The Effects of Fibroblast Growth Factor on Anxiety Induced by Maternal Care

By

Emily Lowry

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Carleton University
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Abstract

Anxiety disorders are the most common mental health concern in Canada, affecting 1 in 4 individuals (Anxiety Disorders Association of Canada, 2003). Over the years, anxiety and mood disorder research has focused on genetic and environmental factors that could leave individuals vulnerable to such illnesses. Specific environmental factors include acute and chronic early life stressors, trauma, and maternal care. Variations in rat maternal care have been shown to be specifically influential in offspring stress response throughout life (Francis et al., 1999). Pups that are raised by high licking/grooming and arched-back nursing (LG-ABN) mothers show decreased glucocorticoid levels, increased glucocorticoid receptor expression, and decreased hypothalamic-pituitary-adrenal (HPA) axis activation in adulthood (Salmaso et al., 2016; Champagne, 2013). These findings have been attributed to the tactile stimulation (TS) of licking and grooming, which has been associated with epigenetic changes in stress axis responsivity (Richards et al., 2012). FGF2 is a part of the fibroblast growth factor (FGF) system, a complex neuroprotective family involved in development, neuronal repair, protection, and plasticity (Riva et al., 2005). FGF2 has also recently been implicated in the expression of anxiety behaviour, such that brain FGF2 levels are inversely related to levels of anxiety, suggesting an anxiolytic role. We hypothesized that FGF2 levels are also increased in response to licking and grooming stimulation. Therefore, we investigated the efficacy of FGF2 administration in reversing an anxiety-like phenotype in offspring raised by low LG-ABN mothers. This was accomplished by randomly assigning 79 offspring from both high and low LG-ABN mothers into two treatment groups, one receiving vehicle solution and the other FGF2. Three behavioural tests (Open field, elevated plus maze, and forced swim test) were performed following injections. We found no significant effect of FGF2 treatment, and where maternal care was found to significantly affect behavioural phenotype, it was in the opposite direction than hypothesized and appeared to be increasing anxiety behaviour. Potential limitations to the current methodology are explored.

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Table of Contents

List of Tables **Page**

List of Figures **Page**

The Effects of Fibroblast Growth Factor on Anxiety Induced by Maternal Care

Anxiety

Anxiety is a normal part of life, and we all experience it in varying degrees. For some individuals however, anxiety can control their life, hindering their ability to participate in daily activities. Anxiety disorders are the most common mental health concern in Canada, affecting 1 in 4 individuals (Anxiety Disorders Association of Canada, 2003). In 2013, a reported estimate of 3 million Canadians (11.6%) aged 18 and older were affected by mood and anxiety disorders (Statistics Canada, 2013). Of the affected population, 27% reported that the disorder obstructed daily life including recreational and leisure activities, such as social events with friends and family (Statistics Canada, 2013). Daily life can be interrupted by anxiety disorders as they are characterized by overwhelming fear, avoidance of feared environments and situations, in combination with physiological reactions (i.e., increased heart rate, shortness of breath). These disorders are also chronic, affecting individuals for up to six months or more, and worsen without adequate treatment (Statistics Canada, 2013).

Anxiety is a treatable illness, typically with pharmacological and psychological treatments. Pharmacological treatment involves the use of prescribed antianxiety or antidepressant medications designed to reduce symptoms (Anxiety Disorders Association of Canada, 2003). These medications work on specific neurotransmitters in the central nervous system (CNS), to either reduce or increase their efficacy at specific receptors. Psychological treatment involves meeting routinely with a registered therapist who specializes in anxiety disorders with a specific technique (i.e., Cognitive-behavioural therapy). When combined, these two treatments significantly reduce symptoms of anxiety. Alone however, pharmacological treatments such as benzodiazepines and selective-serotonin-reuptake inhibitors (SSRIs) are not

as effective. Only a subset of patients respond to medication, and there remains a delay in therapeutic effects (approximately 4-6 weeks) (ElSayed, Bansar, Duric, Fournier, Licznerski, & Duman, 2012). More efficient pharmacological options are needed to treat anxiety disorders as our understanding of the molecular changes associated with such illnesses continues to grow. Molecular changes involve gene transcription, expression, and function. Identifying changes in affected genes is crucial to treatment, however it is also important to note how these changes come about.

Early Environmental Interactions, Epigenetics, and Anxiety

We are all born with genetic mutations, leaving us vulnerable to develop certain character traits or illnesses at different points in our lives. While these mutations are significant, genetic factors do not account for the entirety of individual variability in illnesses (McEwen, 1998). Furthermore, the relationship between genotype and phenotype is not simple or direct (Meaney, 2010). There is not one gene that codes specifically for intelligence or creativity, nor is it correct to say that in lacking a gene the individual lacks an entire ability or characteristic. The determining factors which cause a trait or illness to appear are complex, and often consequence of our early environment and interactions (i.e., epigenetics). Epigenetics are a variety of processes that alter gene activity, without changing the DNA sequence (Weinhold, 2006). These processes include DNA methylation and histone modifications, which encourage or discourage gene expression, creating vulnerabilities to illnesses through altered biological responses (Weinhold, 2006). Studies from developmental psychobiology report that early changes in gene expression and function persist into adulthood (Meaney, 2010). A variety of events can cause changes in genes, including acute and chronic early life stressors (i.e., food scarcity, environmental instability/insecurity, unpredictable events), trauma, and parental care.

At such a time in development, stressful events alter the genetic processes associated with the consolidation of our stress response, the hypothalamic-pituitary-adrenal (HPA) axis. This pathway is an important area of study as stress is a risk factor for many physical and mental illnesses (Francis & Meaney, 1999). The earliest points in life are when the HPA axis is undergoing critical development. This pathway and others, through which stressful events promote the development of illnesses, involve the same hormones that ensure survival during a period of stress (Francis et al., 1999). Chronic activation of these pathways increases the presence of stress hormones, and develops a greater sensitivity for stress-induced illnesses over the lifespan (Heim, Owens, Plotsky, & Nemeroff, 1997; Francis et al., 1999). When activated, the hypothalamus releases corticotropin-releasing factor (CRF), causing the release of adrenocorticotropin hormone (ACTH) from the pituitary. At the end of this pathway, the adrenal glands release catecholamines (i.e., adrenaline and norepinephrine) and glucocorticoids, which assist in the fight-or-flight reaction. Heart rate increases, blood flows quickly, and our body uses stored glucose for energy in fighting the stressor. This response not only increases the amount of stress hormones working in our CNS, yet also directs our attention toward the stressor and environment. The level of attention directed to the environment hinders our ability to concentrate on tasks not associated with the stressor (Francis et al., 1999). The result is disturbed episodic memory through increased glucocorticoid (GC) activity on the hippocampus and amygdala (Lupien, Sharma, Nair, Hauger, McEwen, de Leon, & Meaney, 1998). This produces a sensitivity to future stressors by strengthening memory for emotional stimuli (Quirarte, Roozendaal, & McGaugh, 1997; Francis et al., 1999). Increased GC activity also results in hippocampal and prefrontal cortex (PFC) neuronal atrophy, which disturbs the inhibitory control that these areas typically hold over amygdala activation (Meyer & Quenzer, 2005).

There is evidence, however, that supports acute stress as beneficial during development. Meaney and colleagues (1988) found that brief periods of handling in rodents (i.e., 3-15 minutes) decreased their overall stress response. In contrast, prolonged stress was found to increase behavioural and endocrine responses to stressors throughout life (Meaney, Aitken, Bhatnagar, Van Berkel, & Sapolsky, 1988). At the time of this discovery it was apparent that the maternal separation induced with long periods of handling altered the mother-offspring interactions when pups were returned to the cage. Maternal care was thought to be playing some role in mediating stress pathways, however the exact influence was not clear (Francis et al., 1999). This is now better understood, and maternal care is known to be a significant factor in the development of the stress response.

Maternal Care and Epigenetic Changes

The strongest evidence linking environmental changes to the development of stress responses has come from postnatal handling studies (Meaney, 2001). These experiments revealed not only a change in offspring stress-response, yet also a change in maternal behaviour towards her pups. Mothers of handled offspring spent significantly more time licking and grooming (LG) them (Francis et al., 1999). This finding realized that like human beings, there is a natural variation in maternal care amongst rodents (Champagne, 2013). A dam who spends significant time performing LG and arched-back nursing (ABN) behaviours is said to be high in maternal care. Evidence supports variations of maternal care alter the formation of the stress response in offspring (Francis et al., 1999). CRF-containing neurons, both in the paraventricular nucleus of the hypothalamus (PVNh) and in the central nucleus of the amygdala (CnAmy), are considered target sites for the effects of variations in maternal care (Francis et al., 1999). These systems are crucial in determining the emotional, autonomic, and endocrine responses to fear and

anxiety (Meaney, 2010). Champagne (2013) noted several changes often found in offspring from high LG-ABN versus low LG-ABN mothers. Male offspring from low LG-ABN dams have higher ACTH and corticosterone levels in response to stress (greater HPA axis activation), and take longer to return to baseline GC levels (Champagne, 2013). They also show increased startled responses, decreased exploration in the open field (OF) test, and increased latencies to eat in novel environments. In contrast, males from high LG-ABN dams show lower levels of GCs, and greater hippocampal GR mRNA expression (Francis et al., 1999). These effects are proposed to be, in part, the result of the tactile stimulation received from the licking and grooming behaviour (Richards, Mychasiuk, Kolb, & Gibb, 2012). More importantly is the support of an increase in fibroblast growth factor-2 (FGF2) because of such tactile stimulation (Richards et al., 2012).

Implications of Fibroblast Growth Factor

Fibroblast growth factor-2 (FGF2) is a part of the fibroblast growth factor (FGF) system, a complex neuroprotective family consisting of 22 ligands and five receptors (Turner, Akil, Watson, & Evans, 2006). FGF receptors (FGFRs) are found in neuronal populations throughout the CNS from early development into adulthood. This includes significant expression in the hippocampus and cortex (Turner et al., 2006). The ligands are predominately expressed in neurons, glial cells, and astrocytes. Considering the locations of FGFRs and ligands, the FGF system has been shown to be of importance in development, neuronal repair, protection, and plasticity (Riva, Molteni, Bedogni, Racagni, & Fumagalli, 2005).

Whether early or late in development, a disruption of the FGF system could leave individuals vulnerable to anxiety and mood disorders (Turner et al, 2006; Salmaso et al., 2016). Evidence suggests that FGF2 treatment early in development alters neurogenesis and

hippocampal gene expression, whereas an antidepressant effect has been found when administered in adulthood (Turner et al., 2006). Furthermore, Salmaso and colleagues (2016) found that FGF2 levels are significantly related to anxiety and depression through hippocampal GR expression. The study investigated the difference in anxiety, depression, and motor behaviour in FGF2 knockout (KO) mice, wild-type (WT), and FGF2 KO mice with adult rescue by FGF2 administration. FGF2 KO mice showed increased basal corticosterone levels and a decrease in hippocampal GR mRNA (Salmaso et al., 2016). HPA axis hyperactivity was found in FGF2 KO mice, a consequence of decreased GR gene expression in the hippocampus. Rescue in adulthood by FGF2 administration restored hippocampal GR levels, and diminished anxiety-like behaviour (Salmaso et al., 2016). These findings support previous research demonstrating increased hippocampal FGF2 levels are correlated with reduced manifestations of anxiety (Eren-Kocak, Turner, Watson, & Akil, 2011; Salmaso et al., 2016).

Maternal Care and FGF2

Bredy and colleagues (2003) investigated whether natural variations of maternal care influenced hippocampal neuronal survival and FGF2 expression in rats at postnatal day (P)21. Maternal care was observed for 75 minutes each day from P1-P8, and no treatment or behavioural testing occurred. At P21 there was a significant effect for maternal care on FGF2 expression in offspring from high versus low LG-ABN mothers (Bredy et al., 2003). Pups from high LG-ABN mothers had significantly more FGF2 expression and greater hippocampal neuronal survival. These results support naturally occurring effects of maternal care on FGF2 expression and neuronal cell survival in areas important for learning and memory. Furthermore, a study from Richards and colleagues (2012) found that TS has several mediating effects on the developing rat brain. Litters were split in half, with one group receiving TS three times a day

from P3-P21, then left to develop further. Although behavioural testing revealed no significant differences between pups receiving TS and those without, widespread effects of TS on synaptic organization in the PFC were found (Richards et al., 2012). The authors suggest this finding might be correlated with the endogenous expression of FGF2 during development, in addition to TS (Richards et al., 2012). Studies using similar techniques support that TS increases FGF2 expression in the skin and PFC (Gibb, 2004; Richards et al., 2012). This research adds further to the literature concerning the role of maternal care and FGF2 in mediating the stress response.

Current Study

Both maternal care and TS have been found to increase the expression of FGF2, and increases of FGF2 have been correlated with greater GR mRNA, lower GC levels, and decreased HPA axis activity (Salmaso et al., 2016; Turner et al., 2006; Francis et al., 1999). In the current study, we hypothesized that it is through the tactile stimulation (TS) of licking and grooming that FGF2 is released and has an effect. We were therefore interested in the efficacy of FGF2 administration in reversing adulthood anxiety produced by low maternal care during development. This investigation was accomplished by injecting FGF2 and vehicle solutions to a total of 79 offspring from low and high LG-ABN mothers between P56-P70. Behavioural tests consisted of the open field test, elevated plus maze, and the forced swim test. Two hypotheses were made: 1) A reversal of the anxiety-like phenotype of pups treated with FGF2 from low LG-ABN mothers, resulting in performance like that of pups from high LG-ABN mothers. 2) There would be little or no effect of FGF2 or vehicle treatment on pups from high LG-ABN mothers.

Methods

Animals

Thirty Long Evans rats (24 dams, 6 studs) were received from Charles River. Nine dams gave birth to 79 offspring. The offspring were placed into one of four treatment groups, partly determined by the maternal score of their mother. If the pup was from a low LG-ABN mother they were randomly placed into one of two treatment groups (vehicle or FGF2 administration), as were the pups from high LG-ABN mothers. The breakdown of each treatment group is shown in Table 1. Each pup was given injections of either FGF2 or vehicle solution, and all were subjected to behavioural testing one day after injections ended. Injections and behavioural testing were staggered with four experimental groups to minimize the number of animals being tested each day. The specifics of each group are shown in Table 2. All animal use procedures were approved by the Carleton University Committee for Animal Care, according to the guidelines set by the Canadian Council for the Use and Care of Animals in Research.

Maternal Behaviour Scoring

Nine dams and their offspring were observed by video recording when the pups were one day old (P1). The critical period for effects of maternal behaviour on offspring is from P1-P7 (first week of life), and maternal behaviour (MB) has been found to be stable over the course of that week. Table 3 and Table 4 respectively show the scores of a high and low dam. Scores were allocated based on the amount of time the dam spent with her litter, and which behaviour she was performing while with them. *Behaviour one* was considered the lowest, with the dam lying on her litter without any licking/grooming or nursing behaviours. For each minute spent performing this behaviour the dam was allotted one point. *Behaviour two* consisted of licking/grooming, and some arched-back nursing, and two points were allotted for every minute spent performing this

behaviour. The *third behaviour* was significant licking/grooming, with pronounced arched-back nursing, and three points were allotted for each minute of this behaviour. Each observation video was 75 minutes in length, recorded once in the morning, and once in the evening.

After completing MB scoring and finding the mean score amongst all dams, three were selected from each end of the spectrum (scoring one standard deviation, or more, above and below the mean) to be high and low moms. These scores are depicted in Figure 1.

Drug Treatments

FGF2 Administration. FGF2 ligand was dissolved in 1 mol/L phosphate buffered saline. This was administered at a dose of 10mg/kg through subcutaneous injections at the same time each day, once per day, for one week.

Vehicle Solution. 0.1% bovine serum albumin was also administered at a dose of 10mg/kg through subcutaneous injections at the same time each day, once per day, for one week. Behavioural testing began on day eight.

Behavioural Testing

Open Field. The open field (OF) test is commonly used to measure anxiety (within the first five minutes), fear, and motor movements (full twenty minutes). The first five minutes allow observations of the animal's response to a novel environment. If the animal is anxious within the first five minutes they will typically remain in the corners. As time goes on, the normal anxiety response subsides and animals begin to explore the environment. Fear is measured in the remainder of the test since a fearful animal will likely remain in a corner. Impaired motor movements can also be observed with greater time once the anxiety passes. It was the first behavioural test completed as it was the least stressful of the three. Two transparent plastic tubs (76.2 cm x 54.61 in) were used to administer the OF test. One side of the tub was blocked with

opaque paper to ensure the rats did not observe one another. The open field was divided into a peripheral and center zone which can be seen in Figure 2. The peripheral zone measured the same width and length of the tub, and was only 11.43 cm wide inside the tub. The remaining space, 43.18 cm x 21.59 cm, constituted the center zone. Two animals were tested simultaneously, and were always placed into the right-hand corner of the peripheral zone to begin the test. The test lasted for 20 minutes, and the open field was wiped down with 70% ethanol between trials. The test was recorded via a mounted video camera above the apparatus.

Elevated Plus Maze. The elevated plus maze (EPM) is similarly designed to measure anxiety in rodents. It was considered the second highest anxiety-producing test of the three. The apparatus measured 3.5 ft x 3.5 ft, with two half-wall covered arms on opposing sides, and two open arms on opposing sides. The maze was divided into three zones for testing which can be seen in Figure 3. The first zone was the closed arms (48.26 cm x 11.43 cm), the second zone as the open arms (48.26 cm x 11.43 cm), and the center zone (11.43 cm x 11.43 cm). Increased latency to approach the open arms and less time spent exploring such zones is representative of a more anxious animal. The maze was elevated on four stilts measuring 39.87 cm tall. Animals began the test by being placed in the center zone, and the apparatus was wiped down with 70% ethanol between trials. The duration of the test was five minutes. The test was also recorded via a mounted video camera above the apparatus.

Forced Swim Test. The forced swim test (FST) is typically used to measure depressive-like behaviours in animals. Three glass cylinders were used, which were covered on one side to prevent animals from seeing one another during testing. Once animals are placed in the water, latency to immobility is used to measure learned helplessness. The height of each cylinder was 60.32 cm, with a diameter of 15.24 cm. Lukewarm water was filled up to approximately 40 cm.

See Figure 4. Three animals were tested simultaneously for five minutes. The test was recorded via a mounted video camera in front of the cylinders.

Behavioural and Statistical Analyses

All behavioural tests were recorded with a video camera, and animal behaviour for the open field and elevated plus maze was scored using AnyMaze Tracking System (Stoelting, USA). Behaviour measures for the forced swim test were performed by a trained lab employee. Statistical analyses were performed individually for each behaviour test using Vassar Statistics online resources.

General Procedure

Twenty-four dams and six studs from Charles River were separated into six cages, with four females and one male in each to allow for mating. Once a female was suspected of being pregnant, she was placed in her own separate cage. Nine dams successfully produced a total 79 offspring. Video cameras constantly monitored the maternal behaviour of each dam from P1 to P6 of the pups. Video recording allowed the pups to remain as undisturbed as possible. Maternal behaviour was analyzed and scored according to the level of their maternal care (i.e., licking, grooming, nursing, and pup retrieval). Three dams whose maternal behaviour scores were at least 1 standard deviation above and below the average score of all the dams were respectively classified as high or low lickers/groomers and arched-back nursing mothers (high/low LG-ABN). See Figure 1. Six dams were selected. The litters from these dams were weaned and allowed to reach adulthood. Between P56-P70, half of the pups raised by low LG-ABN dams were injected with a vehicle solution daily for seven days, while the other half received FGF2 injections. Similarly, half of the pups raised by the high LG-ABN dams received vehicle injections with the remainder receiving FGF2. The pups were divided into four groups that dictated which injection

they would receive (High LG/FGF2, High LG/Vehicle, Low LG/FGFG, Low LG/Vehicle). See Table 1. Furthermore, the pups in each of these groups were randomly placed into one of four experimental groups to help stagger injections and behavioural testing, see Table 2. On day one, we began injections with Group 1, then on day two we injected Group 1 as well as began Group 2 injections. This continued until the fourth group started and ended injections. Following the week of injections, all animals began behavioural testing. The behavioural testing was done in a way to gradually increase the stress that animals experienced. We started with the OF test, followed by the EPM, and ending with the FST. Once all animals had been tested they were sacrificed. Five males and five females from each of the four groups underwent decapitations without anesthesia, and the remaining animals in each group were perfused.

Results

Open Field

Four 2x2 ANOVAs were completed for both males and females separately for the OF test. Independent variables were Maternal Behaviour (Low/High), and Treatment (Vehicle/FGF2). The dependent variable changed with each analysis. Within the first five minutes of the test we measured latency to center zone, time spent in center zone, time immobile in center zone, and distance travelled in center zone. Mean distance travelled was measured for the full 20 minutes.

In the analysis of mean latency to center zone for females there was no significant effect of maternal behaviour, $F(1, 39) = .99$, $p = .32$, or of treatment, $F(1, 39) = .13$, $p = .72$. Similarly, for males there was no effect of maternal behaviour, $F(1, 34) = .34$, $p = .56$, or of treatment, $F(1, 34) = .68$, $p = .41$. Results for females and males are shown in Figure 5 and Figure 6 respectively. The mean time spent in the center zone for females showed no significant effect of

maternal behaviour, $F(1, 39) = 2.66$, $p = .11$, or of treatment, $F(1, 39) = 1.77$, $p = .19$. There was also no effect of maternal behaviour, $F(1, 34) = .01$, $p = .92$, or of treatment $F(1, 34) = .89$, $p = .35$, for male mean time in center zone. These results are shown in Figure 7 for females, and Figure 8 for males. For mean time immobile in the center zone, there was no effect of maternal behaviour, $F(1, 39) = .67$, $p = .41$, or of treatment, $F(1, 39) = .79$, $p = .38$, for females. There was a significant main effect of treatment for male mean time immobile, $F(1, 34) = 5.29$, $p = .02$, however not of maternal behaviour, $F(1, 34) = 2.28$, $p = .14$. Male pups from low LG-ABN mothers treated with vehicle solution appear to have the greatest mean immobility times. There is no difference between high LG-ABN offspring treated with vehicle or FGF2, and offspring from low LG-ABN treated with FGF2 have the lowest immobility time. Results for female mean time immobile in center zone are shown in Figure 9, and results for males are shown in Figure 10. For distance travelled in the center zone, there was no effect of maternal behaviour, $F(1, 39) = .05$, $p = .82$, or of treatment, $F(1, 39) = .11$, $p = .74$, for females. There was also no effect of maternal behaviour, $F(1, 34) = 1.23$, $p = .27$, or of treatment, $F(1, 34) = .27$, $p = .6$, for males. Figure 11 and Figure 12 show these results for females and males respectively.

For the full 20 minutes, female mean distance travelled showed no significant effect of maternal behaviour, $F(1, 29) = .32$, $p = .57$, or of treatment, $F(1, 29) = 1.12$, $p = .29$. For males there was also no significant effect of maternal behaviour, $F(1, 12) = .98$, $p = .34$, or of treatment, $F(1, 12) = 0$, $p = 1$. Female results are shown n Figure 13, and results for males are shown in Figure 14.

Elevated Plus Maze

Six 2x2 ANOVAs were completed for both males and females separately for the EPM. Independent variables were Maternal Behaviour (Low/High), and Treatment (Vehicle/FGF2).

The dependent variable changed with each analysis. Mean distance in open arms, latency to open arms, mean time in open arms, mean distance in closed arms, and mean time in closed arms were measured over five minutes.

For female distance in open arms, there was no significant effect of maternal behaviour, $F(1, 30) = 2.07$, $p = .16$, or of treatment, $F(1, 30) = .38$, $p = .54$. There was no significant effect of maternal behaviour, $F(1, 30) = 1.13$, $p = .29$, or of treatment, $F(1, 30) = 0$, $p = 1$, for male distance in open arms. Results for females and males are shown in Figure 15 and Figure 16 respectively. A significant main effect of maternal behaviour was found for female latency to open arms, $F(1, 30) = 4.92$, $p = .03$, although not for treatment, $F(1, 30) = .02$, $p = .88$. While there was no difference between treatment groups, it appeared that female offspring from high LG-ABN mothers had significantly greater latencies to enter the open arms of the maze. There was no significant difference between latency times for treatment groups of low LG-ABN offspring. For male latency to open arms, there was no significant effect of maternal behaviour, $F(1, 29) = .03$, $p = .86$, or of treatment, $F(1, 29) = .04$, $p = .84$. Latency to open arms results for females are shown in Figure 17, and males in Figure 18. There was no significant effect of maternal behaviour, $F(1, 29) = 2.76$, $p = .1$, or of treatment, $F(1, 29) = .21$, $p = .65$, for female mean time spent in open arms. For males, there was no significant effect of maternal behaviour, $F(1, 30) = .61$, $p = .44$, or of treatment, $F(1, 30) = .32$, $p = .57$. Results for females are shown in Figure 19, results for males are shown in Figure 20. Mean distance in closed arms for females showed no significant effect of maternal behaviour, $F(1, 32) = .33$, $p = .57$, or of treatment, $F(1, 32) = .01$, $p = .92$. For males, there was also no significant effect of maternal behaviour, $F(1, 31) = .3$, $p = .58$, or of treatment, $F(1, 31) = .42$, $p = .52$. Results for females are shown in Figure 21, and for males in Figure 22. For the mean time spent in closed arms, there was a significant effect

of maternal behaviour, $F(1, 32) = 5.09$, $p = .03$, yet not of treatment, $F(1, 32) = .68$, $p = .41$, for females. The results support female offspring of high LG-ABN mothers spent more time in the closed arms of the maze, however there was no significant difference between treatment groups for both high and low LG-ABN offspring. There was no significant effect of maternal behaviour, $F(1, 31) = .22$, $p = .64$, or of treatment, $F(1, 31) = .01$, $p = .92$, for male mean time spent in closed arms of the EPM. Results for females are shown in Figure 23, and males in Figure 24.

Forced Swim Test

Four 2x2 ANOVAs were completed for both males and females separately for the FST. Independent variables were Maternal Behaviour (Low/High), and Treatment (Vehicle/FGF2). The dependent variable changed with each analysis. Total immobility and latency to immobility were measured over the ten-minute test

For female total immobility, there was a significant main effect of maternal behaviour, $F(1, 36) = 6.62$, $p = .01$, and of treatment, $F(1, 36) = 9.23$, $p < .05$. Female offspring from high LG-ABN spent the least amount of time immobile, however the significant effect of treatment shows an increase in immobility in both high and low offspring treated with FGF2. There was also a main effect of maternal behaviour for males, $F(1, 37) = 10.95$, $p < .05$, yet not of treatment, $F(1, 37) = .18$, $p = .67$. Male offspring from low LG-ABN mothers had significantly greater immobility times, with no significant difference between treatment groups. Results for females are shown in Figure 25, and for males in Figure 26. There was no significant effect of maternal behaviour for female latency to immobility, $F(1, 36) = .98$, $p = .32$, or of treatment, $F(1, 36) = .63$, $p = .43$. No significant effect of maternal behaviour, $F(1, 40) = .26$, $p = .61$, or of treatment, $F(1, 40) = 2.4$, $p = .12$, was found for male latency to immobility. Figure 27 and Figure 28 show results for females and males respectively.

Discussion

The current study aimed to replicate and expand on previous findings showing a natural influence of maternal care on anxiety-like phenotypes in adult rats. The overall goal of the research was to reverse anxiety-like behaviour in adult rats raised by low maternal-scoring dams, through late injections of FGF2. Three behavioural tests were conducted on all adult offspring from high and low LG-ABN mothers after one week of daily subcutaneous injections. The behavioural tests were chosen specifically to measure anxiety and depressive behaviours. Two hypotheses were made: 1) A reversal of the anxiety-like phenotype of offspring treated with FGF2 from low LG-ABN mothers would occur, resulting in performance like offspring from high LG-ABN mothers, and 2) Little or no effect of FGF2 or vehicle treatment on offspring from high LG-ABN mothers. The results do not fully support the hypotheses of the current experiment, and are not consistent with previous literature.

Overall, significant effects of maternal behaviour were found, however it was often that the offspring from high LG-ABN mothers performed worse, or appeared more anxious, than offspring from low LG-ABN mothers. The significant effect of treatment on male immobility in the OF test contrasted with much of our findings, and supports the expectation that offspring from low LG-ABN mothers will be more anxious within the first five minutes of the OF test. Previous literature supports that offspring from low LG-ABN mothers show decreased open-field exploration, and greater latencies when placed in novel environments (Francis et al., 1999). The male offspring from low LG-ABN mothers treated with the vehicle solution were significantly more immobile compared to any other treatment group. Low offspring treated with FGF2 also appeared to spend the least amount of time immobile than other treatment groups. There was no difference between time spent immobile in the offspring from high LG-ABN

mothers treated with vehicle or FGF2. Although these results are consistent with previous findings, they were not consistent throughout the study. Female results from the OF test showed in both high and low offspring that FGF2 treatment increased immobility. As there is a lack of research using female offspring, and from previous work showing males and females often perform in opposite fashion under stressful conditions, this finding should be investigated in future work. Additionally, while FGF2 treatment increased immobility, male and female offspring from high LG-ABN mothers showed significantly low immobility times. It was thought that these offspring were hyperactive, and total distance travelled over the full twenty minutes of the OF test was measured. The analysis of total distance travelled revealed no significance of maternal behaviour or of treatment in males or females, however male offspring from high LG-ABN mothers had higher mean distances with little difference between vehicle and FGF2 treatment. Female results showed offspring treated with FGF2 from both low and high LG-ABN mothers had greater mean total distance travelled. Unfortunately, these results did not support hyperactivity of offspring from high LG-ABN mothers.

A significant effect of maternal behaviour was found in female offspring when measuring latency to the open arms in the elevated plus maze. While maternal behaviour appeared significant, it was the offspring from high LG-ABN mothers who had the greatest latency times, suggesting increased anxiety in those animals. This finding has not been supported by previous research. There was also no significant difference between treatment groups of female offspring from low LG-ABN mothers for immobility times. This suggests the treatment of FGF2 did not reverse the expected anxiety-like behaviours in such animals. Similar findings came from the results of mean time spent in closed arms, with a significant effect of maternal behaviour found in females. The offspring from high LG-ABN mothers were found to spend significantly more

time in the closed arms, and there was no difference between time spent in closed arms in either treatment group of offspring from low LG-ABN mothers. This further supports results showing no effect of FGF2 treatment on offspring from low LG-ABN mothers.

The forced swim test results showed significant effects of maternal behaviour and treatment for females, and a significant effect of maternal behaviour for males. Female offspring from high LG-ABN mothers showed significantly less immobility, however FGF2 treatment increased immobility time for both groups of offspring from low and high LG-ABN mothers. Male offspring from low LG-ABN mothers showed significantly greater immobility, however there was no significant difference between treatment groups. Previous studies have demonstrated that FGF2 has antidepressant-like effects in behavioural models such as the FST (ElSayed et al., 2012). This effect has specifically been shown in adulthood (Turner et al., 2006), and our results from low LG-ABN male offspring support possible depressive behaviours, however FGF2 treatment did not have a therapeutic effect. Similarly, FGF2 treatment did not reduce total immobility in female offspring, contrasting much of the previous literature.

Limitations

There were several limitations to the current study. The first concerns the dosage of FGF2 and vehicle treatments. Following injections and behavioural testing it was revealed that an error was made in the calculation of the appropriate dosage for the animals used in the current study. The FGF2 solution was diluted 10fold more than the known effective dose. This error would account for the lack of effect of FGF2 administration on offspring from low LG-ABN mothers.

Maternal behaviour was also assessed by a method that was not peer reviewed, and only scored behaviour based on two 75 minute videos. Future research should use a method of

maternal behaviour scoring like that developed by Myers and colleagues (1989). It is also possible that a longer observation time is needed to assess maternal behaviour. The current study observed behaviour from a single day, while previous work has assessed maternal-offspring interactions throughout P1-P10. There was also no baseline measure of anxiety for offspring from low or high mothers, which might have highlighted any misinterpretations of maternal behaviour. Previous work suggests that baseline measures are not necessary, as maternal behaviour is consistent throughout offspring development, however since scoring occurred only on one day there was room for misunderstanding. Baseline behavioural tests might have shed light on which animals were exhibiting anxiety-like behaviours, however this would also cause a confounding variable in that the animals might become familiar with the stress testing.

While the study incorporated females into the analysis, there was no control for estrous cycle. Much of previous research has used only male offspring and there is not as much literature to refer to when interpreting female results. The lack of effect from FGF2 treatment can account for some variance, however further work is needed to control for the estrous cycle in female stress research.

The last limitation of the current work is that not all 79 animals were included in each behavioural analysis. Due to technical issues with the recording device, some behaviour videos were not recoverable, and approximately 15 animals were not consistently accounted for on various behaviour measures. This resulted in larger variances in behaviour scores and unequal group numbers, making it difficult to compare separate behaviours from the same animals.

Future Work

Female results from the OF test showed that FGF2 treatment increased immobility in both high and low LG-ABN offspring. As there is a lack of research using female offspring, and

from previous work showing males and females often perform in opposite fashion under stressful conditions, this finding should be investigated in future work. Furthermore, as suggested, future work should investigate the role of the estrous cycle and account for it.

A replication of the current study is needed, however with a change in FGF2 and vehicle dosage. While the dose being administered was believed to be 10 mg/kg, this was not the case and should be recalculated. When the dose is corrected, future work should also take immunohistochemical analyses of the brains of offspring from both low and high LG-ABN mothers. This is an important step in understanding the mechanisms behind the efficacy of FGF2 to reverse anxiety-like behaviour. Previous work supports FGF2-mediated increases in GR mRNA and decreased GC levels, resulting in less anxiety (Salmaso et al., 2016; Turner et al., 2006). Therefore, future work should investigate FGF2 expression, GR mRNA expression, and GC levels in offspring from both low and high LG-ABN mothers, who were treated with both vehicle and FGF2 ligand.

Conclusions

While the current study failed to find support for the research hypotheses, the results are consistent with what might be expected from animals given an inadequate dose of treatment. As stated, future work will replicate the current study and perform immunohistochemical analyses. It is possible that the current study also touched on the differences in male and female responses to stressors in adulthood, however greater research is needed to assess the ability of FGF2 treatment in mediating the stress responses in both sexes.

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Table 1. Offspring from both low and high maternal care receiving FGF2 or vehicle injections.

	Low Maternal Care		High Maternal Care	
	Female	Male	Female	Male
FGF2	12	9	8	11
Vehicle	12	9	8	10
Total	24	18	16	21

Table 2. Number of offspring in each experimental group.

		Low Maternal Care	High Maternal Care
Group 1	FGF2	6	5
	Vehicle	5	4
Group 2	FGF2	5	5
	Vehicle	5	5
Group 3	FGF2	5	5
	Vehicle	5	5
Group 4	FGF2	5	4
	Vehicle	6	4

Table 3. Maternal Behaviour for a High-Scoring Dam.

RAT: F3	Time ON/OFF	Behaviour 1	Behaviour 2	Behaviour 3	TOTAL
6-6:10	10 ON		8 MIN	2 MIN	22
6:10-6:20	10 ON		4 MIN	6 MIN	26
6:20-6:30	10 ON		9 MIN	1 MIN	21
6:30-6:40	10 ON		8 MIN	2 MIN	22
6:40-6:50	10 ON		10 MIN		20
6:50-7	10 ON		8 MIN	2 MIN	22
7-7:15	15 ON		9 MIN	6 MIN	36
TOTAL	75 ON				169

***Table 4*.** Maternal Behaviour for a Low-Scoring Dam.

RAT: F1	Time ON/OFF	Behaviour 1	Behaviour 2	Behaviour 3	TOTAL
6-6:10	10 ON		8 MIN	2 MIN	22
6:10-6:20	10 ON		5 MIN	5 MIN	25
6:20-6:30	1 ON 9 OFF		1 MIN		2
6:30-6:40	10 OFF				0
6:40-6:50	10 OFF				0
6:50-7	10 OFF				0
7-7:15	15 OFF				0
TOTAL	21 ON 54 OFF				49

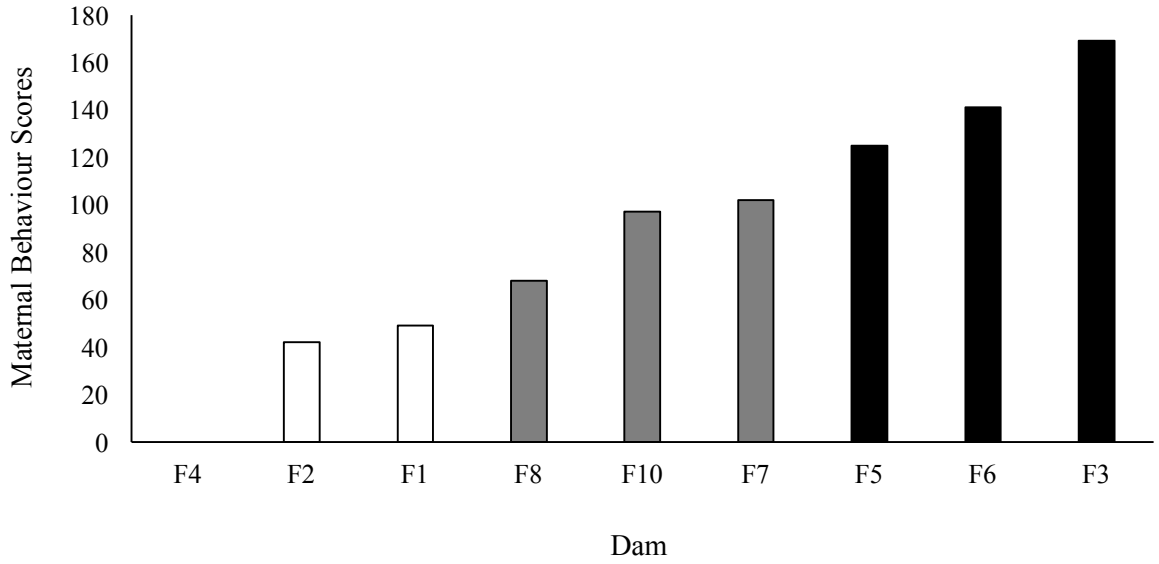

Figure 1. *High and Low Maternal Behaviour Scores.* The first three outlined bars represent the three lowest scoring maternal dams (the first rat scoring zero) used in the study, as well as the top three highest scoring maternal dams indicated by the black-filled bars.

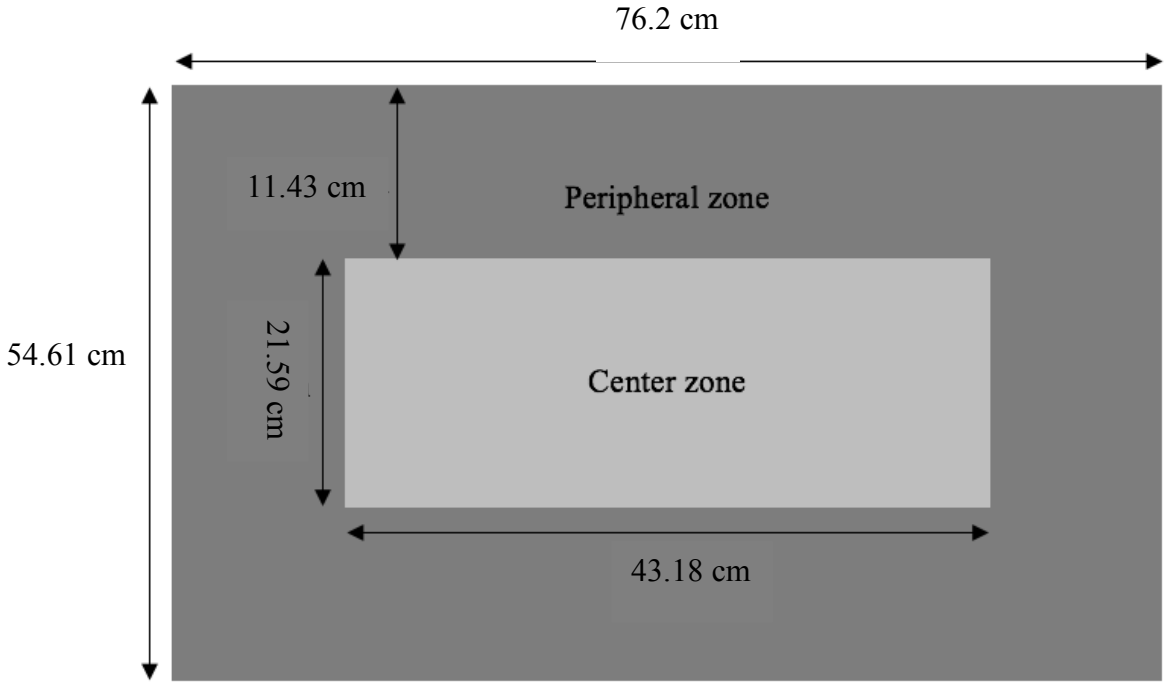

Figure 2. *Open Field.* The darker surround area represents the peripheral zone, while the lighter grey represents the center zone.

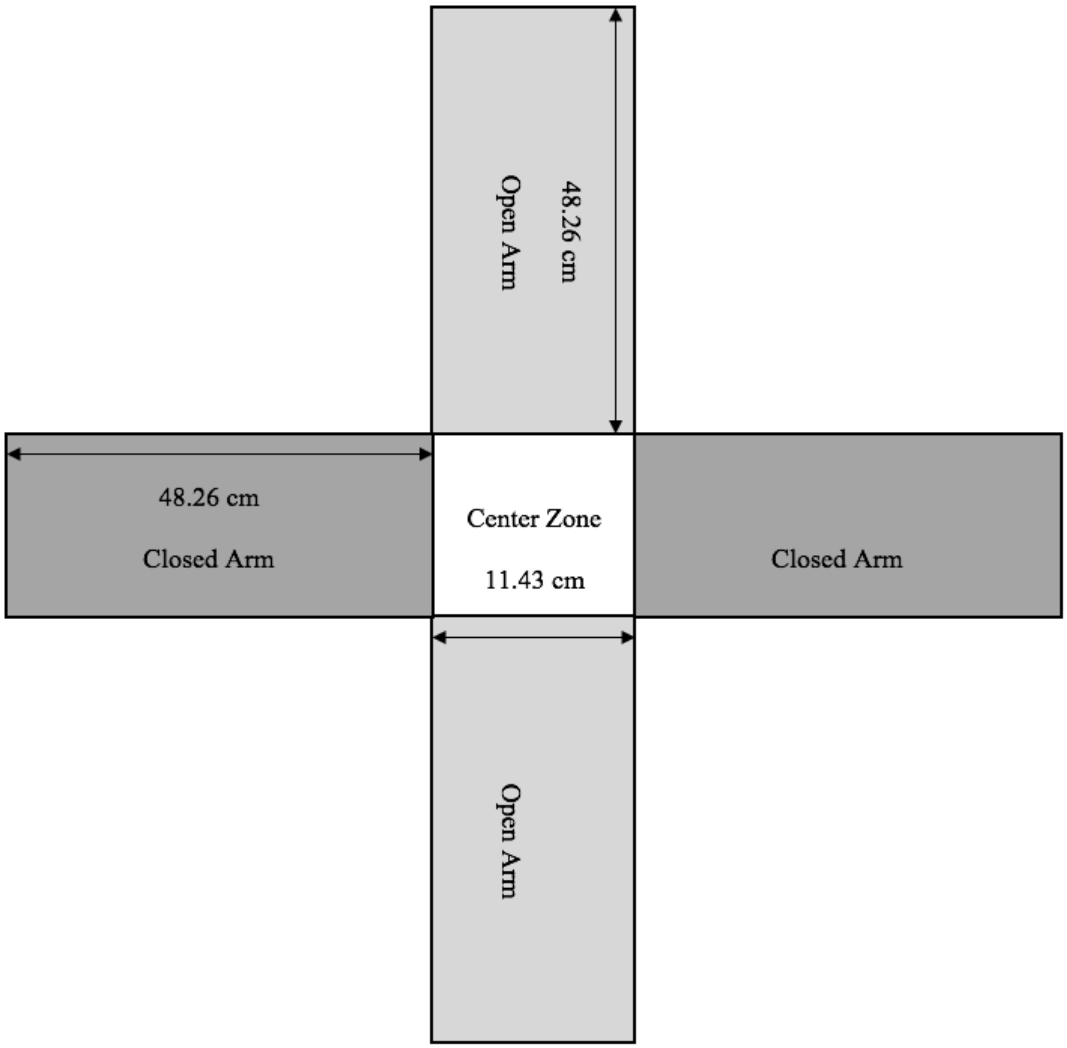

Figure 3. *Elevated Plus Maze.* The measurements of opposing closed and open arm zones, as well as the center zone.

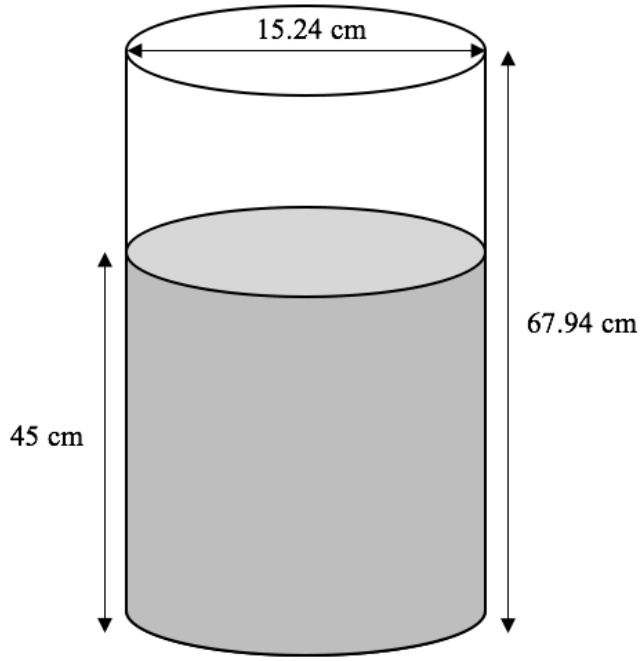

Figure 4. *Forced Swim Test.* The measurements of the cylinder in which the forced swim test took place.

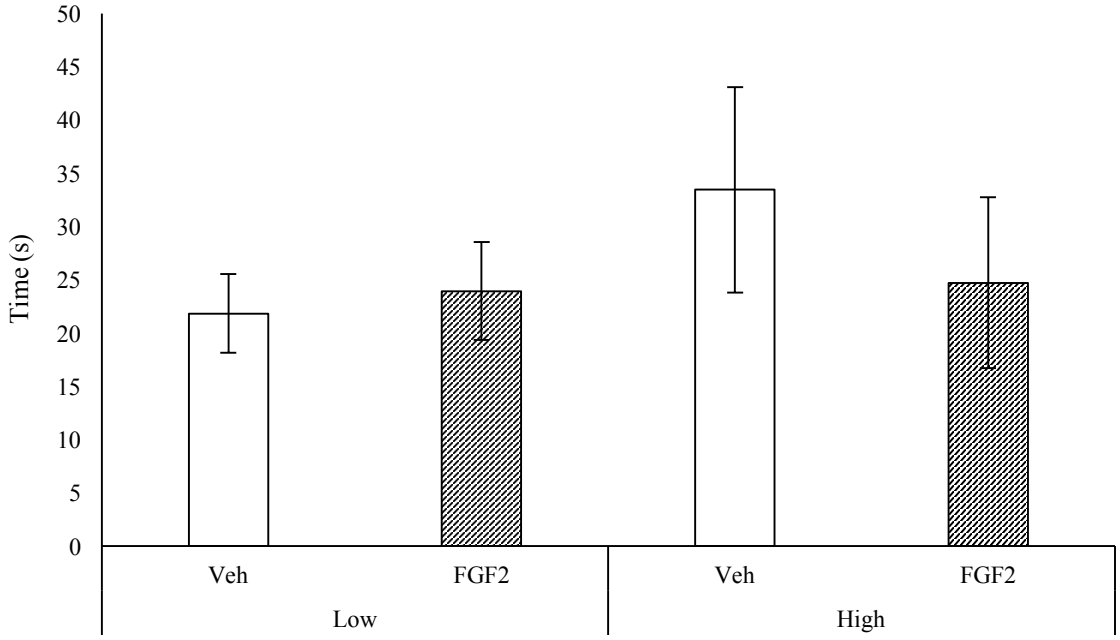

Figure 5. *Female Mean Latency to Center Zone in Open Field Test.* Data bars denote group means, and error bars as +/- SEM.

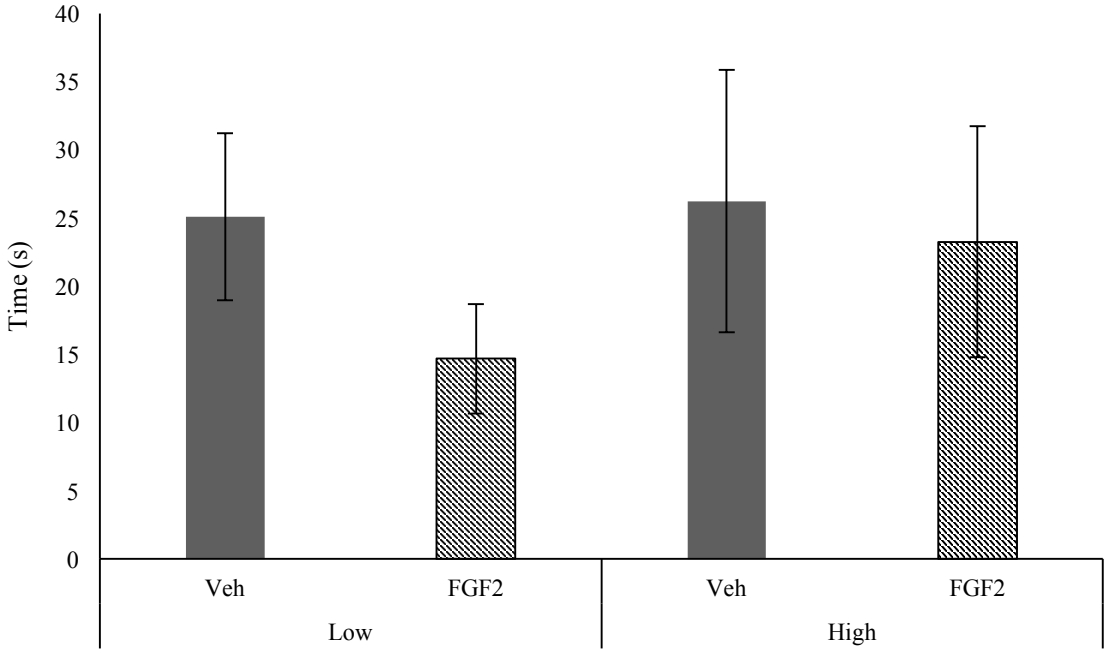

Figure 6. *Male Mean Latency to Center Zone in Open Field Test.* Data bars denote group means, and error bars as +/- SEM.

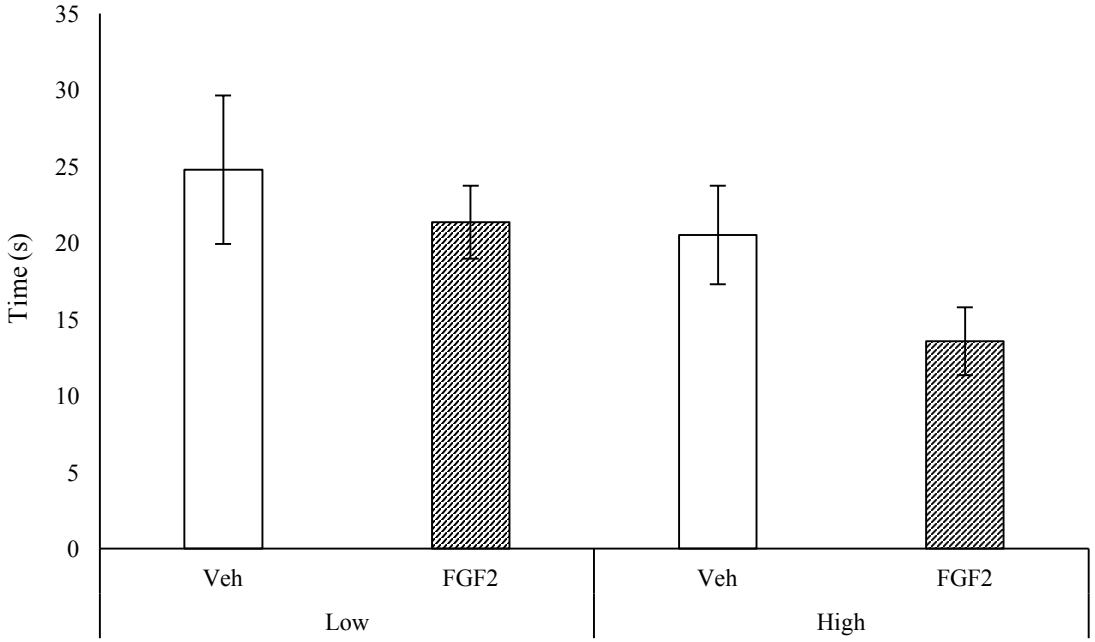

Figure 7. *Female Mean Time in Center Zone of Open Field Test.* Data bars denote group means, and error bars as +/- SEM.

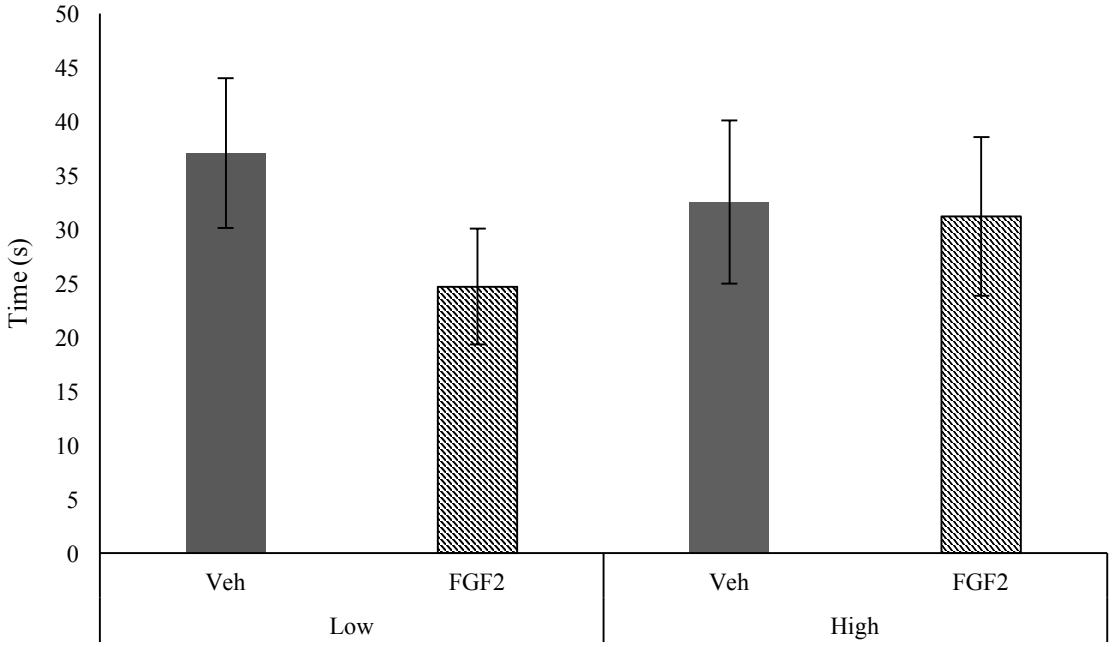

Figure 8. *Male Mean Time in Center Zone of Open Field Test.* Data bars denote group means, and error bars as +/- SEM.

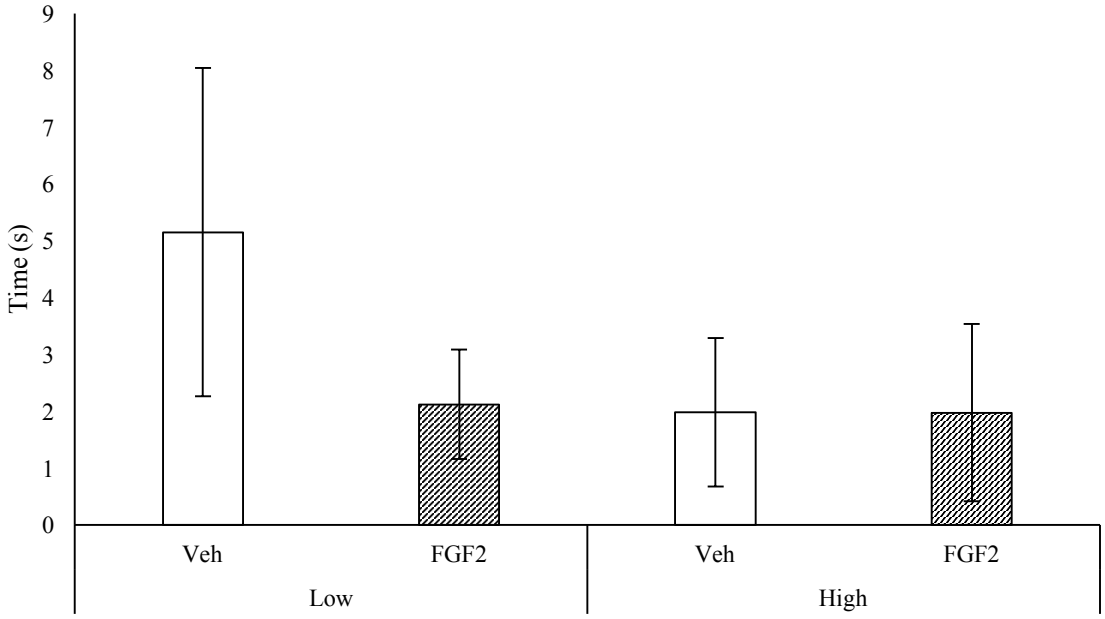

Figure 9. *Female Mean Time Immobile in Center Zone of Open Field Test.* Data bars denote group means, and error bars as +/- SEM.

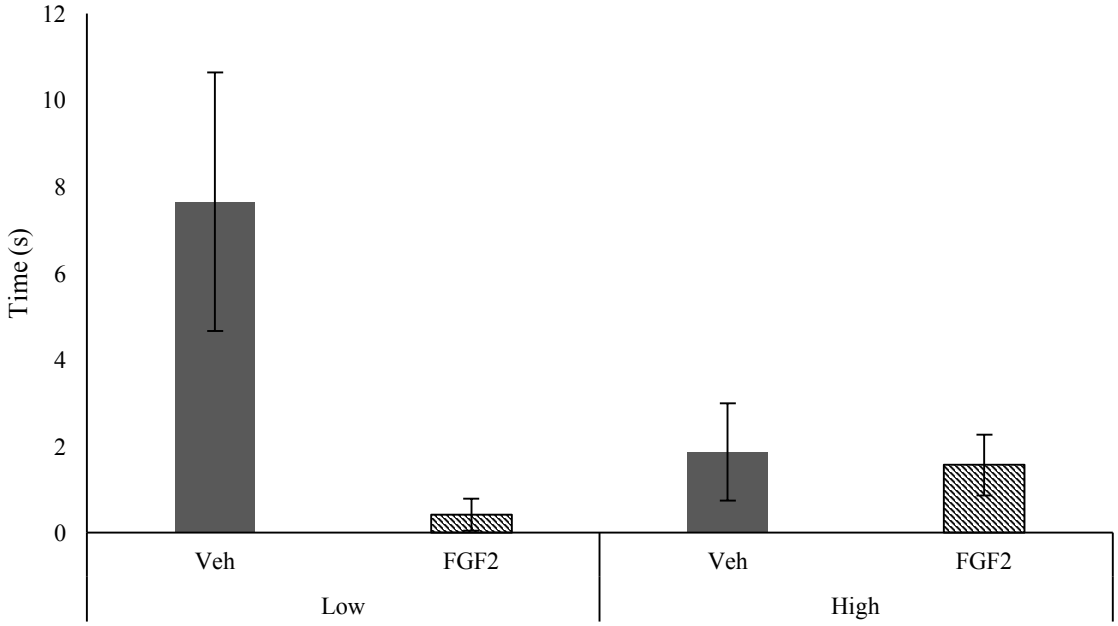

Figure 10. *Male Mean Time Immobile in Center Zone of Open Field Test.* Data bars denote group means, and error bars as +/- SEM.

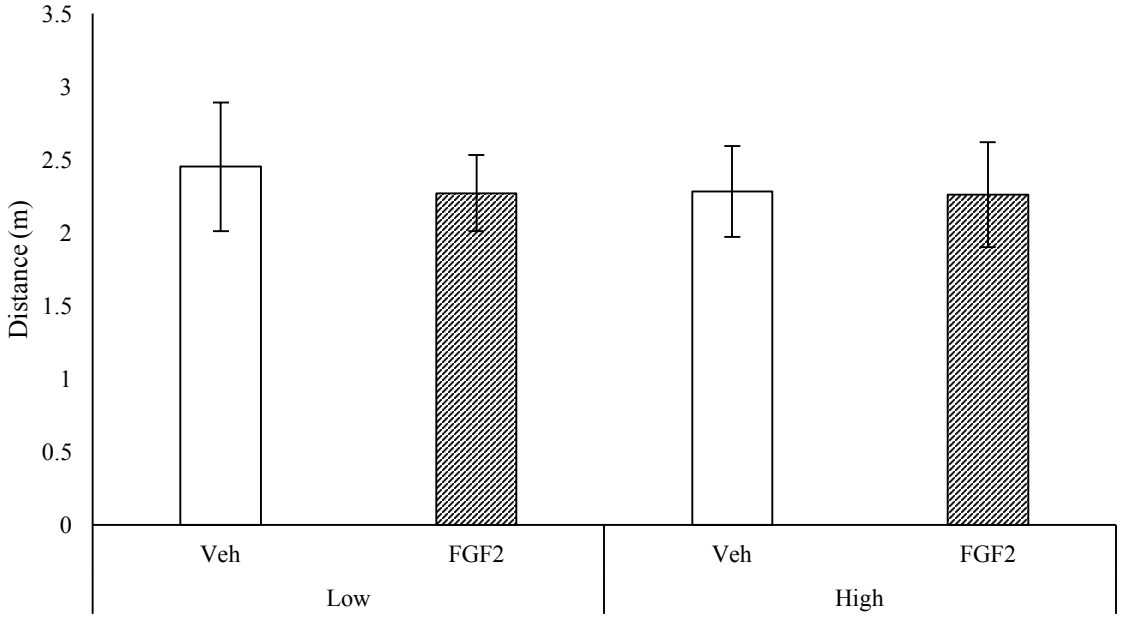

Figure 11. *Female Mean Distance in Center Zone of Open Field Test.* Data bars denote group means, and error bars as +/- SEM.

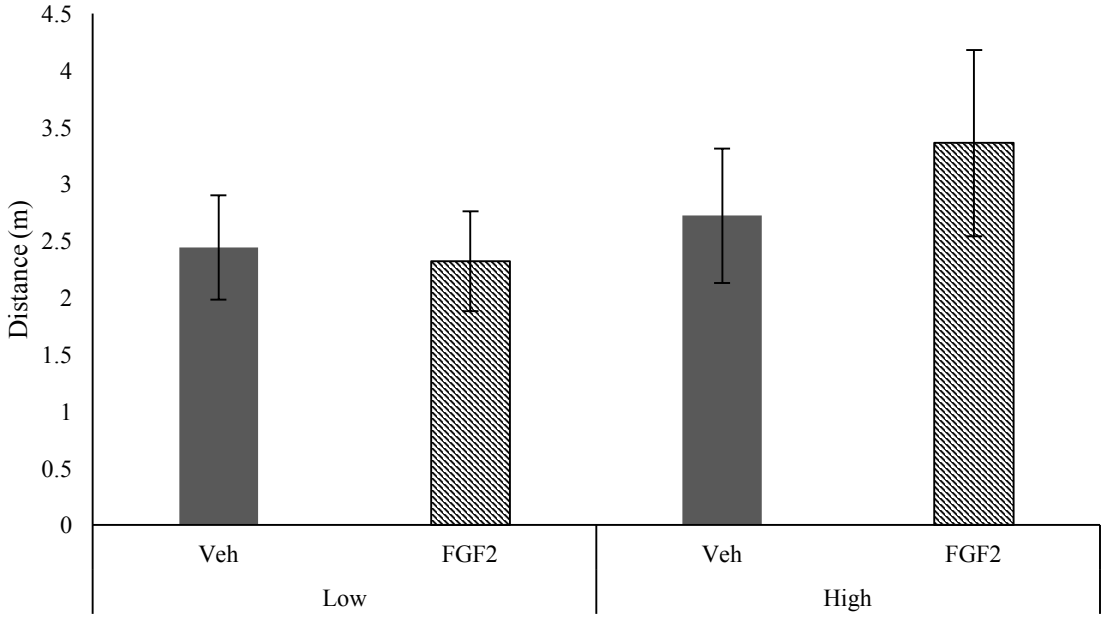

Figure 12. *Male Mean Distance in Center Zone of Open Field Test.* Data bars denote group means, and error bars as +/- SEM.

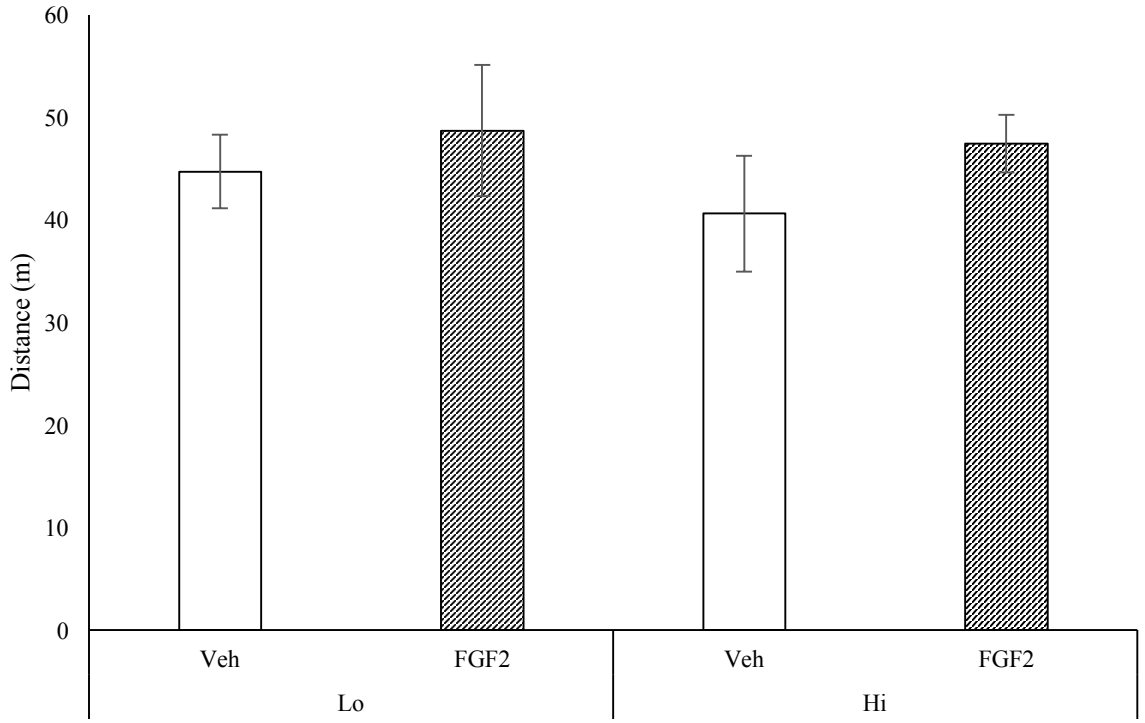

Figure 13. *Female Mean Total Distance in Open Field Test.* Data bars denote group means, and error bars as +/- SEM.

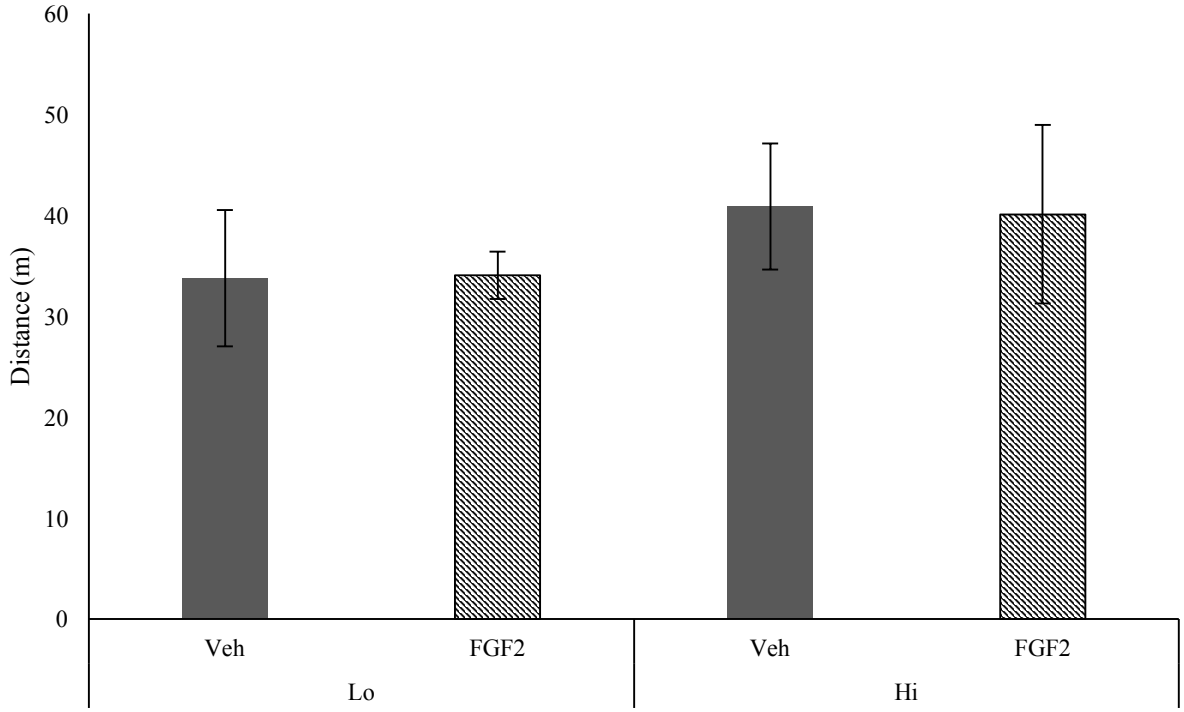

Figure 14. *Male Mean Total Distance in Open Field Test.* Data bars denote group means, and error bars as +/- SEM.

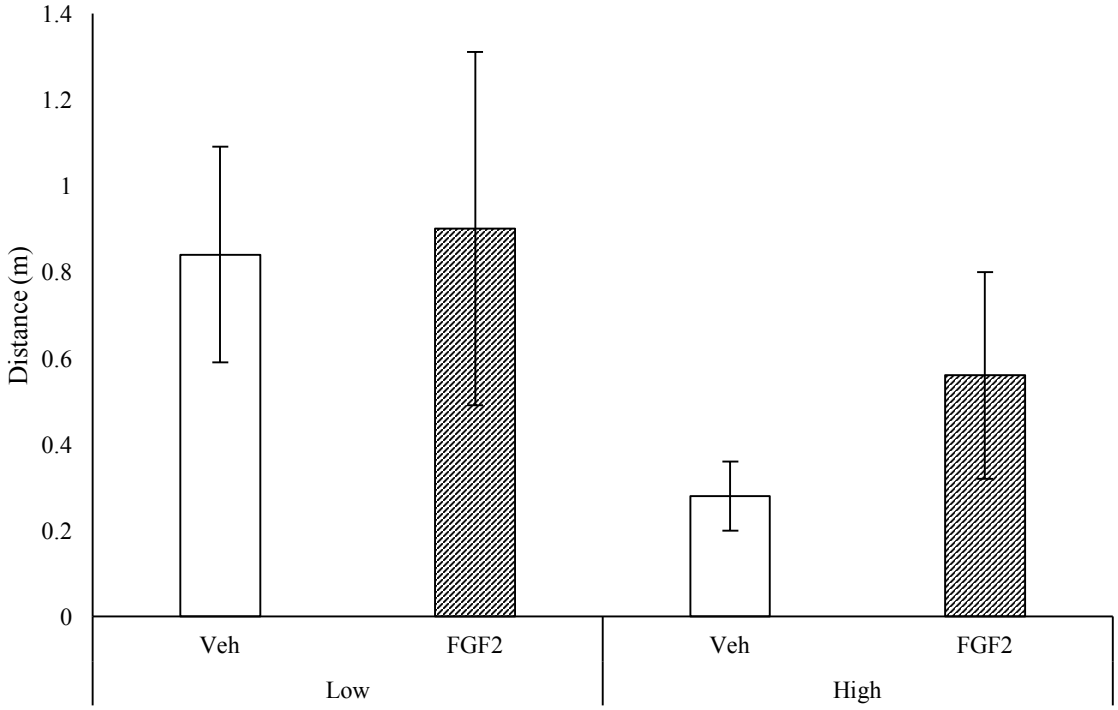

Figure 15. *Female Mean Distance in Open Arms of Elevated Plus Maze.* Data bars denote group means, and error bars as +/- SEM.

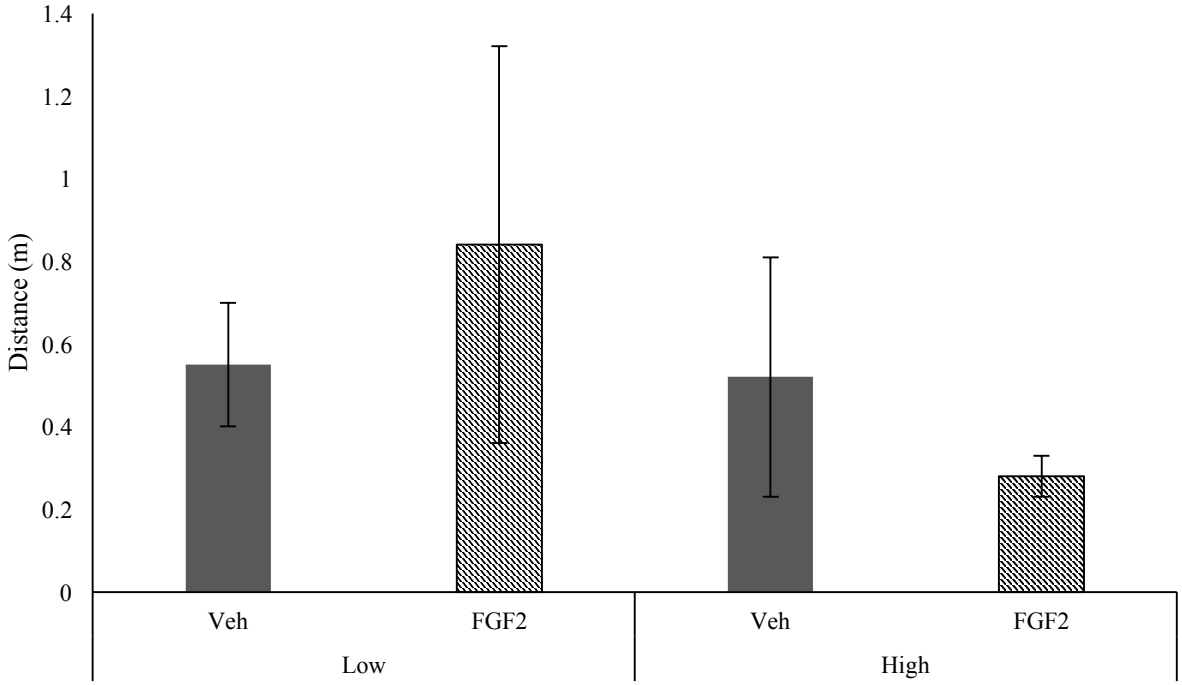

Figure 16. *Male Mean Distance in Open Arms of Elevated Plus Maze.* Data bars denote group means, and error bars as +/- SEM.

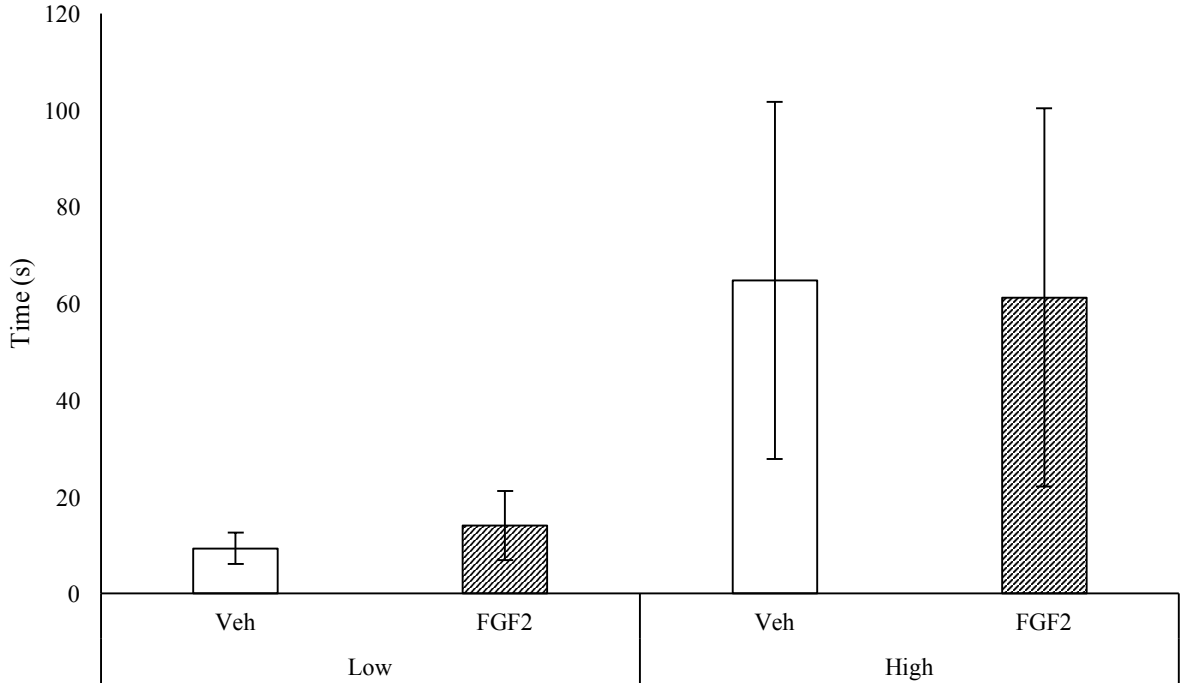

Figure 17. *Female Mean Latency to Open Arms of Elevated Plus Maze.* Data bars denote group means, and error bars as +/- SEM.

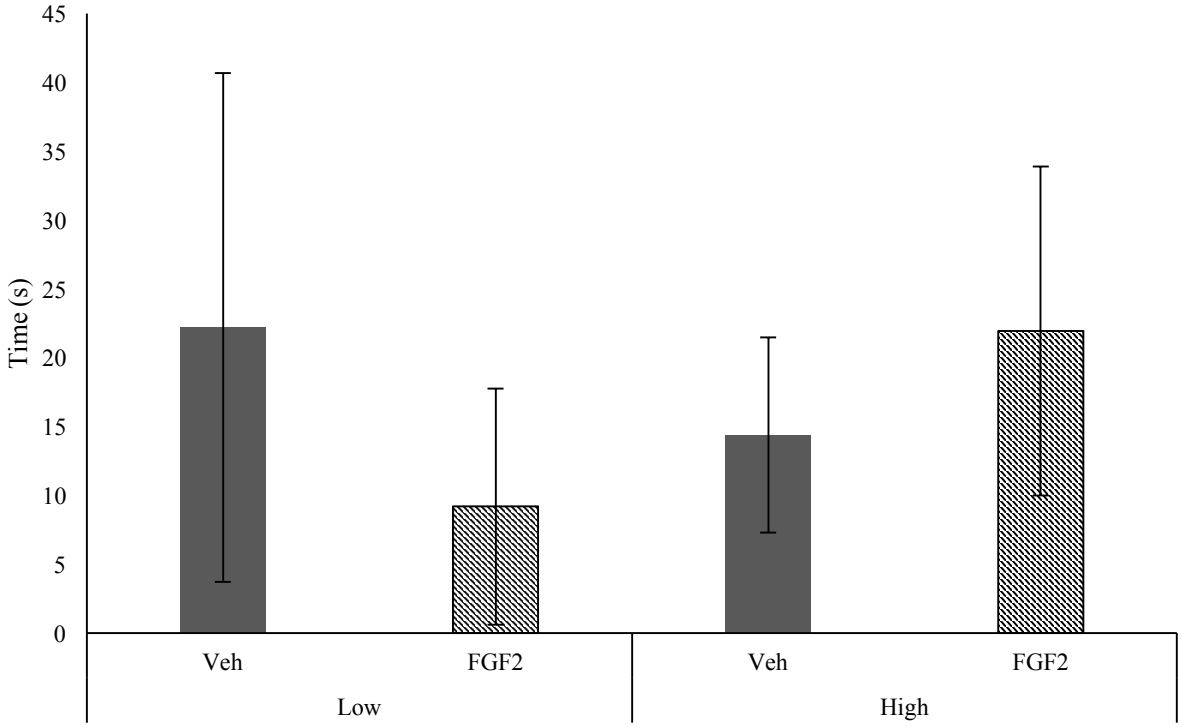

Figure 18. *Male Mean Latency to Open Arms of Elevated Plus Maze.* Data bars denote group means, and error bars as +/- SEM.

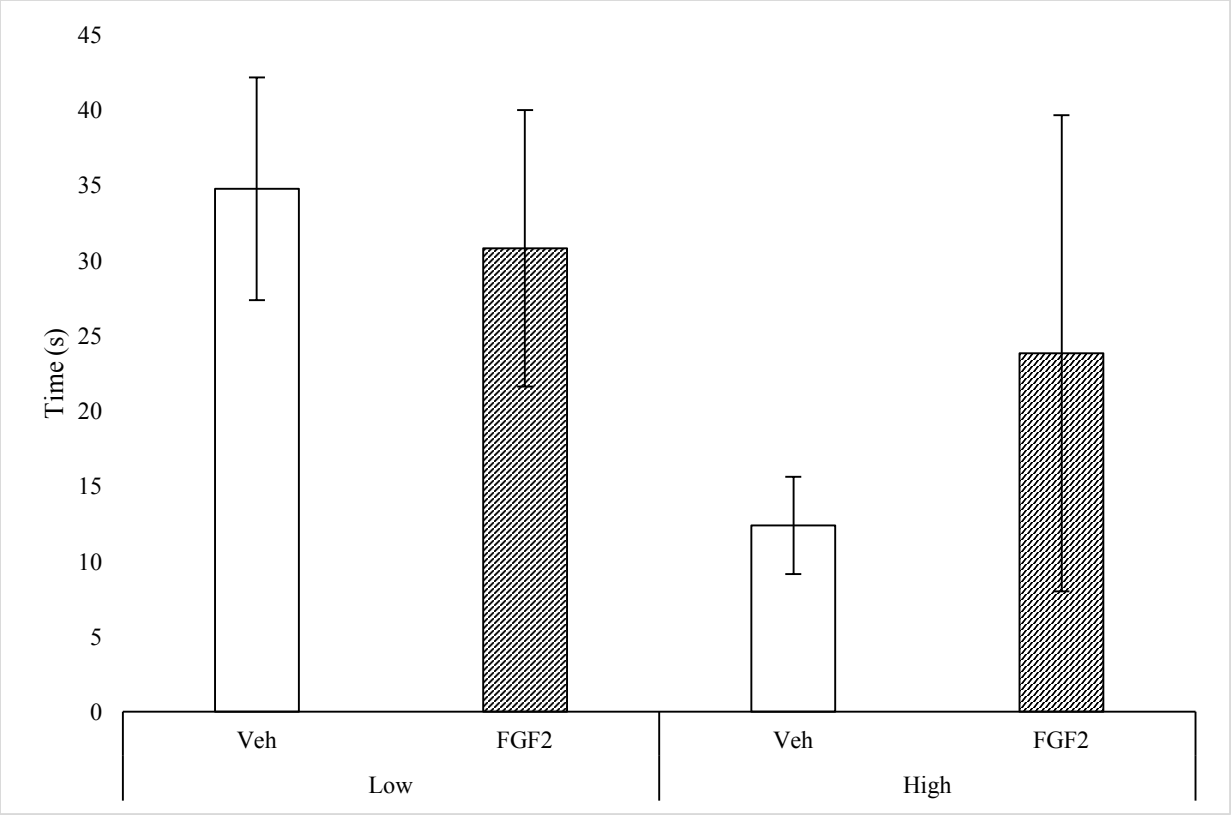

Figure 19. *Female Mean Time in Open Arms of Elevated Plus Maze.* Data bars denote group means, and error bars as +/- SEM.

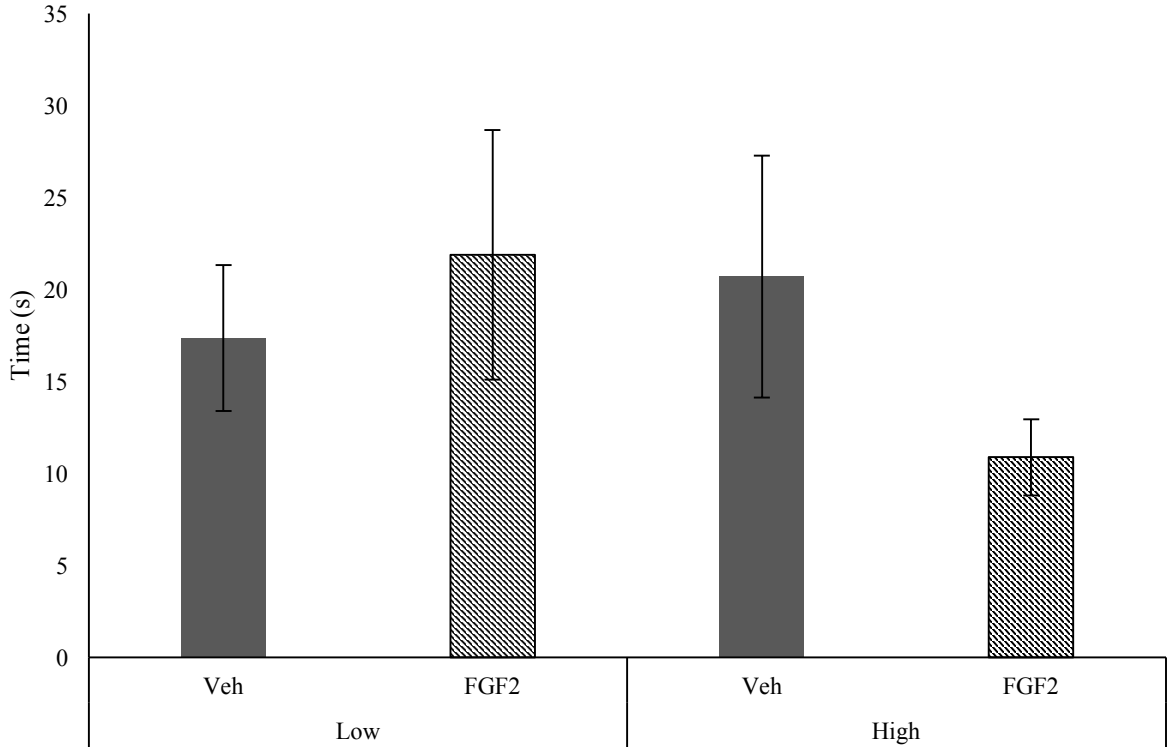

Figure 20. *Male Mean Time in Open Arms of Elevated Plus Maze.* Data bars denote group means, and error bars as +/- SEM.

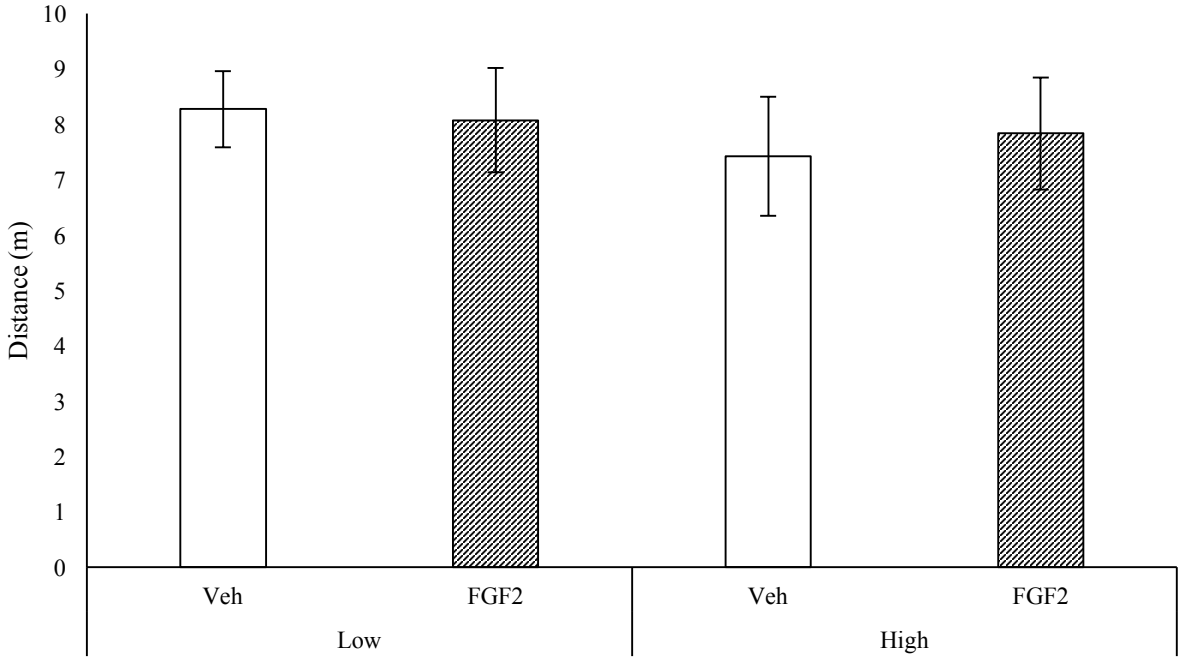

Figure 21. *Female Mean Distance in Closed Arms of Elevated Plus Maze.* Data bars denote group means, and error bars as +/- SEM.

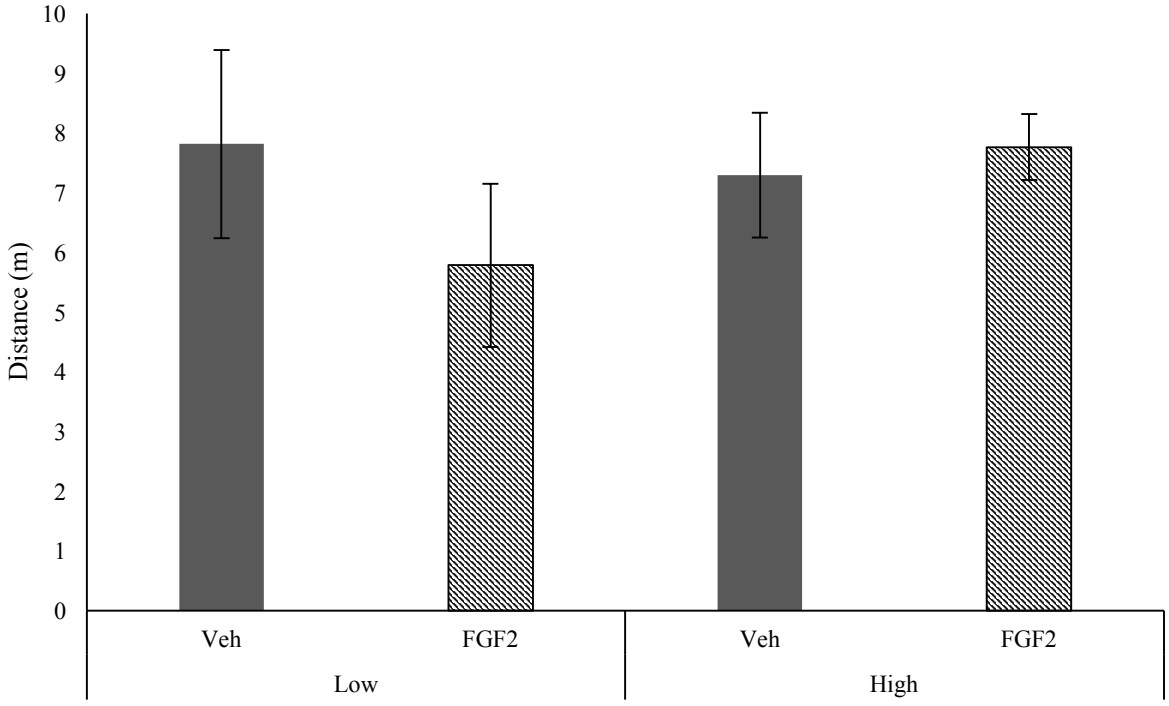

Figure 22. *Male Mean Distance in Closed Arms of Elevated Plus Maze.* Data bars denote group means, and error bars as +/- SEM.

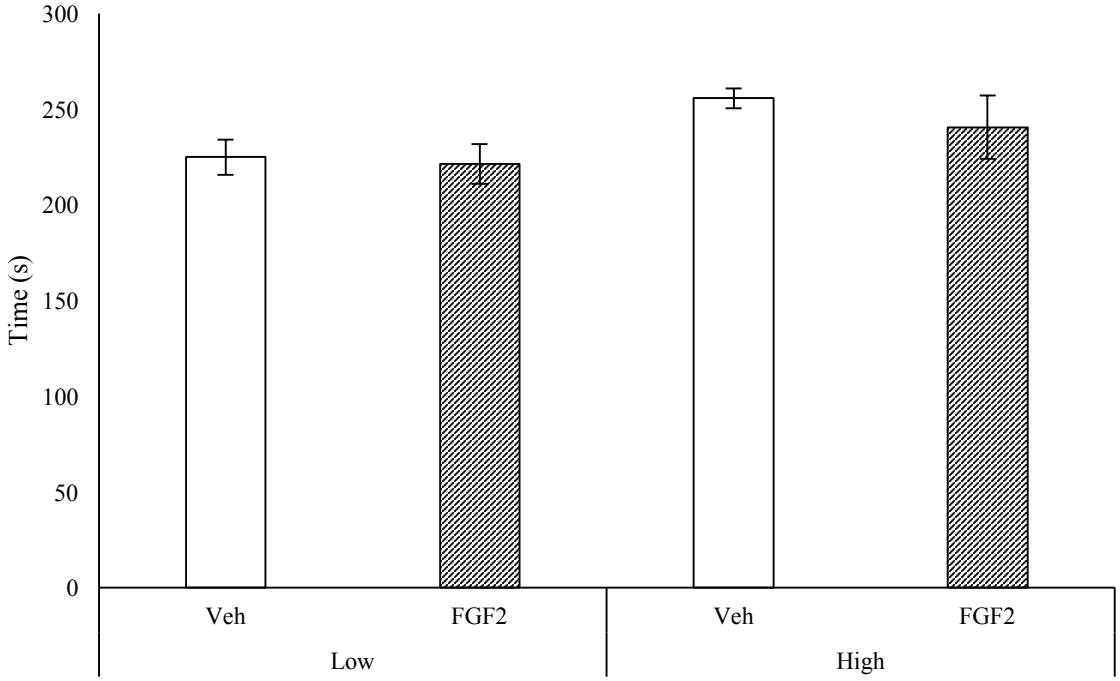

Figure 23. *Female Mean Time in Closed Arms of Elevated Plus Maze.* Data bars denote group means, and error bars as +/- SEM.

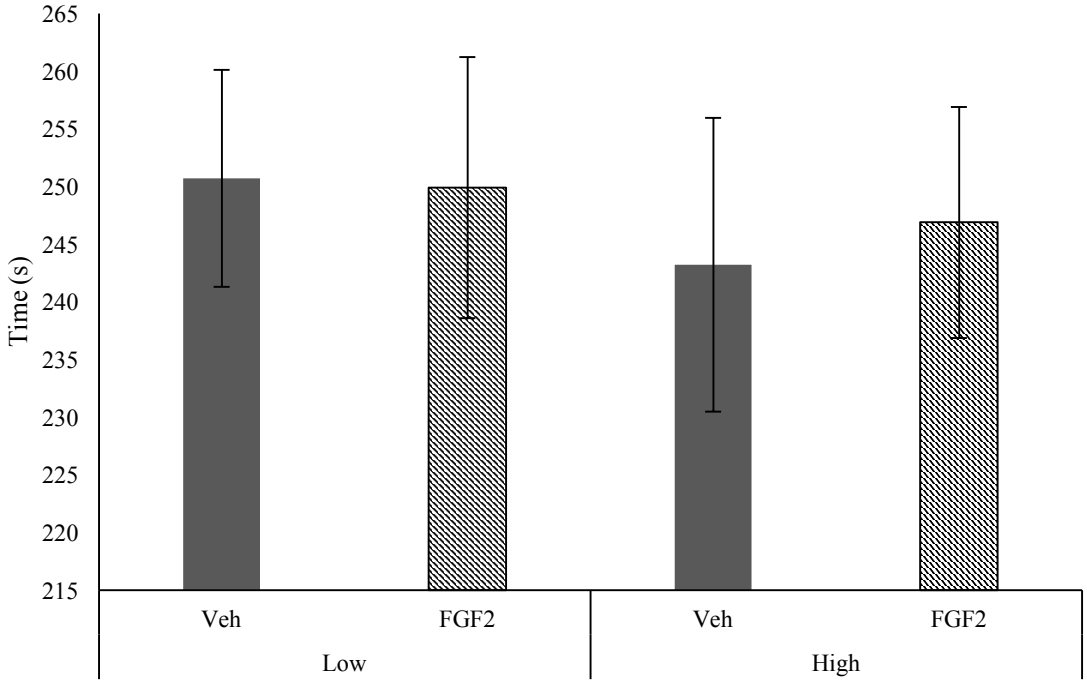

Figure 24. *Male Mean Time in Closed Arms of Elevated Plus Maze.* Data bars denote group means, and error bars as +/- SEM.

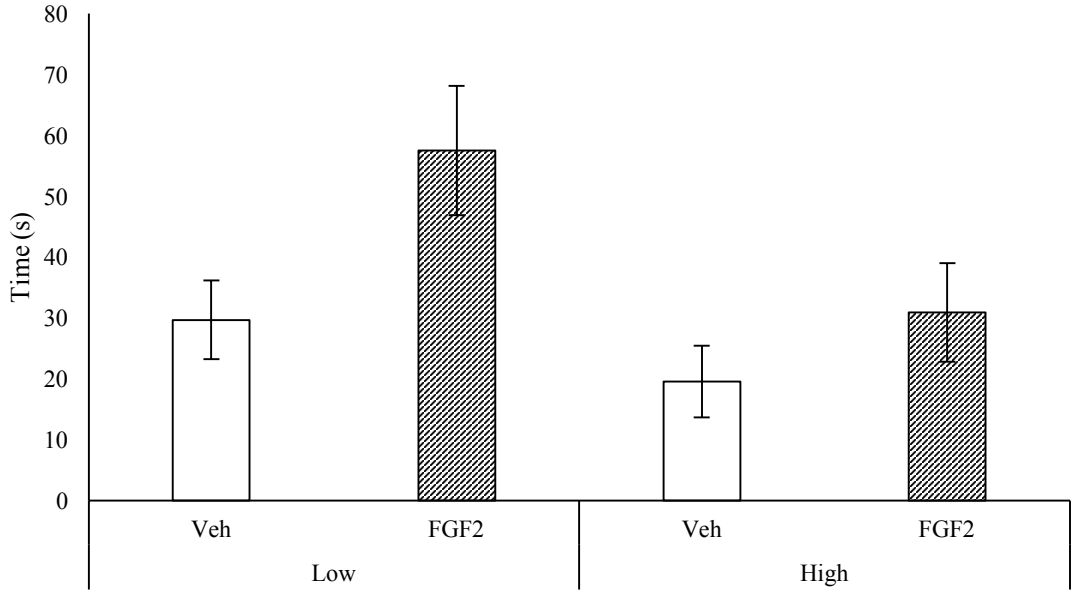

Figure 25. *Female Mean Total Time Immobile in the Forced Swim Test.* Data bars denote group means, and error bars as +/- SEM.

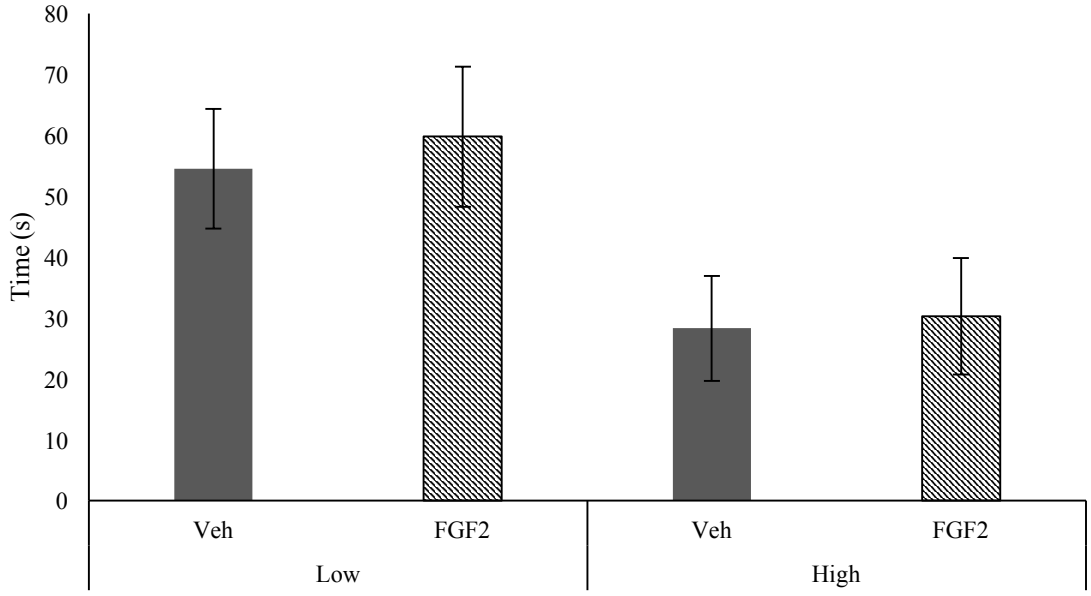

Figure 26. *Male Mean Total Time Immobile in the Forced Swim Test.* Data bars denote group means, and error bars as +/- SEM.

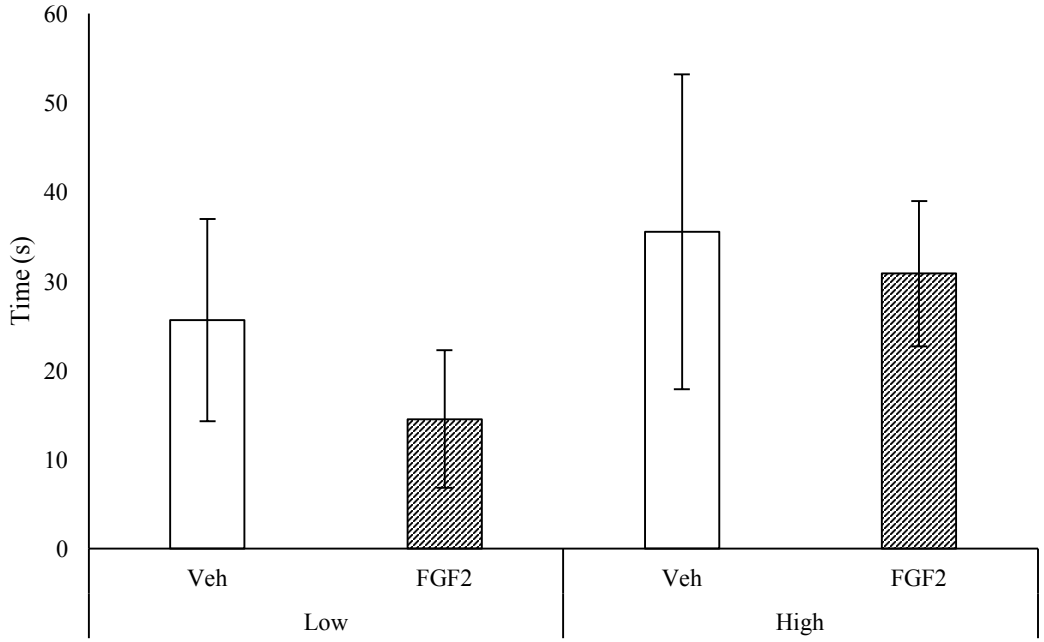

Figure 27. *Female Mean Latency to Immobility in the Forced Swim Test.* Data bars denote group means, and error bars as +/- SEM.

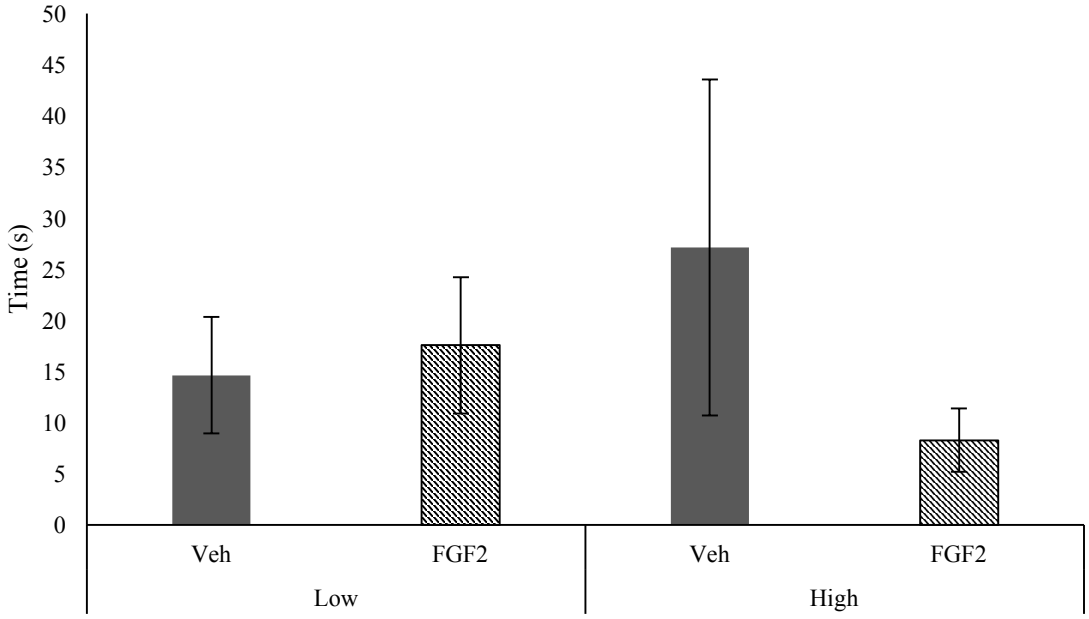

Figure 28. *Male Mean Latency to Immobility in the Forced Swim Test.* Data bars denote group means, and error bars as +/- SEM.