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Do Zebrafish Exhibit a Placebo Response? Fluoxetine Conditioning in Chronically Stressed Adult Zebrafish (*Danio rerio*)

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DO ZEBRAFISH EXHIBIT A PLACEBO RESPONSE? FLUOXETINE
CONDITIONING IN CHRONICALLY STRESSED ADULT ZEBRAFISH (DANIO
RERIO)

by

Kanza Musarrat Khan

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in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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ABSTRACT

Placebo responses are a widely observed phenomenon in humans and animals alike. In humans, placebo responses are largely attributed to expectancy processes, and conditioning (Stewart-Williams, & Podd, 2004). In the clinical setting, the placebo response is very useful as it has the power to improve physical and/or psychological states, without the need for treatment with a higher drug dose. In clinical trials however, researchers must control for potential placebo effects. Still, despite the experimental control, placebo responses are widely observed during phase 2 and phase 3 clinical trials, resulting in a weak drug effect. In the effort to improve the efficiency of drug discovery and development, it is necessary to better understand the placebo response. The current study tested a protocol that examined the existence of placebo responses in chronically stressed adult zebrafish. Over a 30 day period, animals were subjected to an unpredictable chronic mild stress (UCMS) procedure (Piato et al., 2011). Concurrent with the UCMS protocol, stressed animals were conditioned to receive antidepressant (fluoxetine) treatment in a visually distinct arena to protect against stress in the zebrafish. The conditioned placebo response was then evaluated in the novel tank test following the placebo session (treatment with system water). Exposure to the placebo dose produced a slight anxiety-like response and this compensatory response has been observed in different domains of drug conditioning, supporting the conditioning model. The results of this study contribute to our current understanding of the placebo responses in animal models, specifically in a stressed animal model.

ACKNOWLEDGMENTS

DEDICATION

This dissertation is dedicated to Sarah. You have been my rock and my

cheerleader. Thank you for everything that you have done and continue to do for me.

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CHAPTER I - INTRODUCTION

In the clinical setting, substances or procedures that do not have any inherent therapeutic power can often improve physical or psychological states. The administration of these inert substances or procedures have the potential to reduce pain perception, alter sensorimotor performance, and improve mood (Fillmore, Mulvihill, & Vogel-Sprott, 1994; Peciña et al., 2015; Voudouris, Peck, & Coleman, 1985). The interventions that bring about these effects are referred to as *placebos*, and the improvement in condition that they produce is termed the *placebo response* (Oken, 2008).

The Placebo Response

Despite the inert nature of the placebo, there have been many reports of an improvement in mental or physical state following its administration. Early attempts to understand the placebo phenomenon led researchers to study conditioned responses and the expectations that they produce an improvement in condition (Stewart-Williams & Podd, 2004). In one of the first experimental analyses of the placebo effect in humans, participants were conditioned to have an analgesic placebo response to an inert therapeutic cream (Voudouris et al., 1985). Over three phases, the conditioned placebo response was evaluated. In phase one, baseline responses to a painful stimulus were measured. In phase two, participants experienced a pairing of the 'analgesic' cream in which the participant was told that the pain induction level would remain constant, but in reality, the researcher reduced pain induction levels. Thus, the participant was conditioned to associate a reduction in pain levels with the administration of the cream. In phase three, perceived pain levels with and without the cream were measured. The results showed that individuals conditioned to associate the cream with a reduction in

pain levels reported a lower pain perception level in phase three, than the non-conditioned group.

In their study, Voudouris et al. (1985) evaluated self-reports of pain, though placebo responses may also be observed in objective measures. For instance, following third molar extractions, patients receiving a placebo treatment (an ultrasound massage, but the machine was turned off) showed a reduction in facial swelling (Ho, Hashish, Salmon, Freeman, & Harvey, 1988). Similarly, placebo treatment may prevent bronchoconstriction in asthma prone individuals, and alter prefrontal cortex activity in clinically depressed patients (Butler & Steptoe, 1986; Leuchter, Cook, Witte, Morgan, & Abrams, 2002).

These experimental studies are valuable as they not only demonstrate the existence of a genuine physiological response to a sham treatment, but they also highlight the complex nature of the placebo phenomenon. In clinical settings, the placebo response has been observed in many areas of study, ranging from ulcerative colitis and irritable bowel syndrome, to chronic pain management and the treatment of psychiatric conditions like panic disorder or depression (Furukawa et al., 2016; Lu & Chang, 2011; Petrovic, Kalso, Petersson, & Ingvar, 2002; Su, Lewis, Goldberg, Brensinger, & Lichtenstein, 2007).

Great efforts have been made to identify and characterize factors contributing to the placebo response. Meta-analytic studies have found that psychosocial factors surrounding treatment, study design, and disease / symptom severity play significant roles in the production and strength of a placebo response. Psychosocial factors include having a previous experience with a drug or treatment, as well as having an expectation

(conferred through observational learning or verbal communication) that a treatment will improve condition (Benedetti et al., 2003; McMillan, 1999). Study design has also been found to influence rates of placebo and improvement. Specifically, the way in which the dependent variable is measured may inflate rates of placebo (Rief et al., 2009). Other factors affecting placebo response rates include symptom severity. This is highlighted in comparing response rates of patients diagnosed with dysthymia against those diagnosed with major depression. Typically, patients diagnosed with dysthymia (a chronic depression) have diminished placebo response rates versus patients diagnosed with major depression (which has a more variable and cyclic course). Similar patterns of differing placebo rates based on diagnostic severity have been noted in other mental disorders (Huppert et al., 2004; Rief et al., 2009).

In clinical practice, the placebo effect may be used to enhance the medication response. For instance, by boosting a patient's expectation of drug efficacy, it may be possible to produce stronger drug effects without increasing drug dosage (Stewart-Williams & Podd, 2004). In clinical trials, however, the main aim is to demonstrate drug efficacy above and beyond that afforded by placebo responses. Thus, in clinical trials, researchers must be cautious in their experimental design to adequately control for placebo effects.

Clinical Trials: Drug Versus Placebo Responses

Newly developed drugs must go through several phases of testing in both animal and human trials. The purpose of clinical trial testing is to determine the efficacy of the novel treatment against the placebo response and to identify adverse outcomes. In clinical trials, placebos are routinely used in randomized double-blind placebo-controlled trials to

determine the efficacy of a new treatment. In such a study, the participants are randomly divided into 2 or more groups where they receive either the novel treatment or the sham treatment.

The process begins with preclinical testing. In this phase the drug is tested in animal models to demonstrate safety (i.e., the proposed medication should not cause chromosomal damage, and should not be toxic at the doses that would be considered effective). Based on the findings from the preclinical testing phase, an application may be submitted to the Food & Drug Administration (FDA) to test the investigational new drug (IND). If approved, the IND is followed up with several phases of clinical trials (a brief overview of the process is described below; Lipsky & Sharp, 2001).

Clinical trials follow a series of phases that start out as small-scale studies and progressively get larger in sample size and duration. The process for approving a drug through the FDA is a highly thorough, albeit long and expensive process. On average, for every 5,000-10,000 new molecules that enter preclinical testing, only one gets approved for marketing. Further, this testing and approval process takes 10+ years (Van Norman, 2016).

Once a new drug has been approved through preclinical testing, it is tested in a Phase 1 clinical trial. Over the course of several months, the focal drug is administered in low doses to healthy human volunteers or people diagnosed with the disease (20-100 individuals). The main purpose of Phase 1 testing is to determine drug safety, and effective dosages. Roughly 70% of the drugs that enter Phase 1 get approved for further testing.

In Phase 2, the sample size is increased (up to several hundred volunteers), and the drug is tested in individuals diagnosed with the focal disease / condition. This phase may last up to two years, during which time the efficacy and side effects of the drug are evaluated. Many drugs do not move onto the next phase; approximately only 33% of drugs tested in this phase will move on to Phase 3. This low approval is attributed to a combination of low safety, low efficacy, and intolerable side effects.

Drugs that pass safety and efficacy tests in Phase 2 will graduate to Phase 3 of the clinical trial. Here, the sample size is increased (300-3,000 volunteers diagnosed with the disease). Over the course of 1-4 years, efficacy and adverse reactions to the drug are monitored. Because of the long duration, these studies provide most of the safety data about the drug. Previous testing (Phase 1 or Phase 2), may not be able to show the rare or long-term side effects of the drug. Roughly 25-30% of drugs tested in this phase will move onto Phase 4 of testing. This last phase of testing is following approval by the FDA (U.S. Food and Drug Administration, 2018).

From the initial idea to FDA approval and marketing, the preclinical and clinical testing phases are a long and expensive process. On average, the approval for a single drug takes more than 10 years and costs more than $500 million (Van Norman, 2016). Thus, while the approval process is necessarily thorough, there are certain road blocks that prevent the development of new, safe, and effective drugs. As noted, most drugs tend to fail during Phase 2 and Phase 3. There are many drivers of these failures. Included among these are a flawed study design, poor understanding of the disease biology, type of drug being tested (e.g., oncology treatment, antidepressants, diabetes treatment) and irreproducibility of findings between animals and humans, among others (Grignolo &

Pretorius, 2016). In clinical trials for antidepressant medications for instance, a big culprit for a negative (i.e. failed) trial is that the study yields a weak drug effect – which is in part due to the placebo response (Fava, Evins, Dorer, & Schoenfeld, 2003).

To yield clear and interpretable results, and to minimize the placebo response in clinical trials, researchers have developed many new study designs. Many improvements have focused on the clinical trials at the human level and on integrating computational techniques (Chi, Li, Liu, Lewin, & Lim, 2016; McGuire, Iyengar, & Mercer, 2011). While these methods hold value and provide invaluable insight, they do not take advantage of all available tools, namely animal modelling of disease states and testing the placebo potential of novel drugs in preclinical models.

While animal models are used in preclinical testing to determine mechanisms of action for a drug, there is currently no protocol for testing the placebo potential of a drug in the animal model. By determining whether a drug has a high placebo potential in the preclinical phase, it may be possible to streamline the clinical trial process, thereby allowing time and resources to be focused on drugs that have proven efficacy above placebos. My dissertation aims to fill this gap.

Current Study: Purpose

My dissertation evaluated the placebo effect in the zebrafish animal model. Specifically, I evaluated whether a placebo treatment could reduce the production of stress-related behaviors in chronically stressed zebrafish. In this endeavor, two methods from established protocols in animal (specifically zebrafish) models were combined: Classical conditioning and unpredictable chronic mild stress (UCMS).

Classical Conditioning in Animals

Classical conditioning is a process by which an organism learns to associate an outcome with a paired stimulus. It may include the learned association between the ringing of a bell and the presentation of food or relevant to placebo use, the association between the administration of a drug and symptom relief (Pavlov, 1929; Stewart-Williams & Podd, 2004). This type of learning is observed in humans and animals alike (Pavlov, 1929; Solomon & Turner, 1962; Wikler & Pescor, 1966; Mathur, Lau & Guo, 2011).

In zebrafish animal models, a common use of classical conditioning has been to evaluate the rewarding properties of a drug (Collier, Khan, Caramillo, Mohn, & Echevarria, 2014; Mathur & Guo, 2010). These are typically done through the conditioned place preference (CPP) paradigm (Collier et al., 2014). In this paradigm, a zebrafish is introduced to a tank that has two visually distinct regions. For example, one half of the tank may contain polka dots lining the walls of the tank, while the other may contain stars (Figure 1). The preference between the two regions is measured during a baseline session. After determining the region preference, the zebrafish is subjected to one or more conditioning sessions in which it is conditioned to associate the effects of the drug with the *least* preferred region. In these conditioning sessions, drug administration typically occurs via bath immersion (i.e. the drug is mixed into a solution of system water, and the animal absorbs the drug via the gills). In the conditioning sessions, the zebrafish is physically restricted to either region and is not allowed to travel between the two, which allows the animal to associate the behavioral effects with the environment (Mathur, Berberoglu, & Guo, 2011).
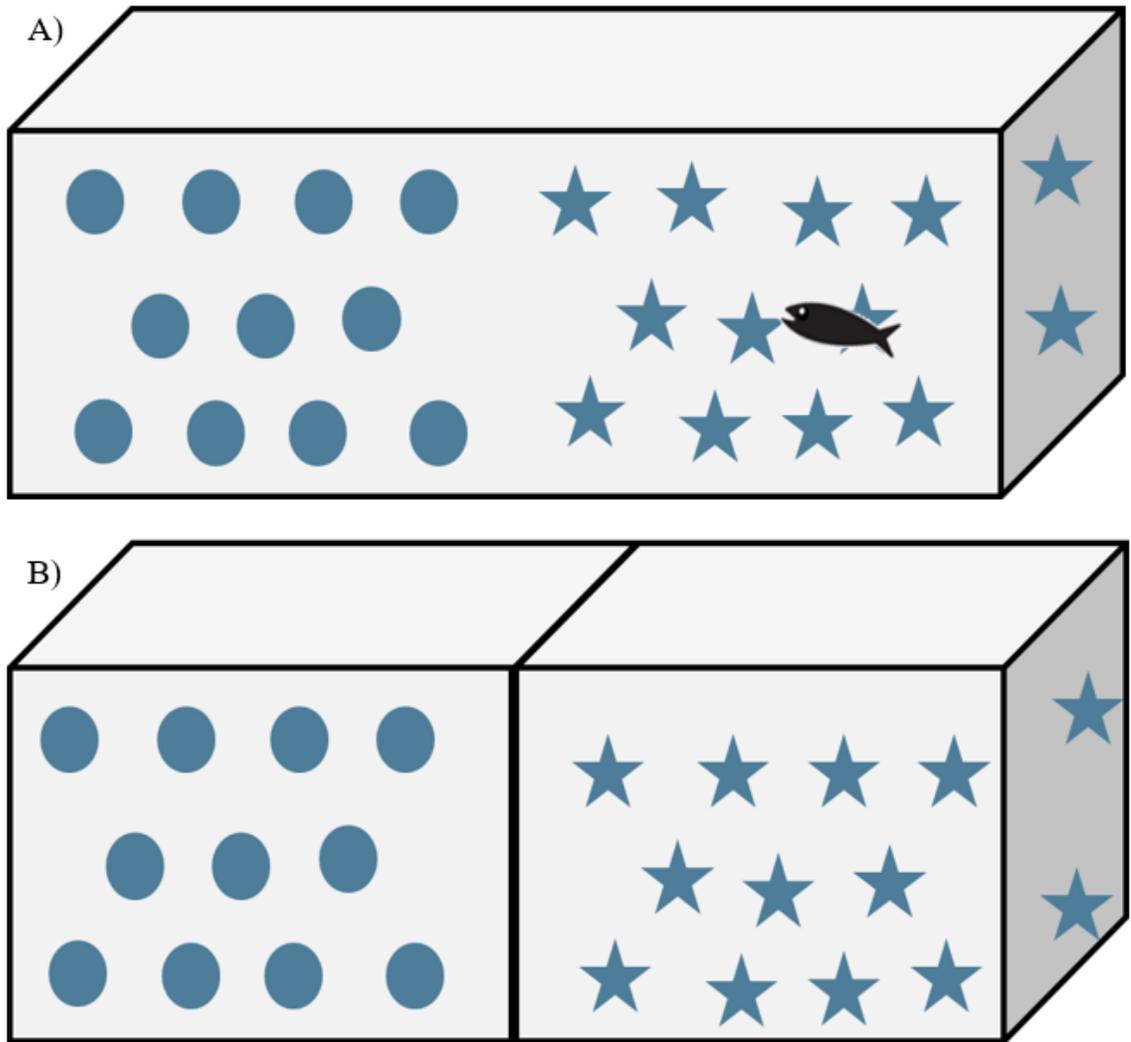
Figure 1. Example of a conditioned place preference task.

(A) In the baseline test phase, and final preference test, the animal is allowed to freely swim between two region of a tank that have different markings on either half. (B) In the conditioning phase, the animal is not allowed to freely move between the two regions. In the least preferred side, the animals will receive drug treatment (via bath solution or intraperitoneal injection). In the preferred side, the animal will be allowed to swim in a bath of system water. Order of exposure to each region is randomized.

When treated with a rewarding drug such as a low dose of ethanol, zebrafish will exhibit a place preference reversal following a single conditioning session (Collier et al, 2014). Nevertheless, researchers often employ multiple training sessions over several days (Faillace et al., 2016). Pertinent to the study of learning, CPP models are very

robust: Zebrafish trained in the CPP paradigm can retain the learned region preferences for up to three weeks after the last training session (Kily et al., 2008).

Placebo Testing in Animals. In the endeavor to study the placebo effect in animal models, many researchers have turned to classical conditioning models. Early examples include Pavlov's (1927) conditioning of a dog to receive morphine injections in a laboratory setting. In this study, dogs were administered a low dose of morphine, which produces several adverse symptoms: Excessive saliva production, vomiting and sleep. After repeated morphine injections in the laboratory environment, the dogs began to exhibit morphine-like symptoms upon return to the environment. The sight of the experimenter, or the sound of the syringe box opening were enough to produce restlessness, saliva secretion, and extreme nausea.

Similar conditioning experiments have been performed in the years since. Hernstein (1962) administered scopolamine hydrobromide (a drug that disrupts learned memory) via injection, to rats. After several conditioning sessions, when the rats were administered an inert substance (saline) via injection, they performed in a scopolamine-like manner. This type of study has been replicated with different types of drugs. Working with adult hooded rats, Pihl and Atlman (1971) characterized the strength and nature of these types of conditioned responses. They found that the strength of the conditioned response was dependent on the number of pairings between the unconditioned (the drug) and the conditioned (e.g., the environment) stimuli. They also discovered that the drug used for conditioning played a role in the placebo response as well. When treating animals with amphetamine (a stimulant) during the conditioning phase, the animals produced stimulant-like behavior during the placebo phase but, when

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treated with chlorpromazine (a tranquilizer) during the conditioning phase, the animals exhibited a significantly greater activity level after receiving a placebo dose (Pihl & Altman, 1971).

Thus, while placebo responses have been documented in animal models, these studies have largely focused on an acute treatment / conditioning phase, that occurred in the absence of a diseased or a stressed state. The current study sought to expand on what is currently understood of the placebo effect in animals by inducing stress states, and then conditioning the animal to receive treatment in a visually distinct environment.

Stress Models in Animals

Animal stress models are widely utilized to study the etiology and neurobiology of affective disorders in humans. Most often, animal stress models are used to replicate and evaluate various symptoms of human depression (Cryan & Holmes, 2005). Rodents are the most widely used model organism for the study of depression; as such, numerous stress models have been developed to evaluate symptoms of the human condition such as depressed mood, decreased pleasure, irritability, weight change, sleep disturbances, and psychomotor disturbances (Castagné, Moser, & Porsolt, 2009). For instance, the chronic mild stress (CMS) model is used to evaluate anhedonia and antidepressant activity (Castagné et al., 2009; Willner, 2016). In this procedure, an animal is exposed to mild stressors such as water restriction, small changes in temperature, or the changing of cage mates, over an extended period of time (Papp, 2012; Willner, 2016). There is no agreed upon minimum for classifying a procedure as chronic, however, the minimum duration for chronic mild stress protocols has been a period of 7 days. As such, the term 'chronic' in this experiment may be defined as any intervention that recurs at least once-daily for a

period of at least 7-days. The CMS procedure generally results in a reduced saccharin intake and impairments in hedonic activity, which are rescued by chronic, but not acute, antidepressant treatment (Papp, 2012). This procedure has generally been shown to have high face validity, predictive validity, and construct validity (Willner, 2005).

Not all stress models are as time consuming as the CMS. For instance, the forced swim and tail suspension test are behavioral despair models and have a higher throughput than the CMS. These and similar tests do not have the same face validity, but provide predictive value in drug screening (Castagné et al., 2009). The forced swim test involves placing the animal in a container filled with water. When first placed in the arena the animal will make efforts to escape but eventually will exhibit immobility, a behavioral sign of despair (Yankelevitch-Yahav et al., 2015). The tail suspension test is a similar despair-based design where the animal is placed in an inescapable and moderately stressful situation. Specifically, mice are suspended by their tails and held in a position that does not allow them to escape or hold onto nearby surfaces (Can et al., 2012; Steru, Chermat, Thierry, & Simon, 1985). The forced swim and tail suspension tests are used to evaluate the effect of genetic, environmental or neurological manipulations on behavioral despair in animals. These tests are also widely used to evaluate the effects of antidepressant treatment on behavioral despair (Can et al., 2012).

Zebrafish Stress Models. The zebrafish stress response begins early in development, as early as 4 days post fertilization (dpf; Griffiths et al., 2012). This reactivity continues into adulthood. Exposure to a stressful stimulus stimulates the secretion of corticotropin-releasing factor (CRF), which in turn, causes the release of

adrenocorticotropic hormone (ACTH). Finally, the head of the kidney (homologous to the human adrenal glands) secretes cortisol, which binds to glucocorticoid receptors, resulting in many stress related behaviors, and the transcription of genes related to immune function (Piato et al., 2011). Zebrafish with a mutation in the gr[357] gene have non-functional glucocorticoid receptors, and present with higher whole body cortisol levels, and greater startle responses (Griffiths et al., 2012). These heightened anxiety-like responses are rescued with treatment with fluoxetine, suggesting this model is amenable to the study of stress disorders via genetic manipulations.

Behavioral models have also been developed for the zebrafish. The unpredictable chronic mild stress (UCMS) protocol, similar to the rodent model, is a chronic stress paradigm that involves subjecting animals to a variety of mild stressors for a period of at least 7 days (Fulcher, Tran, Shams, Chatterjee, & Gerlai, 2017; Piato et al., 2011; Song et al., 2017). Stressors include restraint in a 5mL tube, net chasing, air exposure, dorsal body exposure, changes in water temperature, among others.

A seven-day exposure to the UCMS protocol produces increased CRF expression and increases whole-body cortisol expression. This is met with a reduction in GR expression, similar to the human symptomology (Piato et al., 2011). After 7 days of the UCMS protocol, fish maintain a lower height in the tank, and tend to form a tighter shoal – a pattern suggestive of an adaptive behavior in the face of stressors (Piato et al., 2011).

After 14+ days of the UCMS protocol, there is a marked decrease in shoaling. Since zebrafish are a highly social species, and shoaling is seen as an adaptive behavior, the aberrant loss of shoal cohesion is seen as maladaptive and interpreted as a loss in resiliency (i.e., loss in resiliency to maintain shoal cohesion), and is compatible with a

depressive-like behavior (Chakravarty et al., 2013; Piato et al., 2011). Reduced shoal cohesion is also accompanied by cognitive deficits, whereby stressed animals perform poorly in an inhibitory avoidance task, exhibit an overall reduction in locomotion (interpreted as lethargy), and a reduction in novelty seeking (Chakravarty et al., 2013; Piato et al., 2011). Stressed fish will enter the top regions of the tank (in the novel tank test) fewer times and exhibit a lower latency to enter darker environments (in the light-dark task).

The novel tank is typically a narrow 1.5L tank that mainly affords vertical swimming. It is widely used to evaluate swim patterns following drug exposure, and to study the effects of anxiolytic or anxiogenic agents. Upon exposure to a stressful stimulus, zebrafish will tend to dive to deeper regions of their environment, which is interpreted as an effort to escape potential predation. Since the arena used in the novel tank test affords mainly vertical swimming, the main behavior evaluated in this task is the amount of time the animal spends in the bottom regions (vs. top) of the tank (Stewart et al., 2012). The light-dark test is based on the natural preference of the zebrafish to seek out darker environments following an exposure to a stressful stimulus. Though the novel tank test and light-dark task are typically used to assess anxiety-like behaviors, these tests are also commonly used to evaluate swim patterns following several days of the UCMS protocol. These and others (e.g., shoaling test) allow researchers to evaluate vertical exploration (i.e., determine if the fish prefers to remain in the lower regions of the tank) and novelty seeking (Piato et al., 2011; Fulcher et al., 2017; Stewart et al., 2012). Compared with acute stressors, the behaviors elicited by chronic stress via the UCMS

protocol have been shown to elicit severe anxiety-like behaviors, suggesting that this protocol is useful for the modeling of stress related disorders (Chakravarty et al., 2013).

At 14d+ of stress exposure, aberrant behavioral patterns are met with alterations in molecular marker expression. Chronically stressed zebrafish have elevated levels of pro-inflammatory cytokines, IL-1B and IL-6 (Song et al., 2017). Elevated levels of these neuroimmune biomarkers are associated with clinical depression in humans (Dahl et al., 2014), and chronic stress in rodents (Deng et al., 2015).

The detrimental effects of the UCMS protocol are reduced by antidepressant treatment, further adding to the validity of this model. Chronic treatment with 0.1 μg/L fluoxetine for 7 or 14 days reduces the frequency of anxiety-like behaviors and returns the altered molecular marker profile to normal (Chakravarty et al., 2013; Marcon et al., 2016). Typically, in such paradigms, animals are exposed to the drug in their home tank continuously (24h) for several days (Marcon et al., 2016; Stewart et al., 2014).

Hypotheses

The aim of the current study was to determine the existence of placebo responses in the zebrafish and to characterize these responses. In this effort, animals were subjected to a 30-day UCMS protocol and were classically conditioned to receive treatment with an antidepressant (fluoxetine) in a visually distinct arena. Drug administration occurred via immersion in a solution of the drug. The placebo dose was an exposure to the drug arena that contained only system water (i.e., no drug present). Based on previous research, the current study had the following hypotheses:

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H₁: The UCMS protocol would increase the frequency of the anxiety-like behaviors in the novel tank test; specifically, stressed animals would remain in the lower regions of the novel tank environment and be slow to explore the top region.

H₂: Chronic antidepressant treatment would reduce the frequency of anxiety-like behaviors in stressed animals.

H₃: Experience of the placebo dose would result in a reduced anxiety-like phenotype. Placebo-receiving animals would produce fewer anxiety-like behaviors than stressed animals, but not as few as the stressed and chronically treated animals.

CHAPTER II – METHODS

Animal Care and Maintenance

Adult zebrafish (3 months and older) were purchased from a local pet supplier (Pet Palace, Hattiesburg, MS) and allowed to acclimate to the laboratory conditions for at least 10 days. During acclimation to the aquatic system, zebrafish were group housed (20-30/10L tank) in a water recirculating system. Water temperature was approximately 27^0C, and quality was maintained through mechanical, chemical, and biological filtration.

Fish were maintained, and protocols were carried out in accordance with the Institutional Animal Care and Use Committee of the University of Southern Mississippi, Hattiesburg MS, USA. Animals were kept on a 14:10 light-dark cycle and fed twice per day: In the morning with live brine shrimp (Premium Grade Brine Shrimp Eggs, Brine Shrimp Direct, Ogden, UT), and in the afternoon with flake food (Tetra, Blacksburg, VA). All animals were allowed to acclimate to the aquatic systems for a period of at least 10 days prior to experimental manipulation or testing.

Experimental Groups and Procedures

Prior to experimental manipulation, individual animals were transferred to single housing tanks (20cm length x 13cm width x 14 cm water height) and allowed to acclimate for two days. Animals were randomly assigned to one of the four experimental conditions (n=13-15 per condition): No Stress Group (NSG), No Treatment Group (NTG), Treatment Group (TG), and Placebo Group (PG). Animals in the NTG, TG, and PG experienced a battery of mild stressors. Stressor exposure occurred between 09:50 and 17:00 each day.

No Stress Group (NSG): Animals remained in their home tanks unperturbed for the full duration of the experiment (30d). On the final day of the experiment (day 30), the focal animal was transferred from the home tank to the novel tank test for behavior testing.

No Treatment Group (NTG): Animals were subjected to the UCMS protocol, following the schedule outlined in Table 2, for 30 days. On day 30, the focal animal was transferred from the home tank to the novel tank test for behavior testing.

Treatment Group (TG): Animals were subjected to the UCMS paradigm for 30 days. Beginning on day 15, animals received chronic once daily treatment of fluoxetine. After drug treatment the animal was transferred to a rinse tank containing system water for roughly 8-10s before being returned to the home tank. This ensured that there was no externally bound drug when the fish was returned to the water recirculating system. The once-daily drug treatment continued until the final day (day 30). After drug exposure on day 30, the focal animal was transferred to the novel tank test for behavior testing.

Placebo Group (PG): Animals were subjected to the UCMS protocol for 30 days. On days 15-21, individual animals were transferred to the drug tank for fluoxetine treatment. After drug exposure, animals were transferred to the rinse tank before being returned to their respective home tanks. A washout period from days 22-29 allowed the drug to metabolize and be removed from the body; this was termed the depuration phase (Figure 2).
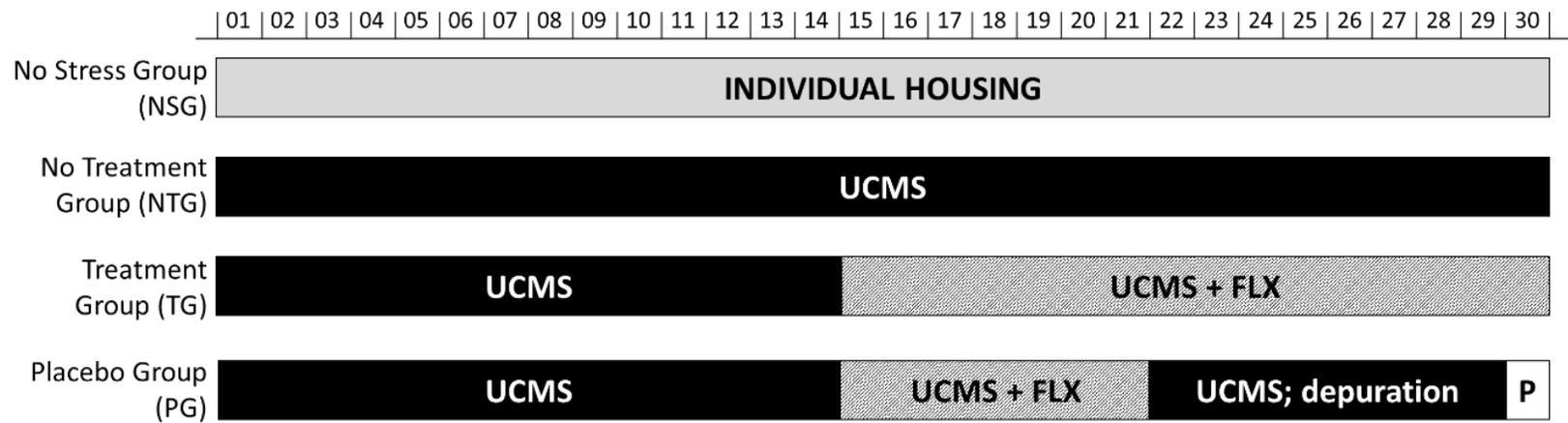
Figure 2. Experimental timeline.

UCMS = Unpredictable Chronic Mild Stress; FLX = fluoxetine treatment (simultaneous with UCMS); depuration = drug washout – animals not transferred to drug arena; P = placebo dose.

Behavior testing in the novel tank test occurred on day 30.

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A treatment period of 7 days was reached based on findings that fluoxetine treatment concurrent with a UCS protocol was effective in reducing the behavioral and physiological effects of the UCS protocol (Marcon et al., 2016). Treatment cessation (8 days) allowed the drug to go through four half-lives, thereby rendering the dose pharmacologically inactive (Chen, Gong, & Kelly, 2017). On day 30, the focal animal was transferred to the drug arena, where in place of fluoxetine, the focal zebrafish remained in a solution of system water. This served as the placebo dose. Immediately following the placebo dose, animals were transferred to the novel tank test for behavior testing.

<div align="center">Drug Treatment</div>

Animals in the TG and PG were treated with the selective serotonin reuptake inhibitor (SSRI), fluoxetine hydrochloride (Alfa Aesar). Individual zebrafish were treated with a 5mg/L dose of fluoxetine in a visually distinct arena (Figure 3). Treatment occurred at roughly 12:00 each day and was a 30-min exposure.

Figure 3. Interior of the drug dosing tank.

Dimensions of the tank are: 17.5cm width x 17.5cm length x 6cm water height. The dots on the interior make the environment visually distinct from any other tanks or arenas. Animals were dosed in a room with illumination at approximately 200 lux.

There is currently little consistency in fluoxetine drug dosing methods for zebrafish. When administering chronic treatment, the drug is added to the home tank water and the fish is allowed to remain in the bath for 24h+ (Marcon et al., 2016; Song et al., 2017). In acute treatment, dosing times and concentrations vary: Some researchers expose the animal to drug for only 3min (de Abreu et al., 2014), while others opt for a 2hr period (Theodoridi, Tsalafouta, & Pavlidis, 2017); dosing concentrations may vary from 0.001mg/L through 10mg/L (de Abreu et al., 2014; Singer, Oreschak, Rhinehart, & Robison, 2016).

A pilot experiment in our lab found that a 30-min exposure at 5mg/L for 7 days was effective in reducing the frequency of anxiety-like behaviors in the novel tank test, compared with 0mg/L and 1mg/L. In this pilot test, animals were transferred once daily to a drug treatment arena, for a treatment of 0 ($N = 4$), 1 ($N = 5$), or 5mg/L ($N = 5$) fluoxetine in a 1L solution. After 30 min of exposure, animals were returned to their home tanks. After 7 days of treatment, anxiety like behaviors were evaluated in the novel tank test; the test occurred 30 min after treatment on day 7 to washout any immediate effects of drug exposure and allow any externally bound drug to become unbound. In the novel tank test, animals receiving the 5mg/L dose maintained a significantly lower swim speed over the 6 min task relative to the non-stressed controls and spent a significantly greater amount of time in the top region of the tank relative to non-treated controls (Table 1).

Table 1 *Pilot data.*

Dose	Total Distance Traveled (cm)	Average Velocity (cm/s)	Top time (s)	Latency to top (s)	Frequency of immobility
0mg/L	2821.2	7.7	123.9	11.4	0.5
1mg/L	2041.9	5.6	155.6	10.4	4.4
5mg/L	2019.6	5.6	222.7	2.6	2.2

Descriptive statistics in the novel tank test to determine the ideal fluoxetine dose. Swim behaviors were evaluated in a 6min NTT after 7-daily 30min drug treatments.

Stress Protocol

Methods were adapted from Piato et al. (2011) and Fulcher et al. (2016). A total of seven stressors were employed. Fish in the NTG, TG, and PG experienced two stressors per day, on a random schedule for a four-week period: Restraint stress, consisting of maintaining each animal in a 2mL Eppendorf tube for 60 min with holes in the tube to allow for water flow; water change, consisting of a ¾ water change three consecutive times, while the animal remained in the tank; cold stress, consisting of a transfer to a 350mL beaker that was maintained at 22^0C for 30 min; heat stress, consisting of a transfer to a 350mL beaker that was maintained at 32^0C for 30 min; chasing the animal in the home tank with a net for 8 min; exposure to atmospheric air (lifting via net) for 2 min; dorsal body exposure, consisting of a transfer to an arena with low water levels to allow the dorsal body to be exposed for 2 min (Table 2).

Table 2 *UCMS Schedule*

Day	Restraint stress	Heat stress	Cold stress	Dorsal body exposure	Elevation with net	Chasing with a net	Water change
Day 1						11:00	16:30
Day 2	13:40		09:50				
Day 3		10:15		14:00			
Day 4					13:45		17:00
Day 5	10:00				16:15		
Day 6			11:15			16:30	
Day 7		16:00		09:45			
Duration / frequency	60 min	30 min	30 min	2 min	2 min	8 min	3 times

Behavioral Task and Tracking

The novel tank was a trapezoidal arena, 28cm top x 23cm bottom x 7 cm width x 15 cm water height (Figure 4). Behaviors were recorded for 20 min via USB camera (Logitech) at 30 frames per second. All videos were recorded in a Quicktime Movie format and converted to an Audio Video Interleave (.avi) file type and processed with ImageJ and idTracker.
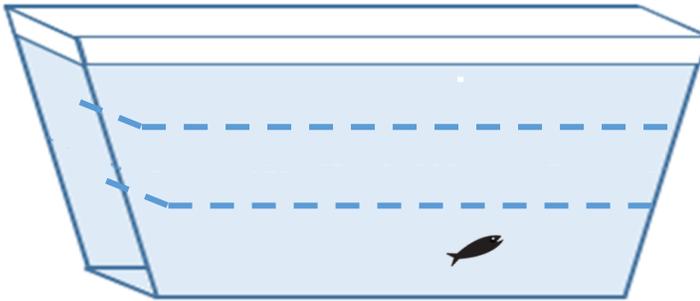
Figure 4. The novel tank test arena.

Dimensions: 28cm top x 23cm bottom x 7cm width x 15cm water height.

idTracker is a MATLAB based software that provides x- and y- coordinates of the animal for each frame in the video (Pérez-Escudero, Vicente-Page, Hinz, Arganda, & de Polavieja, 2014). Data files were then processed through MATLAB to extract behavioral endpoints: Total distance traveled (cm) and average velocity (cm/s); time in the top, and bottom regions of the tank (s); latency to explore the top region of the tank (s); and frequency of erratic swimming and freezing bouts (Table 3).

Table 3 *Endpoint behaviors in the study*

Endpoint	Definition
Total distance traveled	Locomotor activity during the testing session. Measured in cm.

Average swimming speed	Magnitude of speed maintained during the testing session. Measured in cm/s.
High angular velocity	Frequency of incidences when the animal had a turn angle that was in the 95th percentile. Values were based on mean data collected from the animals in the NSG.
Region preference	Amount of time spent in either the top 1/3 or bottom 1/3 of the novel tank arena.
Immobility	Number of times the animal maintained a swim speed of less than 0.5cm/s for at least 1s.

CHAPTER III - RESULTS

Swim behaviors (total distance traveled, average velocity, and region preference) were extracted per minute for each 20-minute video. After behaviors were extracted, a Spearman's correlation was performed for the endpoints Total Distance Traveled and Average Velocity. These two endpoints had a high correlation $r = 0.99$, $p < .001$ (Table 4). Per these results, average velocity was not included in subsequent analyses.

Table 4 *Correlation matrix*

	Total Distance Traveled	Average Velocity	Top Time	Bottom Time
Total Distance Traveled	-			
Average Velocity	.999**	-		
Top Time	.260**	.261**	-	
Bottom Time	-.402**	-.403**	-.864**	-

**denotes that a correlation is significant at the 0.01 level (2-tailed).

The behaviors Frequency of Freezing Bouts and Frequency of High Turn Angle were infrequent (not observed during each minute). Three types of analyses were run on the data: (1) A one-way ANOVA to evaluate differences between groups when animals were first introduced to the tank, (2) repeated measures ANOVAs to evaluate changes in behaviors produced with the passing of time, and (3) a discriminant analysis.

The one-way ANOVA was performed on the first five minutes of the novel tank test. This time period follows from previous studies (e.g., Egan et al., 2009) and evaluates the reaction to the novelty of the environment. The repeated measures ANOVAs was concerned with evaluating behavioral responses with the passing of time; four 5-min time

blocks were evaluated. The discriminant analysis is used to determine which dependent variables are most useful in predicting group membership.

Introduction to the Novel Tank

A one-way ANOVA was performed to identify differences between groups on the mean latency to explore the top region of the novel tank arena (Figure 5). This test violated homogeneity of variance: The Brown-Forsythe correction revealed significant differences across conditions, $F(3, 28.05) = 4.43$, $p = 0.01$, $\eta_p^2 = .21$. The Games-Howell posthoc analysis revealed that the TG animals ($M = 180.42$ sec) had a significantly higher latency to enter the top region of the novel tank relative to the NTG ($M = 216.47$), $p < .001$, $d = 1.98$).
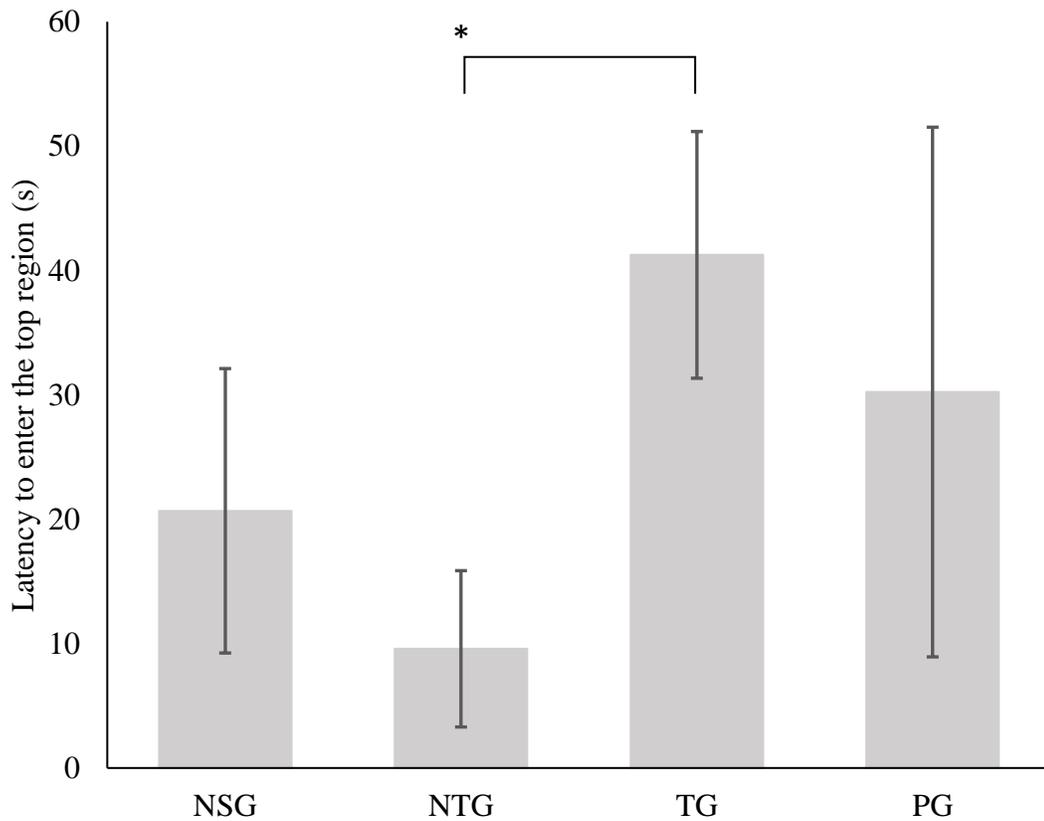

Figure 5. Latency to enter the top region in the novel tank test.

A one-way ANOVA revealed a significant effect of experimental condition on the

total distance traveled during the first five minutes of the novel tank test, $F(3, 80.80) =$

4.87, $p < .01$, $\eta_p^2 = .16$ (Figure 6). The Games-Howell posthoc analysis revealed

significant differences between the TG ($M = 227.30$) and NSG ($M = 314.91$), $p < .001$, d

$= 1.21$, as well as between TG and NTG ($M = 322.22$), $p < .001$, $d = 1.66$.

Figure 6. Total distance traveled in the novel tank test.

One way ANOVA did not reveal differences in the amount of time spent in the

top region during the first five minutes of the test $F(3, 250.20) = 2.57$, $p = 0.06$, $\eta_p^2 = .03$

(Figure 7). One way ANOVA revealed a significant of condition on the amount of time spent in the bottom region during M1-5, $F(3, 237.08) = 3.65$, $p = 0.01$, $\eta_p^2 = .038$. Games-Howell post hoc analysis revealed a significant difference between PG ($M = 28.94$) and NSG ($M = 22.33$), $p = .05$, $d = 0.46$, and between PG and TG ($M = 22.05$), $p = .05$, $d = 0.45$.
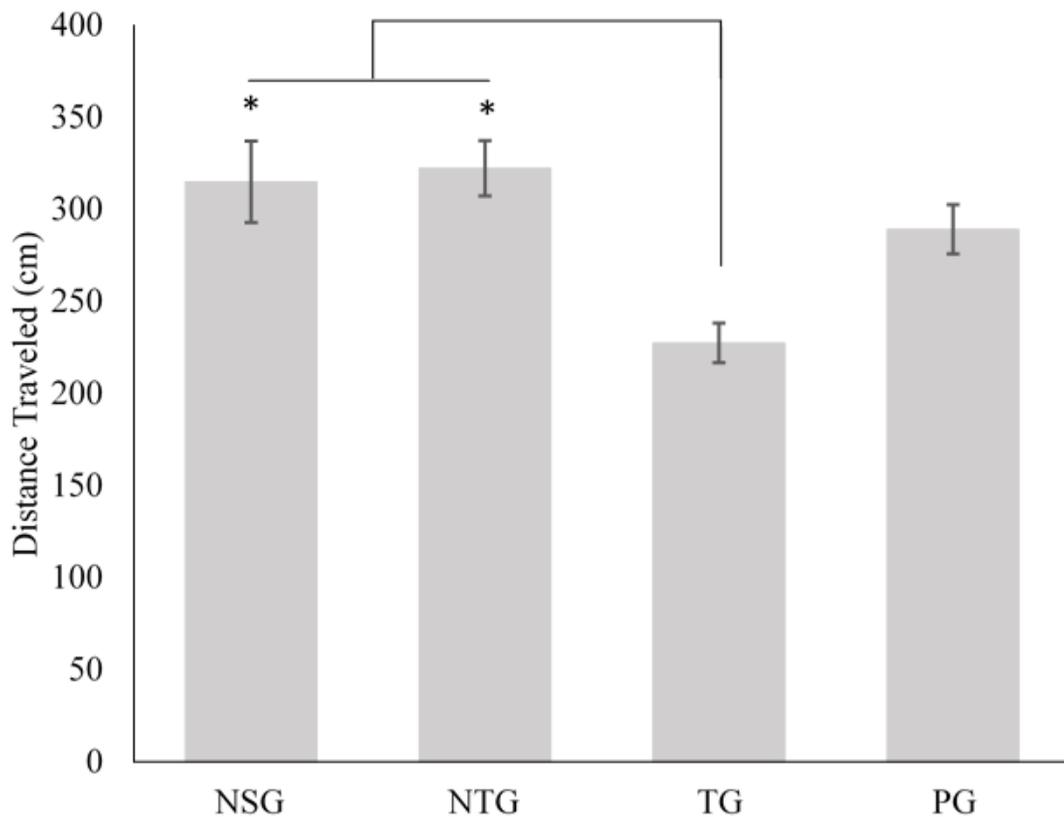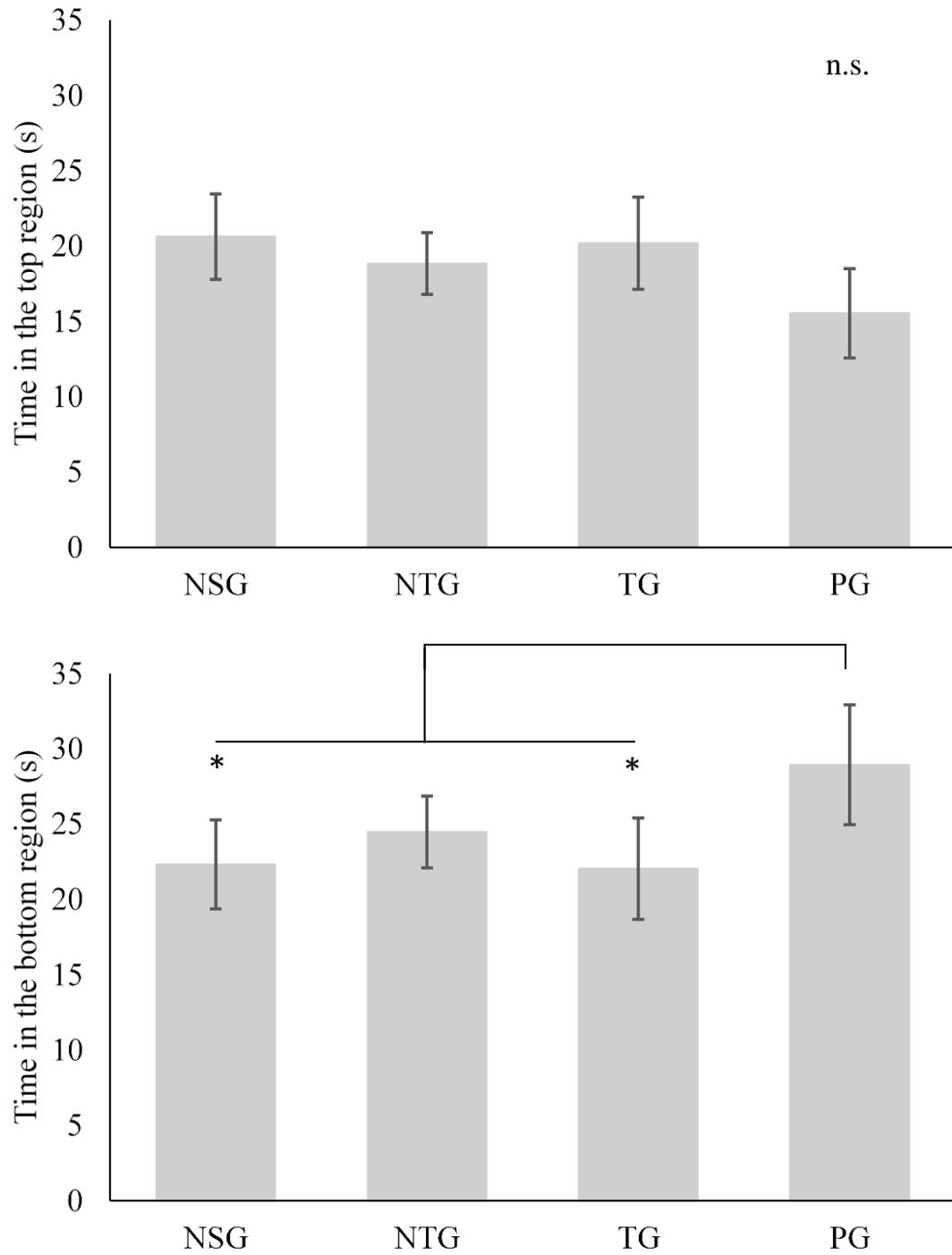
Figure 7. Region preference in the novel tank test.

Values reflect region preference per each minute of the first 5 min of the novel tank test. NSG: No Stress Group; NTG: No Treatment Group; TG: Treatment Group; PG: Placebo Group. Data are expressed as mean +/- 95% CI. $*p \leq .05$.

During the first five minutes of the NTT, there were no observations of high angular velocity, or immobility (parameters outlined in Table 3).

Acclimation to the Environment

A 4 (condition) x 4 (time) repeated measures ANOVA was performed for total distance traveled, top time, and bottom time, to determine whether there was any change in region preference, or locomotor patterns with time. Behaviors were divided into four bins: Min 1-5, 6-10, 11-15, and 16-20.

Total Distance Traveled

Mauchly's test indicated that sphericity had been violated for the main effect of Time, $\chi^2(5) = 224.78$, $p < .001$. Therefore, degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.70$). The repeated measures ANOVA did not reveal a main effect of Time, $F(2.11, 570.86) = 0.99$, $p = .37$, $\eta_p^2 = .004$. But did reveal a main effect of Condition, $F(3,271) = 13.02$, $p < .001$, $\eta_p^2 = .13$ (Figure 8). The TG group ($M = 248.12$) traveled a shorter distance relative to the NSG ($M = 320.86$), $p < .001$, $d = 0.60$; and NTG ($M = 314.29$), $p < .001$, $d = 0.99$; and the PG groups ($M = 294.16$), $p < .01$, $d = 0.34$. The interaction was not significant, $F(6.32, 570.86) = 1.75$, $p = .1$, $\eta_p^2 = .02$.
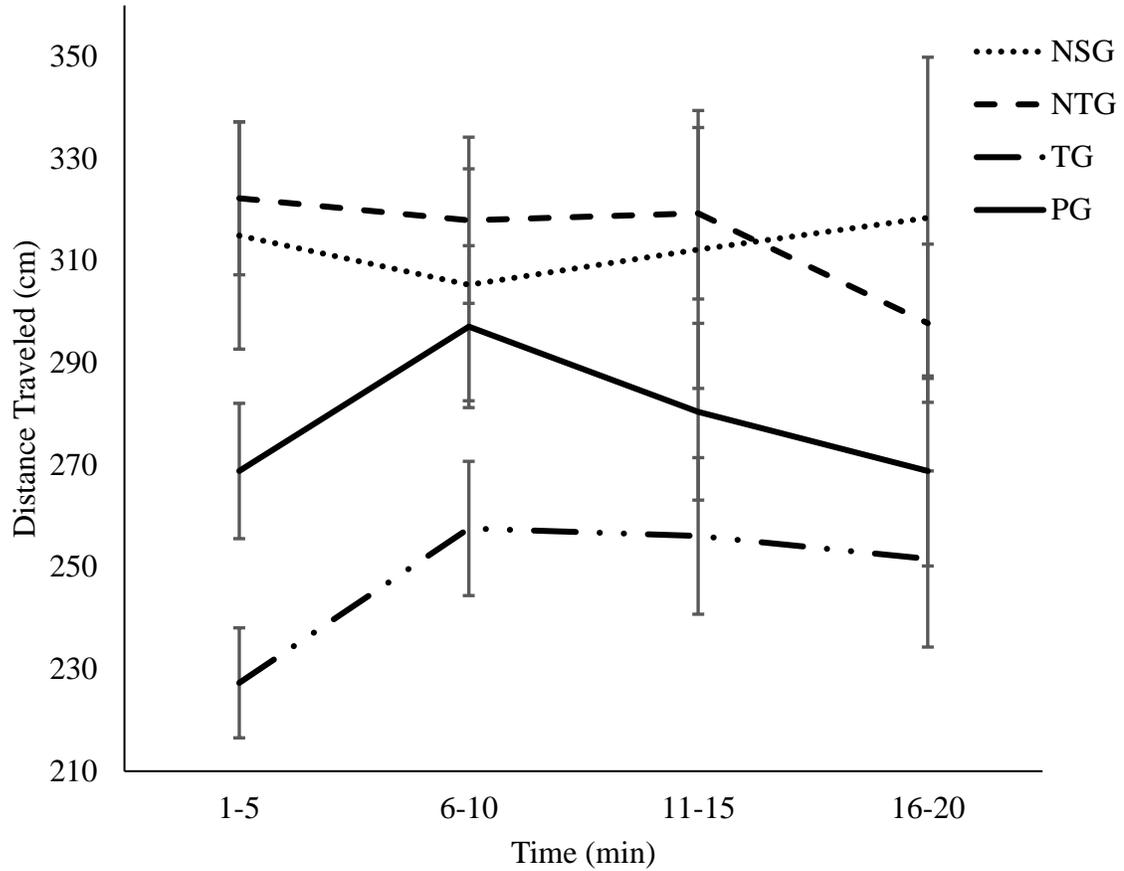
Figure 8. Effect of time on the total distance traveled per minute.

NSG: No Stress Group; NTG: No Treatment Group; TG: Treatment Group; PG: Placebo Group. Error bars are +/- 95% Cis.

Top Time

Mauchly's test indicated that sphericity had not been violated for the main effect of Time, $\chi^2(5) = 7.32$, $p = .20$. The repeated measures ANOVA reveled a significant main effect of Time, $F(3, 813) = 13.47$, $p < .001$, $\eta_p^2 = .047$ (Figure 9). Overall, time in the top region was lowest during the first 5 min. Top time for Min 1-5 ($M = 18.80$) was lower than Min 6-10 ($M = 24.12$), $p < .001$, $d = 0.45$; Min 11-15 ($M = 23.65$), $p < .001$, $d = 0.41$; and Min 16-20 ($M = 23.05$), $p < .001$, $d = 0.36$. There were no significant differences between any other 5-min time blocks.

Figure 9. Effect of time on preference for the top 1/3 region of the novel tank arena.

Values reflect region preference per each minute. NSG: No Stress Group; NTG: No Treatment Group; TG: Treatment Group; PG: Placebo Group. Error bars are +/- 95% CIs.

The ANOVA also revealed a main effect of Condition, $F(3, 271) = 21.91$, $p < .001$, $\eta_p^2 = .20$. Overall, the PG group ($M = 18.03$) had a lower top time relative to the NSG ($M = 22.98$), $p < .001$, $d = 0.41$; NTG ($M = 21.41$), $p = .02$, $d = 0.32$; and TG groups ($M = 27.20$), $p < .001$, $d = 0.75$. The TG group also had a higher top time relative to NSG, $p < .001$, $d = 0.32$; and NTG groups, $p < .001$, $d = 0.50$. The interaction was not significant, $F(8.84, 798.93) = 1.68$, $p = .09$, $\eta_p^2 = .02$.

Bottom Time

Mauchly's test indicated that sphericity had not been violated for the main effect of Time, $\chi^2(5) = 9.19$, $p = .10$. The repeated measures ANOVA reveled a significant main effect of Time, $F(3, 813) = 4.09$, $p < .01$, $\eta_p^2 = .02$ (Figure 10). Overall, time in the bottom region was greatest during the first 5 min. Bottom time during Min 1-5 ($M = 24.45$) was greater than Min 6-10 ($M = 20.89$), $p = .01$, $d = 0.25$; and Min 11-15 ($M = 21.37$), $p = .04$, $d = 0.22$. Time spent in the bottom region during Min 1-5 compared with Min 16-20 were not significantly different ($M = 21.50$), $p = .08$, $d = 0.20$. There were no significant differences between any other 5-min time blocks.
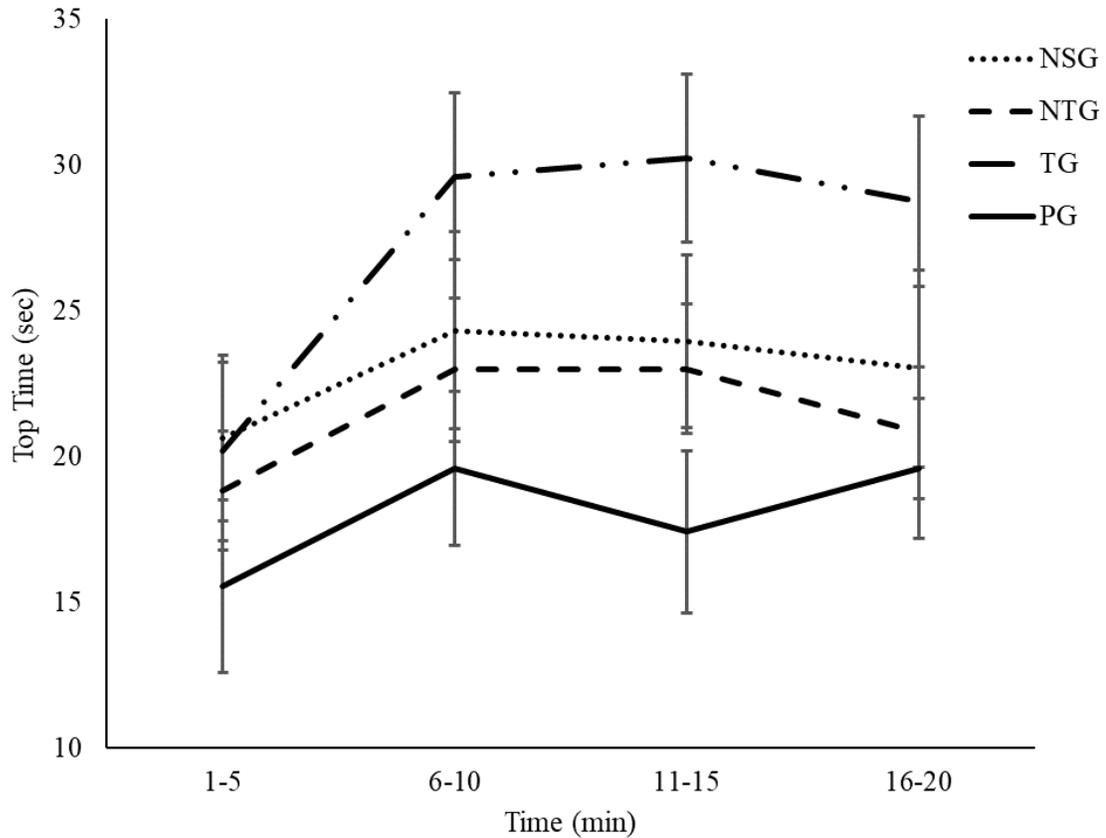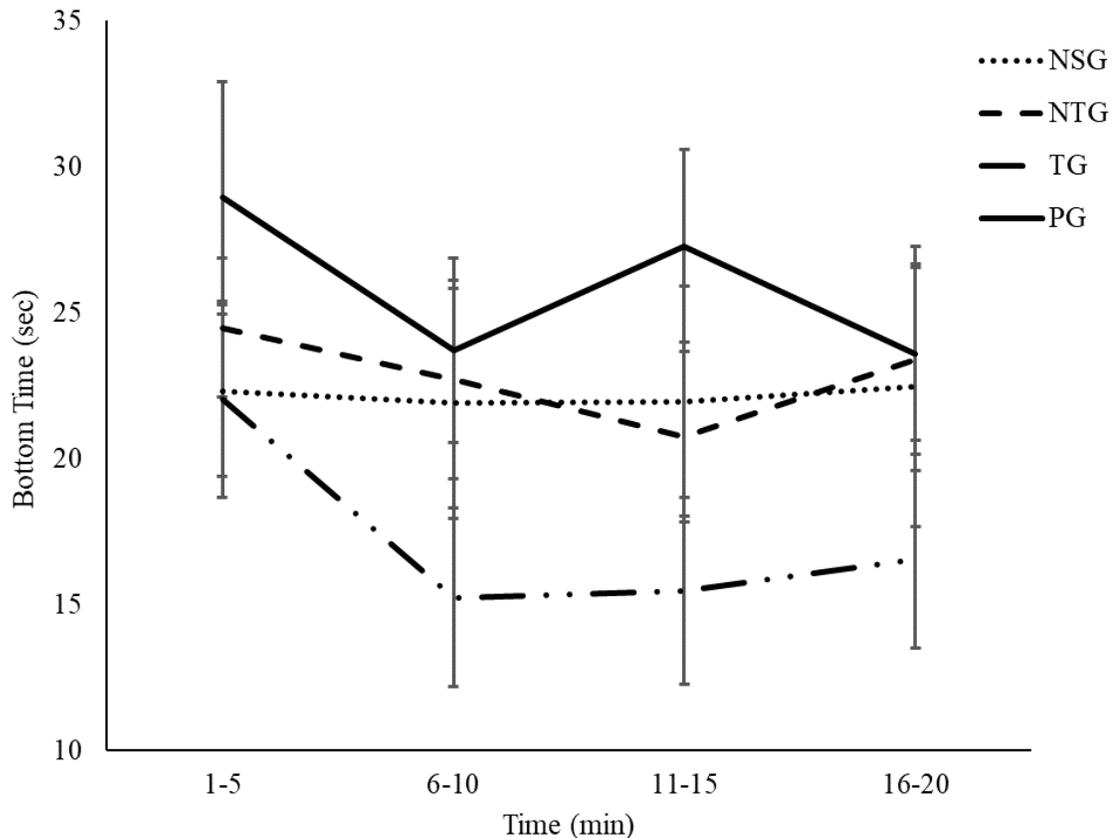

Figure 10. Effect of time on preference for the bottom 1/3 of the novel tank arena.

Values reflect region preference per each minute. NSG: No Stress Group; NTG: No Treatment Group; TG: Treatment Group; PG: Placebo Group. Error bars are +/- 95% CIs.

The ANOVA also revealed a main effect of Condition, $F(3,271) = 11.90$, $p <$ .001, $\eta_p^2 = .12$. Overall, the TG group ($M = 17.33$) spent less time in the bottom region relative to the NSG ($M = 22.16$), $p = .01$, $d = 0.14$; NTG ($M = 22.84$), $p = .001$, $d = 0.19$; and PG groups ($M = 25.88$), $p < .001$, $d = 0.46$. The interaction was not significant, $F(8.80, 794.94) = 1.21$, $p = .28$, $\eta_p^2 = .01$.

Predicting Group Membership

A discriminant analysis was run to predict group membership based on the 20 min swim patterns for total distance traveled, time in the top region and time in the bottom region. Combined, the discriminant functions accounted for a statistically significant percentage of the variance between groups; Wilks' $\Lambda = 0.87$, $\chi(9, N = 1096) = 154.45$, $p < .001$. The second function was also significant, Wilks' $\Lambda = 0.98$, $\chi(4, N = 1096) = 27.34$, $p < .001$. The third function was not significant, Wilks' $\Lambda = 0.99$, $\chi(1, N = 1096) = 0.71$, $p = 0.34$.

The first function yielded an eigenvalue of 0.12, accounting for 10.96% of the total variance, and the second function yielded an eigenvalue of 0.03, accounting for 0.75% of the total variance. The standardized coefficients are presented in Table 5. Based on these values, the first function appears to be guided by lower values of Top Time, and the second function is guided by higher values for Top Time.

Table 5 *Standardized discriminant coefficients*

	Function 1	Function 2
Total Distance Traveled	.696	.732
Top Time	-.726	1.219

Bottom Time	.141	.842

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CHAPTER IV – DISCUSSION

The placebo response is a pervasive phenomenon that is widely observed in human and animal models. In humans, the placebo response is attributed to many factors that include but are not limited to conditioning, the development of expectations conferred through observational learning or verbal communication, and various other psychosocial factors such as the relationship between a physician and the patient (Stewart-Williams & Podd, 2004). In animal models, the placebo response is largely explained via conditioning models. Through classical conditioning, researchers have demonstrated the ability for a placebo treatment to interfere with cognitive processes and immunological responses (Hernstein, 1962; Metalnikov & Chorine, 1926).

Unfortunately, studies of the placebo response in animal models are infrequent, and largely employ healthy animals. That is, in these studies unchallenged animals (e.g., disease free or stress free) are conditioned to associate drug treatment with a neutral stimulus (e.g., environment, route of administration). While these studies highlight the capacity for conditioned placebo responses in animals, they have only focused on a specific subset of animals. To better understand and characterize the placebo response in animal models, it is important that we widen our scope of subjects, so that we may begin to understand the etiology and neurobiology of this phenomenon.

The central aim of the study was to do just this. Over the course of several weeks, animals were subjected to stress protocols that have been previously shown to produce a UCMS-phenotype: Aberrant shoaling patterns, disruptions in cognitive processes, increases in stress related behaviors, and molecular marker profiles that are consistent

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with rodent stress models and human depression (Marcon et al., 2016; Piato et al., 2011). In this experiment, individual zebrafish experienced the stress protocol for two weeks, a period long enough to produce the UCMS phenotype. Beginning in the third week, and concurrent with the stress protocol, stressed zebrafish underwent several conditioning sessions in which an antidepressant drug treatment was paired with a visually distinct arena. Placebo responses were evaluated by analyzing swim behaviors in the novel tank test following the placebo dose.

In addition to evaluating a placebo dose, this study also employed a drug administration method that differed from previous zebrafish chronic stress research. In previous studies, zebrafish were treated with a low dose of the antidepressant in their home tank and allowed to remain in the drug solution continuously for 24h+ (Marcon et al., 2016). The present study, in contrast, employed once-daily treatments of a moderate dose of the antidepressant. This was done in the effort to condition zebrafish to associate drug exposure with the environmental cues. Another deviation from previous protocols was the duration of the stress protocol. Animals in the stress conditions were exposed to the stress protocol for 30 days, roughly 2 weeks longer than previous protocols. This was by design and allowed animals in the placebo group to experience several conditioning sessions, as well as a washout period (Figure 3). It was not anticipated that the increase in stress exposure would produce deviations from previous studies. That is, I was anticipated that relative to the No Stress Group, those in the No Treatment Group would spend a lesser amount of time in the top region of the novel tank test.

In keeping with previous reports, the UCMS protocol in the current study did not have a significant effect on locomotion (Piato et al., 2011). On entry to the novel tank,

animals in the NTG remained in both the top and bottom regions of the NTT a comparable amount of time to the NSG. These region preferences remain somewhat stable over the duration of the 20 min task (Figures 9 & 10). This finding was unexpected; though may have been in part due to the treatment of the animals in the NSG. In this study, NSG animals were individually housed and remained in their home tank unperturbed for the duration of the experiment (30d). In previous studies, when non-stressed animals were individually housed, the test duration was much shorter (7d; Marcon et al., 2016). In longer study durations, animals were group housed in large tanks (Song et al., 2017; Piato et al., 2011). All animals in the current study were individually housed, which allowed us to monitor the health and outward appearance of the animal. The second motivation for individually housing the animals was to limit the isolation stress experienced during the novel tank testing (Egan et al., 2009). However, by individually housing the animals during the study, it is possible that the prolonged isolation blunted the novelty stress of the novel tank testing for the NSG (Giacomini et al., 2015; Piato et al., 2011). The uncertainty of housing condition merits further study. For instance, housing animals in isolation versus in a group for varied durations (e.g., 1d, 7d, 21d, 28d) may provide insight on the production or dulling of stress behaviors, which may guide experimental design and animal housing in future stress research.

On introduction to the novel tank arena, TG animals had a significantly higher latency to the top region of the novel tank relative to the NSG animals. In addition, TG fish remained in the top region a comparable amount of time, relative to other groups during Min 1-5. Taken together, these findings contradict previous reports, and are suggestive of an increased anxiety-like response (Egan et al., 2009; Kalueff et al., 2013;

Wong, Oxendine, & Godwin, 2013). Interestingly, while the treatment group fish did not have a greater preference for the top region initially, the amount of time spent in the top region of the tank increased at Min 6-10 and remained high for the remainder of the behavioral test (Figure 9). This contrasts the swim pattern for the remaining groups: After the initial 5-min introduction to the tank the TG fish maintained a greater overall time in the top region of the tank, while NTG and NSG plateaued at a lower rate, and the PG fish's time in the top region varied with each time period. This would suggest that the drug treatment was effective in reducing anxiety-like behaviors after an introductory period.

This deviation from previous reports (i.e., no increase in top dwelling during introduction to the arena) may in part be due to the mode of drug administration – i.e., 30-min exposure vs 24h+ exposure – as well as the treatment dose. Though it is well established that zebrafish are sensitive to fluoxetine, there has been relatively little study of the dose-dependent effects on behavior. The dose chosen in the current study was guided by a few factors: (1) Mode of administration, (2) currently available information regarding safe fluoxetine doses, and (3) a pilot study.

The current study chose to administer treatment via immersion in a bath solution. Further, because it was the goal of the study to pair drug treatment with a distinct stimulus, animals were treated in the specially designed drug arena. This eliminated the possibility of a chronic drug treatment in the home tank. Studies evaluating the effects of acute fluoxetine treatment in adult zebrafish employ variable doses (0.1mg/L $\rightarrow$ 10mg/L) over a variable amount of time (3min $\rightarrow$ 2h; de Abreu et al., 2014; Singer et al., 2016). With this in mind, the present study chose a dosing period that fell between the two

extremes. Because the goal was for the fish to associate the drug environment with the drug effects, but did not want excessive handling stress, a 30-min dosing period was reached. A pilot study conducted in house evaluated the effects of fluoxetine at 0, 1, & 5mg/L. This study found a reduction in anxiety-like behaviors following 7, 30-min treatments of fluoxetine. It should be noted that in the pilot study, the animals received fluoxetine treatment in the absence of a challenge. That is, the only manipulation in the pilot study was a transfer to a drug arena.

The final hypothesis in this study related to the placebo responses. It was expected that animals in the PG would exhibit fewer anxiety-like behaviors relative to the NTG. During the first five minutes of the NTT, animals in the PG maintained a low height in the tank relative to the other groups, suggestive of an anxiety-like behavior. There have been a few reports of a placebo response producing conditioned responses that are in the opposite direction to the effects of the drug. Working with dogs, Subkov & Zilov (1937) reported that when dogs conditioned to receive epinephrine via injections were administered a saline injection, the animals displayed a bradycardic response. This response contrasts the unconditioned drug effects, which produce a tachycardic response. It has been suggested that this bradycardic reaction is compensatory; and has been replicated in experiments since (reviewed by Wilker, 1973). Thus, it is possible that the current protocol was effective in conditioning the animals to associate the drug arena with drug exposure, and that the resulting (conditioned) response manifested in the form of increased bottom dwelling. To confirm this hypothesis, we would need to run additional experiments. For instance, replicating the drug exposure portion (7

conditioning sessions followed by an 8-d washout phase) in non-stressed animals would provide insight to the conditioned placebo response.

This increase in anxiety-like behaviors may also be in part due to withdrawal from drug treatment. In zebrafish, the cessation of chronic ethanol treatment (0.3% EtOH v/v for 1 week) and chronic morphine (1.5mg/L for 1 week) produces anxiogenic responses in the novel tank test (Cachat et al., 2011). Cessation of treatment produces an increased latency to enter the top region, fewer entries to the top region, and a reduced amount of time spent in the top region of the tank (López-Patiño, Yu, Cabral, & Zhdanova, 2008). This is in addition to an elevated whole-body cortisol expression (Cachat et al., 2011). My dissertation found that the PG group had an increase in anxiety-like behaviors, similar to that observed in animals experiencing withdrawal. However, there are a few important differences between these studies. First, in the study by Cachat et al (2011), animals remained in the drug solution for a 1-week period, whereas in the current study PG animals remained in the drug solution for a 30-min period each day for a 1-week period. Further, behavioral measures were evaluated following a 12-72 h treatment cessation period, whereas the current study evaluated behaviors 9 days following the last drug treatment (8-day depuration phase + 1-day placebo exposure). Thus, it is difficult to compare the behaviors elicited between these studies.

To evaluate any potential withdrawal effects, it would be necessary to evaluate behaviors elicited by the PG animals over multiple timepoints. Such a study may involve comparing the behavioral repertoire of animals 1-, 2-, and 3-days posttreatment against those observed 9 days post treatment. Evaluating the expression of whole-body cortisol in

addition to behavioral measures would provide additional insight into the experience of, and production of withdrawal behaviors in this model of drug administration.

The last analysis performed in this study was the discriminant analysis. This test is typically used to predict categorical group membership (NSG, NTG, TG, PG) based on continuous predictor variables (Total Distance Traveled, Top Time, and Bottom Time). From this analysis, two of the three functions were statistically significant, and accounted for roughly 11% of the total variance in the datasets. Of the predictor variables, the amount of time spent in the top region of the tank was the most important factor in predicting group membership. This was followed by Total Distance Traveled in function 1, and Bottom time in function 2 (see table 5). Thus, though the swim patterns vary by time and by condition, this variability becomes a tool in discriminating between groups. It would be interesting to examine whether the collection of additional predictor variables (e.g,. mean angular velocity per minute) would be useful in improving the predictive power (increase amount of variance accounted for) of the functions.

In conclusion, the central aim of the present study was to determine whether placebo responses could be observed and quantified in a zebrafish-chronic stress model. While it did not yield direct support for the existence of a placebo response, there is some evidence of successful drug conditioning. Some limitations of the current design have already been noted, and future directions discussed; but it is important to note that the current study only evaluated behavior in a single behavioral test. Evaluating behavioral responses along other dimensions (e.g, social behavior, or evaluating molecular marker expression) may provide additional insight into the phenomenon in the animal model. Determining whether the placebo response is observed in a stressed animal model (or an

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animal facing a different kind of challenge) would be very useful in allowing us to begin to study the neurobiology of this behavior. The development of a protocol for testing the placebo response has great value in clinical trials, as it may be used to screen out drugs that have a high placebo response potential.

APPENDIX A – IACUC Approval Letter

THE UNIVERSITY OF SOUTHERN MISSISSIPPI.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.5997 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: 18032703
PROJECT TITLE: Modeling the Placebo Effect in Adult Zebrafish
PROPOSED PROJECT DATES: 03/2018 – 09/2020
PROJECT TYPE: New Protocol
PRINCIPAL INVESTIGATOR(S):   Alan Hajnal
DEPARTMENT: Psychology
FUNDING AGENCY/SPONSOR: N/A
IACUC COMMITTEE ACTION: Full Committee Review Approval
PROTOCOL EXPIRATON DATE: September 30, 2020

_____   March 27, 2018
Jake Schaeffer, PhD   Date
IACUC Chair

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