavior was significantly different between doses at more basic pH levels. Previous research has shown that increasing pH amplifies the uptake of several SSRIs, including sertraline and fluoxetine, into various species of aquatic organisms (Boström et al., 2015; Nakamura et al., 2008; Valenti et al., 2009). While this investigation did not explicitly measure SSRI levels in the zebrafish tissue, it suggests a similar conclusion since swimming behavior is known to be reduced by SSRI exposure (Theodoridi et al., 2017). It follows that if behavior is further reduced at high pH levels, it is plausible that more SSRI is being incorporated into the organism. This offers additional evidence for the modulating effect of pH on SSRI toxicity, since actual

behavior is shown to be affected. The inclusion of citalopram in this study provides further support that SSRIs in general may have a greater impact at higher pH levels, which can have implications on the extent to which they share a common mechanism of action. Therefore, the results of this set of experiments support the hypothesis that the effect of SSRIs can be impacted by external conditions

Alternatively, higher doses of SSRI may also be exacerbating the hypoactive effect that increasing pH has on zebrafish behavior. Studies on mice and of human patient data have suggested that the efficacy of SSRIs is dependent on the environment of an organism (Branchi et al., 2013; Viglione et al., 2017). This can be explained by the possibility that the mechanism by which SSRIs work is through inducing neuroplasticity changes, thereby making organisms more responsive to environmental stimuli (Andrade & Rao, 2010). Since pH by itself has been shown to affect swimming activity (Zahangir et al., 2015), it is possible to conclude that stress-induced hypoactivity caused by alkaline pH is being magnified by high SSRI doses that increase SSRIs' ability to remodel brain circuits in response to environmental change. If this is the case, then it is possible that SSRI dose and pH influence each other bidirectionally.

4.2. No interaction determined between pH and microbiome

The second experiment (Figure 3) tested for any interplay between the environmental and individual factors used in this study, namely pH and microbiome status, respectively. An altered microbiome was shown to have a hyperactive effect on swimming behavior. This is consistent with previous research that has shown increased locomotor behavior in mice and zebrafish (Heijtz et al., 2011). The lack of a significant interaction between pH and microbiome status means that environmental and individual variables do not always affect one another. This may indicate that there are different pathways by which SSRIs can affect behavior.

This set of experiments also showed that the effects of pH and microbiome status on behavior is greater during the acclimation and pre-stimulation stages of the paradigm, indicating that less stressful environments make zebrafish more prone to both external and internal influences.

4.3. Microbiome status determines threshold dose for sertraline's hypoactive effect

This set of experiments (Figure 4) further supports the fact that altering the microbiome and changing sertraline dose affects swimming behavior, as first shown by the previous experiments' results in Figures 2 and 3. In addition, the significant relationship between the two factors may indicate that the status of the microbiome may be able to modulate how sertraline dose affects behavior, or vice versa.

Based on Figure 4, distinctions in behavior between zebrafish of different microbiome statuses were more apparent at 0 µg/L, 1 µg/L and 100 µg/L, as opposed to at 10 µg/L. From 1 µg/L to 10 µg/L, the number of movements decreased significantly in microbiome-reduced zebrafish, but not in the control and conventionalized zebrafish. This may be a result of microbiome-reduced zebrafish being more sensitive to the hypoactive effect of sertraline than zebrafish with an intact or reintroduced microbiome. This is supported by a recent study indicating that certain microbes are able to impact the antidepressive effects of SSRIs, as well as SSRIs being able to impact microbiome composition (Lukić et al., 2019). However, between 10 µg/L and 100 µg/L, decrease in swimming behavior is significant in the control and conventionalized groups, while the microbiome-reduced zebrafish's movements remained relatively constant. This implies that the threshold capacity at which sertraline dose affects zebrafish behavior is lower for zebrafish with reduced microbiomes, and that the movements of those zebrafish are not further affected by increasing sertraline dose after reaching the threshold.

4.4. Influence of pH is dominant over microbiome status

The results from the last experiment (Figure 5) reinforced the hypothesis that pH influences the effect that sertraline has on swimming behavior. A significant effect of pH was found in all three experiments involving pH, along with the significant interaction it had with sertraline dose, creating a magnified hypoactive effect that severely decreased swimming behavior at their highest levels. However, sertraline by itself did not seem to have a significant effect on behavior this time, and neither did microbiome status. This contrasts with the results from the previous experiments. A possible explanation for this is that the combined synergistic effect of alkaline pH and high sertraline dose reduces swimming behavior to an extent in which the hyperactivity induced by reducing the microbiome is masked. In addition, the lack of a significant sertraline effect but the presence of a significant effect when combined with pH adds

support to a proposed mechanism of SSRIs, which states that they affect brain plasticity, allowing environmental stimuli to have a greater influence on an organism (Andrade & Rao, 2010).

5. Conclusion

In this study, the effect of SSRIs on behavior was shown to be impacted by both microbiome composition and pH, presenting new evidence that the efficacy of these drugs can be affected by both individual and environmental variables. Increasing pH resulted in hypoactivity, while reducing the microbial population also impacted the mechanism by which SSRIs affect larval zebrafish behavior, shown by an increase in activity. It was consistently determined that high pH and high SSRI doses create a synergistic hypoactive effect in which both factors possibly magnified each other. On the other hand, pH and microbiome status did not affect each other, signifying that the pathways in which they influenced the hypoactive effect of sertraline were mutually exclusive. The combined effect of basic pH and high sertraline dose were shown to abolish the hyperactive effect of reducing the microbiome. These results provide further support that the mechanism of action of SSRIs is involved in many complex networks such as the gut-brain axis, and that their efficacy is dependent upon both individual traits as well as environmental conditions. The possibility of a threshold SSRI dose that varies with microbiome composition, as demonstrated by this investigation, may have implications for the way these drugs are prescribed to patients suffering from depression. Additional tests that assess different animal behaviors should be carried out in order to provide further support for these results. Research should also be conducted on the cellular and molecular end of the spectrum to determine the biological mechanism by which these phenomena occur. Through these efforts, a more comprehensive understanding of the efficacy of SSRIs can be attained, leading to more specialized treatment for patients that considers their individual characteristics as well as their environment.

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