Sex differences in the behavioural response to FGF2 administration.

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Abstract

Anxiety is one of the most prevalent psychiatric disorders in Canada, with 1 in 4 adults meeting the criteria for diagnosis in their lifetime. Although many of the mechanisms involved in the etiology of anxiety are not yet fully known, there are several environmental and genetic factors that have been identified to increase the risk of developing anxiety in later life, including familial history, severe physical or psychological trauma, and early life stress. The current available treatments for anxiety disorders are far from ideal in that they have delayed therapeutic effects, show selective treatment of symptoms, and approximately half of patients do not respond to current treatment options. This outlines the necessity for further research into the biological basis of anxiety disorders in addition to novel therapeutic targets. Recent research has implicated fibroblast growth factor 2 (FGF2) dysregulation in the pathogenesis of depressive disorders, and has shown considerable promise in being an endogenous anxiolytic factor (Salmaso & Vaccarino, 2011).

The present study aimed to examine a preclinical model of anxiety using a maternal separation paradigm of early life stress to induce an anxious adult phenotype. Maternal separation stress in wild-type Long-Evans rats was used to mimic early life parental separation/neglect in humans, an environmental factor that has been implicated with increased risk for developing anxiety in later life (Kessler, Davis, & Kendler, 1997). FGF2 was administered in adulthood to rescue this anxious phenotype. To our knowledge, studies of FGF2's anxiolytic potential in animal models have to date only been conducted in male subjects, therefore, in the current study we employed both male and female subjects. Following adult treatment with either FGF2 or vehicle control, rats were tested on several measures of depressive and anxiety-like behaviours. Unexpectedly, results did not show an effect of maternal separation stress on the anxiety behavioural tests conducted. There was, however, an interesting sex-specific response to FGF2 in control conditions, such that females appeared to respond better to FGF2 administration than males. Importantly, future research will be needed to delineate these sex-specific differences in FGF2's anxiolytic potential in order to understand the generalizability of its therapeutic potential.

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INTRODUCTION

Anxiety

Anxiety is one of the most prevalent psychiatric disorders in Canada, with one in four adults diagnosed with an anxiety disorder in their lifetime (McLean, 2003). The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) defines anxiety as a group of disorders characterized by the presence of excessive and persistent apprehension, worry, and fear, which ultimately results in behavioural disturbances (American Psychiatric Association, 2013). This group includes specific phobias, panic disorder, and generalized anxiety disorder, among others. The rate of comorbidity between these and other psychiatric disorders is high, with the presence of one often being a risk factor for the development of others (American Psychiatric Association, 2013; McLean, 2003). Among these, depressive disorders have the highest rates of comorbidity with anxiety disorders, with reported rates of prevalence as high as 50% in patients (Beekman et al., 2000; Hirschfeld, 2001). Depressive and anxiety disorders often present themselves with similar, and sometimes overlapping, clinical symptoms, making it difficult to properly diagnose in patients (Hirschfeld, 2001). Similarly, anxiety disorders may also manifest with many physiological disturbances, such as gastrointestinal disease, respiratory conditions, and other chronic illnesses (Sareen et al., 2005). Both anxiety disorders themselves in addition to their high comorbidity with other chronic conditions may lead to high rates of disability in patients, severely affecting their quality of life.

The etiology of anxiety disorders is not known, but many risk factors, ranging from genetic predispositions to environmental triggers or a combination thereof, are thought to contribute to the manifestation of anxiety in an individual. Twin studies have revealed that anxiety disorders, specifically general anxiety disorder and panic disorder, are more prevalent in

individuals with a family history of such disorders, indicating a genetic contribution (Skyre, Onstad, Torgersen, Lygren, & Kringlen, 1993). Stressful and traumatic experiences, particularly in childhood, can also contribute to developing an anxiety disorder. Sexual abuse, parental separation or abandonment, and social isolation are all environmental situations that have been identified as major risk factors for developing anxiety in childhood, which may consequently persist into adulthood if left untreated (American Psychiatric Association, 2013; Kessler et al., 1997). Current treatments for anxiety disorders include antidepressant and anxiolytic pharmaceuticals, cognitive and behavioural psychotherapies, and combinations of both; however, despite this range of available treatments, their efficacy is concerningly low, with only 50-60% of patients experiencing some form of relief (Campbell-Sills et al., 2016; Roy-Byrne, 2015). Both anxiolytic and antidepressant pharmaceuticals have considerable limitations. Many anxiolytic treatments provide only temporary relief of acute symptoms, with few traditional anxiolytics able to provide more long-term alleviation of core symptoms. Antidepressant treatments, such as selective serotonin reuptake inhibitors (SSRIs), have seen success in treating symptoms of anxiety, and are often used as a first line treatment (Bystritsky, Khalsa, Cameron, & Schiffman, 2013; Roy-Byrne, 2015); however, their therapeutic actions are often delayed in nature. Additionally, an anxiolytic or antidepressant treatment may not be successful in a patient, resulting in the patient and clinician being forced to adjust the treatment and repeat the process until a successful therapy is found. To further complicate the process, the treatments themselves are not without side effects, and they are often discontinued by the patient due to these negative consequences (Cowley, Ha, & Roy-Byrne, 1997). The uncertainty surrounding current treatments for anxiety highlights the importance and necessity of continuing research into

the pathophysiology and neural mechanism underlying these disorders, which may expose potential targets that future treatments can successfully act upon in a more curative fashion.

Early Life Maternal Separation Stress

Animal models of anxiety have taken many forms in research, with everything from specially bred transgenic lines, to environmental-based stress procedures. Transgenic models are beneficial in that they provide consistent and predictable phenotypic changes in the animals that can make them more predisposed to high anxiety behaviour; for example, one wild-type dam will produce a litter of high responders to novel situations that are less anxious, whereas a different transgenic dam will produce a litter of low responders to novel situations that are more anxious (Turner, Clinton, Thompson, Watson, & Akil, 2011). However, transgenic models are limited in their translational capabilities. In this respect, more appropriate models are those that attempt to mimic the complex etiology of human anxiety disorders. Although many facets of the etiology of anxiety disorders are not known, environmental adversities can predispose an individual to developing anxiety. Adverse early life experiences, such as parental loss or separation, family dysfunction, social isolation, abuse, or trauma, are considered major risk factors to developing later life anxiety (Kessler et al., 1997; Tata, 2012).

Many paradigms of early life stress have been developed in animals to study the underlying mechanisms of this adversity in adulthood. One of the most common animal protocols is known as maternal separation (MS). This protocol involves the separation of the pups (usually rodents) from their mother and home cage for extended periods of time, before ultimately being returned at the end of each period. This manipulation has been extensively researched, and is widely accepted as a reliable and relevant animal model of how the adverse experience of parental separation in early life can lead to acute and chronic negative effects on

behaviour, emotionality, and physiology (Tata, 2012). The protocols for MS vary greatly, and many studies suggest that the frequency, length, and age of the animals at separation will influence the results. In general, there are two common protocols distinguished by their overall behavioural and physiological effects; a short separation period referred to as early handling (EH), and a long separation period referred to as maternal separation. Early handling involves a separation period of 15 minutes before the pups are returned to their mother and home cage. Interestingly, this method has been repeatedly shown to be protective and increase resilience to later life stress, such that these pups display a hypo-responsive hypothalamic-pituitary-adrenal (HPA) axis, more efficient patterns of glucocorticoid receptor (GR) binding and negative feedback, and lower levels of circulating corticosterone (CORT) in adulthood (Meaney et al., 1996; Pryce, Bettschen, & Feldon, 2000). These molecular results are further supported by decreases in anxiety behaviour measures in the open field test and elevated plus maze compared to non-handled controls (Lehmann & Feldon, 2000).

On the other hand, protocols involving a long separation period have been shown to produce negative effects, and increase susceptibility to stress in later life (Lehmann & Feldon, 2000). These protocols take on many forms, with varying frequencies and lengths of separation, however, all adhere to a few common guidelines. Previous work has found that a separation time of at least 2 hours is necessary to produce immediate effects on HPA axis responsiveness (Kuhn, Pauk, & Schanberg, 1990), and at least 8 hours at one time to produce more long-term consequences (Rosenfeld, Wetmore, & Levine, 1992). Protocols of MS therefore use separation periods of 1-24 hours in length, with these periods being performed on either repeated consecutive days, repeated non-consecutive days, or, with respect to a 24-hour period, on one single day only. The severity of the consequences is also influenced by the age of the pups at the

time of separation, with the earlier in age yielding the most damaging effects in adulthood (Lehmann & Feldon, 2000). The time from postnatal day 4 to 14, an important, highly sensitive developmental period known as the stress hypo-responsive period (SHRP), is a common target at which to perform MS. This period is defined by a significant decrease in basal CORT levels, such that the pups display increased resilience against most environmental stressors; however, any significant increase in CORT during this time can cause permanent, damaging effects on the developing brain and HPA axis (Meaney et al., 1996). Maternal interactions during the SHRP play a vital role in programming the HPA axis of the pups for adulthood, and disruption of the maternal care during this period has been found to cause significant increases in adrenocorticotropic hormone (ACTH) and CORT (Kuhn et al., 1990). This suggests that MS is a highly potent environmental stressor that is strong enough to overload the blunted stress response of pups during SHRP, thereby producing more anxious adults.

Fibroblast Growth Factor 2 & Anxiety

Fibroblast growth factor 2 (FGF2), also known as basic fibroblast growth factor (bFGF), is a ligand in the FGF family that is expressed in astroglial cells throughout the brain (Gonzalez, Berry, Maher, Logan, & Baird, 1995). The mitogenic function of FGF2 is well documented, with much evidence supporting its prominent role in proliferation, differentiation, growth, and survival of neurons and glial cells throughout pre- and postnatal development (Ford-Perriss, Abud, & Murphy, 2001). In addition to these early developmental effects, FGF2 remains one of the few active growth factors in the adult brain, where it has been implicated as a necessary factor in maintaining and influencing the survivability of the adult stem cell pool. Evidence from FGF2 knockout (KO) mice demonstrates a 50% reduction in the dividing neural progenitor

population, and a significant decrease in slow dividing neural stem cells in the subventricular zone compared to their wild-type counterparts (Zheng, Nowakowski, & Vaccarino, 2004).

All members of the FGF family exert their effects through binding with one or more of the five FGF receptors (FGFRs) in the brain. Each receptor has been implicated in specific functions related to growth and survivability. All FGFRs are present in the developing brain; however, only FGFR1, FGFR2, FGFR3, and the more recently discovered FGFR5 are prominent and widely expressed in the adult brain, while FGFR4 is solely expressed in the medial habenula (Itoh et al., 1994). FGF2 has a high affinity with all five receptors, however, it binds with the highest affinity to FGFR1 (Reuss & Von Bohlen Und Halbach, 2003). This receptor is found mostly on neurons and neural stem cells, where it has been shown to play a key role in hippocampal and cortical development, and growth and proliferation of neural stem cells (Frinchi et al., 2008). FGFR2 has been found on cortical radial glial cells during development, where, in conjunction with FGFR1, it has been implicated primarily in the proper development of the medial prefrontal cortex (mPFC), as well as influencing the survivability of specific subcortical projections of the mPFC (Stevens et al., 2010). In the adult brain, FGFR2 has been found on the dividing neural progenitor cells in the subventricular zone, suggesting a role in survivability of these neural stem cells (Frinchi et al., 2008). FGFR3 is predominantly expressed by astrocytes, and appears to be involved in the proper development of the cortex and hippocampus. FGFR3 knockout mice show reduced volumes, but no change in morphology, in both of these areas, as well as smaller overall brain sizes compared to their wild-type counterparts (Moldrich et al., 2011). FGFR5, also known as FGF receptor-like 1 (FGFRL1), is a more recently discovered addition to the FGF receptor family (Sleeman et al., 2001), but is still poorly defined in its functions. FGF2 has been found to bind to FGFR5 with a lower affinity

than the other four receptors, but evidence suggests that alternative ligands in the FGF family will bind with higher affinities to this receptor (Sleeman et al., 2001).

FGF2 is expressed in the periphery by fibroblasts, and has a myriad of functions including angiogenesis (Okada-Ban, Thiery, Jouanneau, Nugent, & Iozzo, 2000), injury recovery (McGee et al., 1988), bone growth (Ornitz & Marie, 2015), and overall growth and maturation of various cell types throughout the body. Interestingly, the expression of FGF2 both in the brain and skin has previously been observed to increase in response to tactile stimulation (Kolb et al., 2012). This relationship is thought to underlie processes such as injury recovery, where animals that received tactile stimulation to treat postnatal injury of the prefrontal and motor cortices showed better functional and anatomical improvements than those that received exogenous FGF2 administration (Gibb, 2004). Beneficial aspects of tactile stimulation in early postnatal life have also been shown in animal models involving stress. Early life tactile stimulation in the forms of handling or maternal behaviours of licking, grooming, and arch-backed nursing results in pups that are more resilient to stressful or novel situations in later life compared to pups that did not experience a high level of tactile stimulation (for example, being reared by mothers that display low licking and grooming behaviours) (Francis, Diorio, Liu, & Meaney, 1999; Levine, Haltmeyer, Karas, & Denenberg, 1967). It thus seems fitting to suggest that increased anxiety behaviour observed as a result of maternal separation may, in part, be due to the disruption of tactile stimulation and subsequent decrease in FGF2 expression.

The relationship between FGF2 and mood disorders was originally identified through post-mortem brain tissue of patients with major depressive disorder. These patients exhibited pronounced dysregulation of the FGF system compared to healthy controls, with a significant decrease in FGF2 levels in the cortex (Evans et al., 2004), and hippocampus, as well as an

upregulation of FGFR1 mRNA in this area (Gaughran, Payne, Sedgwick, Cotter, & Berry, 2006). Transgenic animal models have since provided evidence to further support this relationship. Previous studies have found that basal levels of FGF2 are inversely correlated to natural anxiety behaviour, such that animals with higher levels of FGF2 in the brain exhibit less anxious behaviour on the elevated plus maze (Eren-Kocak, Turner, Watson, & Akil, 2011). Additionally, the same study revealed that a knockdown of FGF2 mRNA in the hippocampus significantly increased the time spent in the closed arms, and decreased the time spent in the center zone of the elevated plus maze compared to normal controls, both of which are behaviours indicative of high anxiety.

The inverse relationship of FGF2 levels and anxiety behaviour begs the question as to whether raising FGF2 levels can prevent or rescue an anxious phenotype. To investigate this question, one study used bred lines of rats identified as high-responders (bHRs) and low-responders (bLRs) to novel situations, with the bLRs exhibiting higher baseline levels of anxious behaviour (Turner et al., 2011). A single injection of FGF2 on postnatal day 2 was found to rescue anxious behaviour in these bLRs in adulthood, with the FGF2 treated group spending significantly more time in the open arms of the elevated plus maze, as well as having a shorter latency to enter the open arms compared with the vehicle treated bLRs. It is important to note that there was not a complete rescue of the anxious phenotype in the FGF2 bLRs, as they still performed worse and exhibited higher anxiety behaviour than bHRs (Turner et al., 2011). Interestingly, this study found no effect of FGF2 on bHRs, suggesting that prophylactic treatment with FGF2 can act in a neuroprotective way for those predisposed to developing high anxiety, but have no effect on others.

The mechanism of action of FGF2 in neurogenesis and mood disorders is not fully understood, but recent research has suggested it may interact directly with the HPA axis to exert its effects. In addition to anxious behaviour, FGF2 knockout mice were found to have decreased hippocampal GR expression and increased HPA axis activity, and administration of exogenous FGF2 in adulthood was able to rescue this entire phenotype (Salmaso et al., 2016). Additionally, a blockade of GR expression in these FGF2 KO mice prevented this therapeutic action, suggesting that FGF2's regulation of hippocampal GR expression in some way underlies its relationship to the HPA axis and anxiety disorders (Salmaso et al., 2016). It is necessary for future studies to continue investigating this mechanism to form more solid conclusions about this relationship.

Rationale & Objectives

Previous research has made inspiring progress into decoding the mechanism of action of FGF2's anxiolytic effects using transgenic animal models. However, many identify the need to use non-transgenic animal models of anxiety to further expand their conclusions in a more translational approach. The present study will use a maternal separation protocol aiming to mimic chronic early life stress in animals, which is hypothesized to lead to an etiologically accurate anxious phenotype in adulthood. Additionally, late life injections of FGF2 will be used as a therapeutic treatment to this phenotype. We hypothesize that chronic administration of exogenous FGF2 in adulthood will rescue this anxious phenotype, as observed by positive changes in anxiety-like, locomotor, anhedonic, and learned helplessness behaviours.

METHODS

Animals

All protocols were reviewed and approved by the Animal Care Committee of Carleton University under the regulations set by the Canadian Council for the Use and Care of Animals in Research. Long-Evans pregnant dams were single-housed in conventional cages (44cm length X 22.5cm width X 20.5cm height), and monitored daily until birth of the litter. Pups were left with their mothers until weaning age, after which they were housed with their siblings (maximum 4 animals of the same sex per cage) in conventional cages with standard enrichment until the start of the treatment and behaviour procedure in adulthood. Apart from during the sucrose consumption test, all animals had *ad libitum* access to food and water throughout the experiment, and were housed in a temperature and humidity controlled room on a 12-hour light/dark cycle (light from 08:00 to 20:00).

Procedure

Figure 1 depicts the complete timeline for the study. All male and female pups from each litter were kept for experiments. At the time of birth, litters were randomly assigned to either the "NO STRESS" (control) or "STRESS" intervention. Pups were left undisturbed with their mothers until postnatal day 6 (P6), at which time the litters in the stress group were moved to a separate procedure room to undergo maternal separation. Pups were removed from their mother and home cage, and placed together in a separate cage with Enviro-dry bedding and nesting material. The separation was performed daily from postnatal day 6 to 10 for three hours (11:00 – 14:00). After the separation, pups were returned to their mother and home cage, and all cages returned to the original holding room.

Animals were then left undisturbed, apart from weaning at postnatal day 21, until adulthood after six weeks of age. The animals were weighed and single-housed two days prior to the start of the treatment and behaviour procedure (denoted as procedure day 1). This procedure began with seven consecutive days of subcutaneous injections of either a vehicle solution, a low dose of FGF2, or a high dose of FGF2. The sucrose consumption test was run during the injection period from procedure days 5 – 9. Two behaviour days followed the last injection day, with the open field and elevated plus maze conducted on day 10, and the forced swim test conducted on day 11. The procedure concluded on day 12 with animal sacrifice, and tissue and blood collection.

Injection Protocol

Three treatment groups were used in this procedure: (i) 1% BSA vehicle solution, (ii) low dose of FGF2 (1µg/kg), or (iii) high dose of FGF2 (10µg/kg). Appendix 1 details the recipe of each injection solution. All injections were given at an amount of 0.1mL per 100g of body weight. To control for litter effects, pups from each litter were randomly assigned to one of the treatment groups. Figure 2 denotes the overall assignment of the animals to each intervention and treatment group. The injections began on procedure day 1 in adulthood, and occurred at the same time (11:00) daily for seven consecutive days.

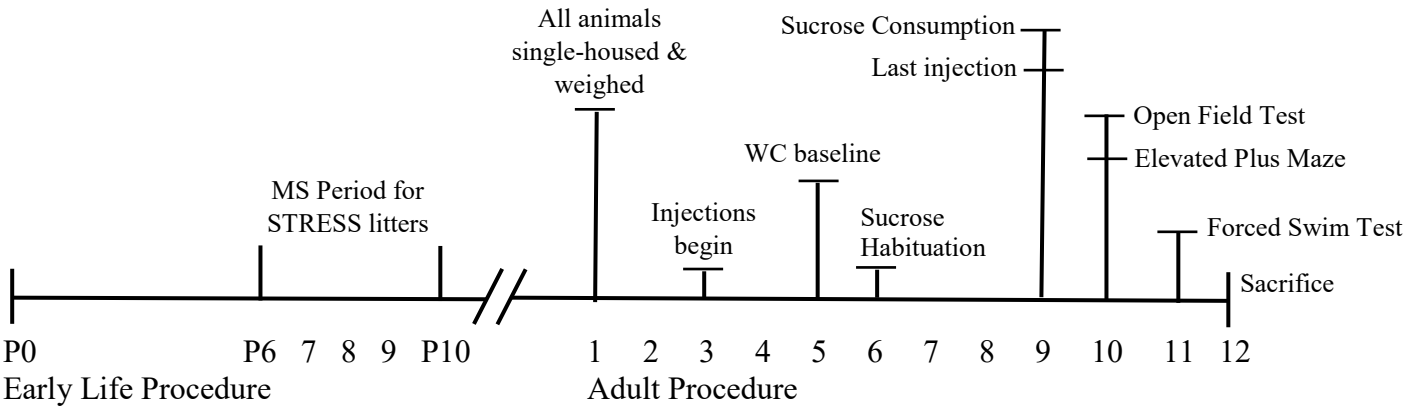
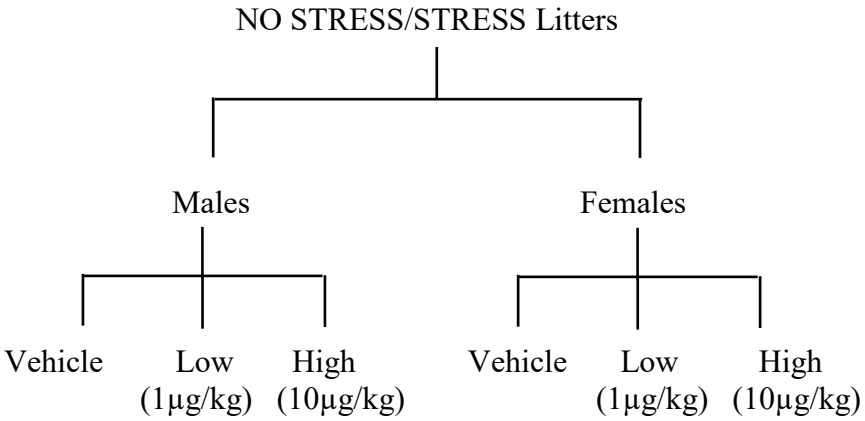


Figure 1. Study timeline. The experimental procedure stretches from birth of the pups into adulthood. The break in the timeline represents the time lapse between the early life stress intervention, and the treatment and behaviour protocol in adulthood. The days are arbitrarily numbered from 1 to 12 for this procedure in adulthood, and are not representative of the age of the animals. Litters denoted as "STRESS" received early life maternal separation daily from P6-P10 (inclusive). Animals were then single-housed in adulthood two days prior to the start of injections, which occurred daily from procedure day 3 to 9. The sucrose consumption test was conducted from procedure day 5 to 9, and the other open field test, elevated plus maze, and forced swim test were conducted on days 10 and 11. All animals were sacrificed via rapid decapitation on day 12. (MS, maternal separation; WC, water consumption)

(a)



(b)

	No Stress (Control)	Stress
Vehicle	13 Male 10 Female	15 Male 14 Female
Low FGF2 Dose (1µg/kg)	6 Male 7 Female	7 Male 7 Female
High FGF2 Dose (10µg/kg)	14 Male 9 Female	15 Male 13 Female

Figure 2. Animal assignment and overall sample size per group. (a) Litter separation into the three treatment groups to control for litter effects. (b) Overall sample size per group.

Sucrose Consumption Test

The sucrose consumption test (SCT) was the first behavioural test conducted, and occurred during the treatment period. This test is a measure of anhedonia, a common depressive symptom in rodents defined as the loss of pleasure in something that was once enjoyable. The test began on procedure day 5 with the initial water baseline measure. All water bottles were removed at 16:00 the previous day so that the animals did not have access to water throughout the dark cycle. The bottles were weighed and returned at 09:00 the following morning. After one hour, the bottles were reweighed, and the difference in weight recorded as water consumption. The water was then substituted for a 1% sucrose solution that the animals habituated to for 48 hours, after which the sucrose solution was switched back to water. On procedure day 8, bottles were again removed at 16:00 for the dark cycle, and the following morning at 09:00 the bottles with the 1% sucrose solution were weighed and placed in the cage. After one hour, the bottles were reweighed, and the difference in weight recorded as sucrose consumption. The original water bottles were then returned for the duration of the protocol.

Open Field Test

The open field test (OF) was the first of the two behaviour tests to occur on procedure day 10. This test is both a measure of anxiety, and locomotor and exploratory behaviour for rodents in a novel open space. Animals were placed in the corner of a large, transparent plastic bin (68cm length X 46cm width X 38.5cm height) and allowed to move freely for 20 minutes. The animals' movements were live tracked by an integrated ANY-maze video camera (ANY-maze USB Camera, Stoelting Co., U.S.A) mounted overhead, and analyzed by ANY-maze Video Tracking system (ANY-maze version 4.99, Stoelting Co., U.S.A). The apparatus was thoroughly cleaned with a 70% ethanol solution between trials.

Anxiety measures were taken within the first 5 minutes of the test, and are based on movements between two previously defined areas: the center zone, and the peripheral zone (Figure 3). Measures of time spent in each zone, as well as locomotor behaviour in each zone were recorded, with less time spent in the center zone being indicative of more anxious behaviour. Locomotor and exploratory behaviour measures were taken independent of zones for the full 20-minute test.

Elevated Plus Maze

The elevated plus maze (EPM) was the second behaviour test conducted on procedure day 10 as an additional measure of anxiety behaviour. The apparatus consisted of a large, plus-sign shaped maze, with two opposing arms being closed in by opaque walls (the closed arms), and the two remaining arms being devoid of walls (the open arms). The arms are all elevated 52cm off the ground (Figure 3). Animals were placed in the centre of the apparatus facing one open arm to begin, and allowed to move freely for 5 minutes. The same ANY-maze Video Tracking set-up as the open field test was used for the elevated plus maze. As before, the apparatus was wiped down with a 70% ethanol solution between trials. The time spent in each zone was recorded, as well as the corresponding locomotor behaviour as secondary measures. The less time an animal spends in the open arms is indicative of more anxious behaviour.

Forced Swim Test

The forced swim test (FST) occurred on procedure day 11. This test is used to measure learned helplessness, a common depressive symptom in which an animal will stop trying to avoid or escape an adverse situation. Animals were placed in a large glass cylinder (68cm height X 15.5cm diameter) filled up to two-thirds with temperature controlled tap water (23-35˚ Celsius) for a period of 10 minutes. They were recorded with a handheld video recording device (Sony

HD Handycam, 9.2 megapixels). At the end of the test, the animals were dried with a towel and returned to their home cage.

The videos were scored for the time mobile (actively swimming side to side or diving), the time immobile (floating, propping up against the side of the cylinder, or making the minimal movements to keep their heads above the water), and the latency to immobility (time at which animal first shows signs of immobility) of each animal.

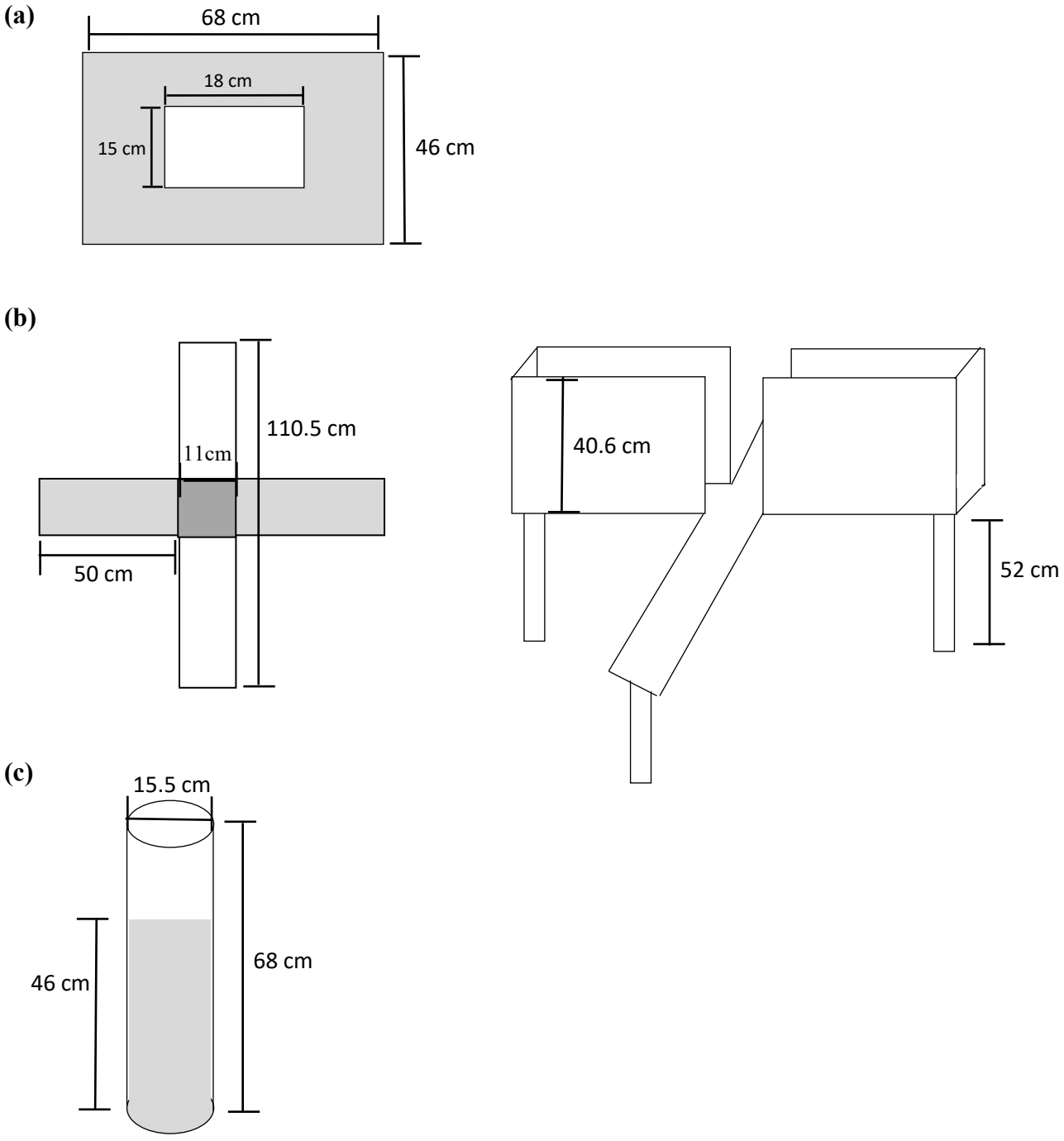


Figure 3. Behaviour tests apparatus. (a) Overhead view of the open field test apparatus. The light grey area represents the peripheral zone, while the white area represents the center zone. (b) Overhead and side view of the elevated plus maze. The Light grey area denotes the closed arms of the maze, while the white area represents the open arms. The darker grey box indicates the center zone. (c) Forced swim test cylinder. The grey area represents the water line.

Animal Sacrifice & Tissue Collection

Animals were sacrificed by live decapitation on procedure day 12. Brains were extracted, and flash frozen at -80° Celsius. Trunk blood was collected in 1.5mL Eppendorf tubes lined with 20µL of tris-EDTA buffer solution (Fluka Analytical), and spun in a centrifuge at 6000rpm and 4° Celsius for 10 minutes. The serum was then separated into sterile Eppendorf tubes, and frozen at -80° Celsius.

Statistical Analysis

All data were analyzed using a 2 X 2 X 2 (early life stress [control vs. stress] by treatment [vehicle vs. high FGF2 dose] by sex [male vs. female]) between-subjects analysis of variance (ANOVA). After a preliminary analysis, two other analyses took place in attempt to create a more complete picture of the data. Firstly, behaviour over the two longer tests, the open field test and forced swim test, were expanded into intervals over the course of the test to get a better idea of if and how behaviour changed over time. A one-way repeated measures ANOVA with three between-subjects factors (early life stress [control vs. stress] by treatment [vehicle vs. high FGF2 dose] by sex [male vs. female]) was used to test this data. Additionally, male and female data were then analyzed separately using a 2 X 2 (early life stress [control vs. stress] by treatment [vehicle vs. high FGF2 dose]) between-subjects ANOVA to observe any sex-specific effects. If a significant interaction was found, post hoc comparisons for main effects were performed with Bonferroni correction method for non-orthogonal tests. Differences were considered significant at $p<0.05$. All analyses took place in SPSS Statistics 20 (IBM, Chicago, Illinois).

RESULTS

For the results displayed below, the low FGF2 groups for both control and stress animals were not included in the analysis. A combination of small sample sizes in the low FGF2 group compared to the vehicle and high FGF2 groups, as well as a change in facilities mid-way through the study resulted in highly skewed data in the low FGF2 groups. Low FGF2 was thus excluded, and only the vehicle and high FGF2 (now just denoted as the 'FGF2' group) are used in the analysis.

Anxiety-Like Behaviour

Open Field Test

A three-way ANOVA was completed to determine the effects of early life stress (control vs. stress), treatment (vehicle vs. FGF2), and sex (male vs. female) on anxiety-like and locomotor behaviour from the 5-minute open field test. There were no significant three-way interactions found for any measure; time in centre zone ($F(1,93)=0.150$, $p=0.699$), distance travelled in centre zone ($F(1,93)=0.08$, $p=0.778$), latency to enter centre zone ($F(1,93)=0.021$, $p=0.885$) (Figure 4), total distance travelled ($F(1,93)=0.331$, $p=0.566$), centre/total distance ($F(1,93)=0.000$, $p=0.989$), average speed ($F(1,93)=0.324$, $p=0.570$), and total time freezing ($F(1,93)=1.156$, $p=0.285$) (Figure 5). There were significant two-way interactions found between early life stress and sex for total distance ($F(1,93)=5.370$, $p=0.023$) and average speed ($F(1,93)=5.274$, $p=0.024$), as well as between treatment and sex for distance travelled in the centre ($F(1,93)=4.190$, $p=0.043$). Post hoc tests on the latter interaction showed that overall, FGF2 treated females travelled more distance in the centre zone than their male counterparts. There were no main effects found for any of the other measures. Because there were significant sex differences, we subsequently analyzed males and females separately, using two-way (early

life stress vs. treatment) between-subjects ANOVAs to identify sex specific interactions. Results showed that control females travelled more distance and had a faster average speed overall than stress females (Figure 5).

Locomotor and exploratory behaviour measures of total time mobile, total distance travelled, average speed, and total time freezing were taken from the 20-minute open field test independent of zone, using the same three-way ANOVA design. There were no significant three-way interactions found for any measure: total time mobile ($F(1,93)=0.078$, $p=0.780$), total distance travelled ($F(1,93)=0.044$, $p=0.834$), average speed ($F(1,93)=0.032$, $p=0.858$), and total freezing time ($F(1,93)=0.513$, $p=0.476$) (Figure 6). Significant two-way interactions between early life stress and sex were found for the following measures: total time mobile ($F(1,93)=6.583$, $p=0.012$), total distance travelled ($F(1,93)=13.059$, $p=0.000$), average speed ($F(1,93)=13.318$, $p=0.000$), and total freezing time ($F(1,93)=6.216$, $p=0.014$). When males and females were analyzed separately, we found that control females spent more time mobile, travelled farther, had a faster average speed, and spent less time freezing overall compared to stress females (Figure 5).

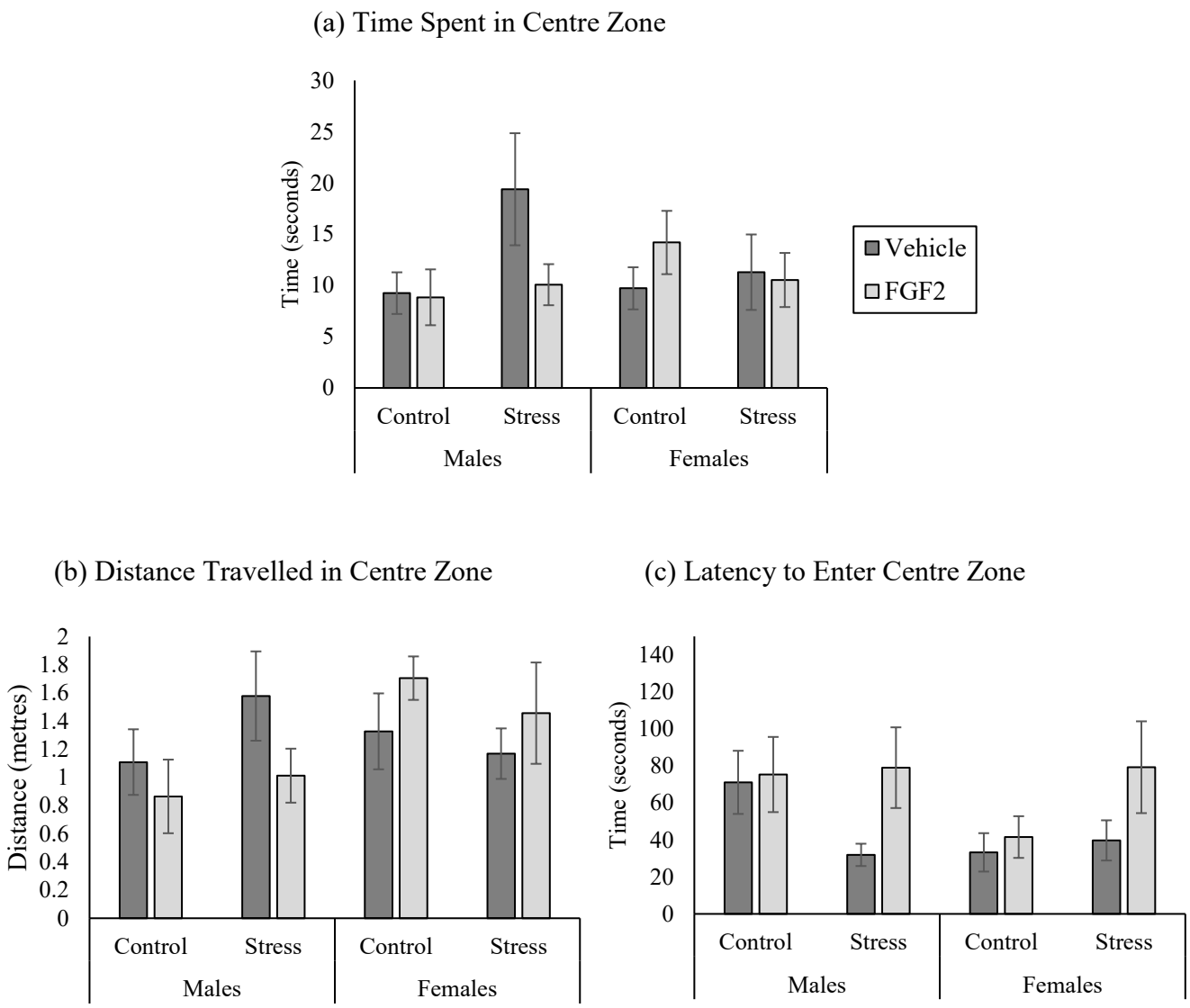


Figure 4. Anxiety measures on the 5-minute OF. (a) There were no significant interactions or main effects for the time spent in the centre zone. (b) There was a significant interaction between treatment and sex, such that overall, FGF2 treated females travelled more distance in the centre zone than their male counterparts. (c) There were no significant interactions or main effects for the latency to enter the centre zone. (M/Veh/Ctrl, n=12; M/Veh/Stress, n=14) Data expressed as mean ± SEM.

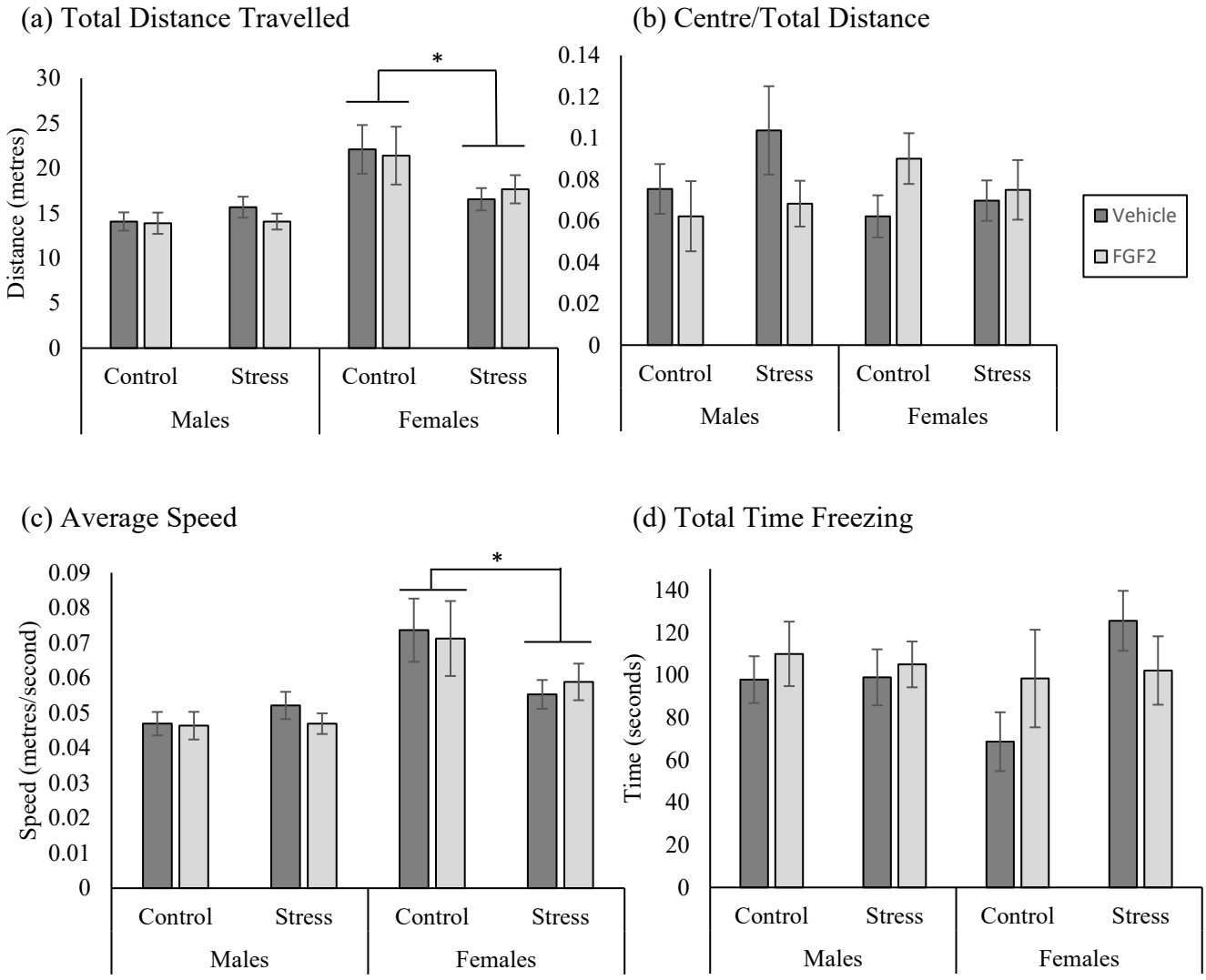


Figure 5. Locomotor measures on the 5-minute OF. (a) There was a significant two-way interaction for the total distance travelled, such that control females were found to travel more overall than stress females. (b) There were no significant interactions or main effects for centre/total distance ratio. (c) There was a significant two-way interaction for the average speed, such that control females were found to have an overall faster average speed than stress females. (d) No significant interactions or main effects were found for the total time freezing. (M/Veh/Ctrl, n=12; M/Veh/Stress, n=14) Data expressed as mean ± SEM. *$p<0.05$

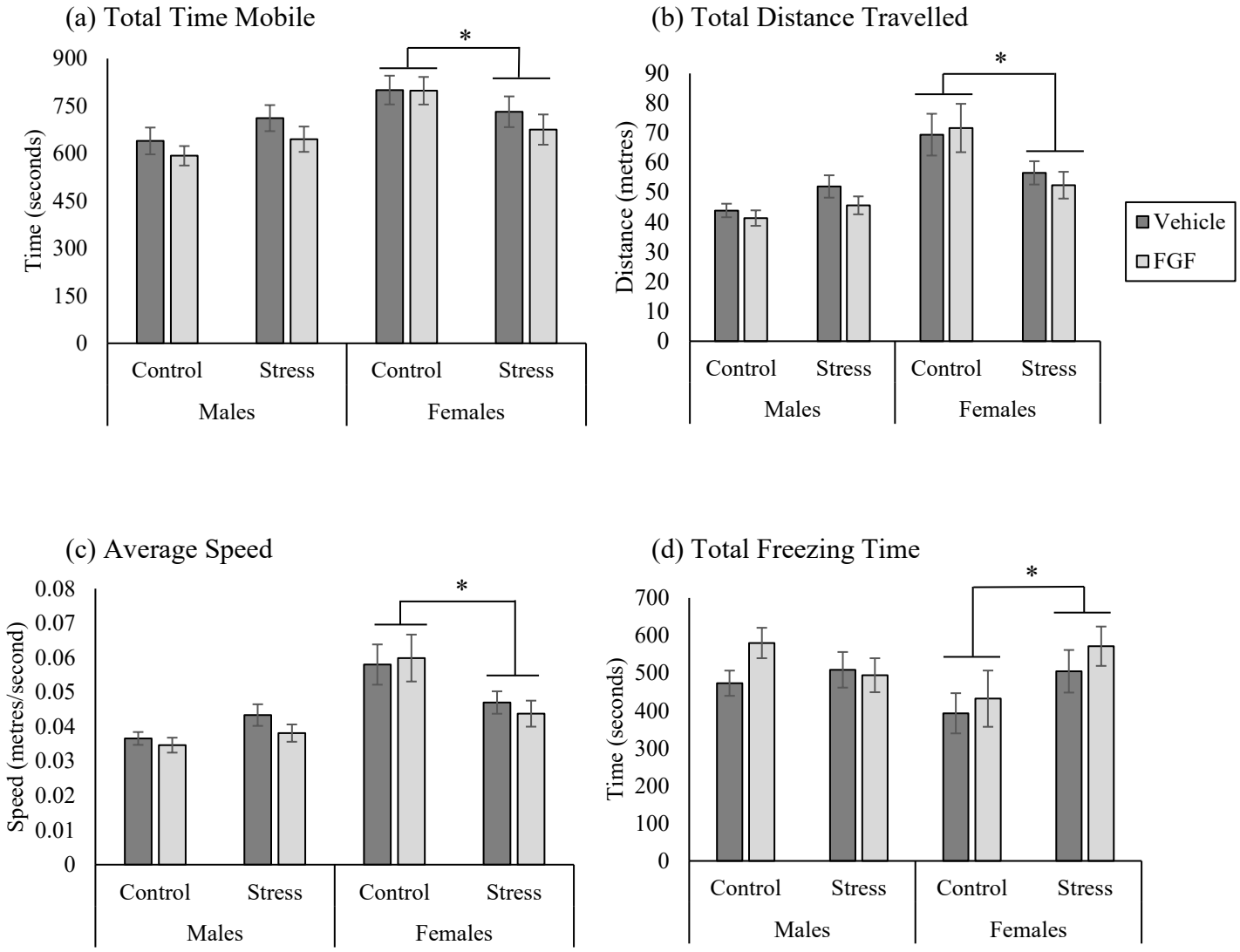


Figure 6. Locomotor measures on the 20-minute OF. (a) There was a significant two-way interaction for the total time mobile, such that control females were found to spend more time mobile overall than stress females. (b) There was a significant two-way interaction for the total distance travelled, such that control females were found to travel more overall than stress females. (c) There was a significant two-way interaction found for the average speed, such that control females were found to have an overall faster average speed than the stress females. (d) There is a significant two-way interaction found for the total freezing time, such that control females spent less time freezing than stress females. (M/Veh/Ctrl, n=12; M/Veh/Stress, n=14) Data expressed as mean ± SEM. *$p<0.05$

In order to understand if anxiety behaviours changed over time, anxiety measures were further broken down into four 5-minute intervals across the full 20-minute OF test using a one-way repeated measures ANOVA design with three between-subjects factors (early life stress, treatment, and sex). There were no significant three-way or two-way interactions across time for the following measures: time in centre ($F(3,279)=0.368$, $p=0.776$), distance travelled in centre ($F(3,279)=0.288$, $p=0.834$), total distance travelled ($F(3,279)=2.042$, $p=0.108$), centre/total distance ($F(3,279)=0.438$, $p=0.726$), and average speed ($F(3,270)=2.068$, $p=0.105$). However, there was a significant three-way interaction found for the total time spent freezing over the task ($F(3,279)=4.370$, $p=0.005$) (Figure 7). Males and females were then analyzed separately using a one-way repeated measures ANOVA with two between-subjects factors (early life stress and treatment). There were no significant two-way interactions found for any measure over time in males, but females showed a significant interaction between early life stress and treatment for the total time spent freezing over time interval ($F(3,126)=4.554$, $p=0.005$), such that FGF2 treated females in the stress condition spent more time freezing than their control counterparts during intervals 10-15 minutes and 15-20 minutes.

There were no other significant two-way interactions or main effects of early life stress and treatment found over time for either males or females. However, there was an overall main effect of time interval on total distance ($F(3,153)=39.685$, $p=0.000$), average speed ($F(3,153)=39.940$, $p=0.000$), and total time freezing ($F(3,153)=32.498$, $p=0.000$) for males, and on distance in the centre ($F(3,126)=3.760$, $p=0.013$), total distance ($F(3,126)=48.310$, $p=0.000$), average speed ($F(3,126)=43.293$, $p=0.000$), and total time freezing ($F(3,126)=30.642$, $p=0.000$) for females (Figure 8). Males and females were found to travel significantly less total distance, had a slower average speed, and spent the most time freezing overall during interval 15-20

minutes than any other interval. Females were also found to have travelled significantly less distance in the centre during interval 15-20 minutes than intervals 0-5 minutes and 5-10 minutes.

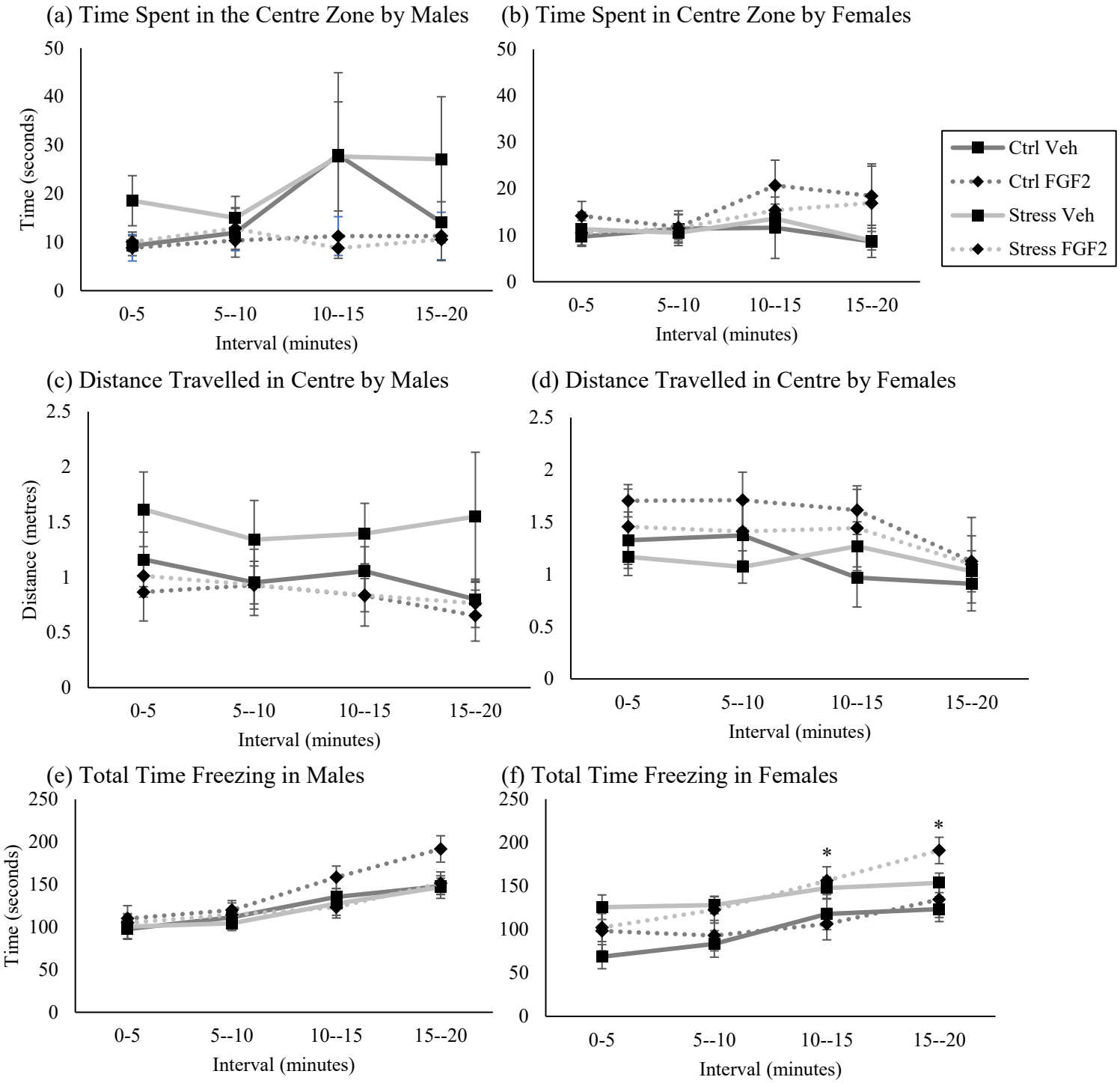


Figure 7. Anxiety and locomotor measures over 4x5-minute intervals on the OF. (a/b) There were no significant interactions or main effects on time spent in the centre zone. (c/d) There was a significant main effect of time interval on distance travelled in the centre for females, such that they travelled significantly less distance during interval 15-20 minutes than interval 0-5 minutes. (e/f) There is a significant three-way interaction on the time spent freezing, such that female FGF2 treated stress group spent more time freezing than their control counterparts during intervals 10-15 and 15-20 minutes. (M/Veh/Ctrl, n=12; M/Veh/Stress, n=14) Data expressed as mean ± SEM. *p<0.05

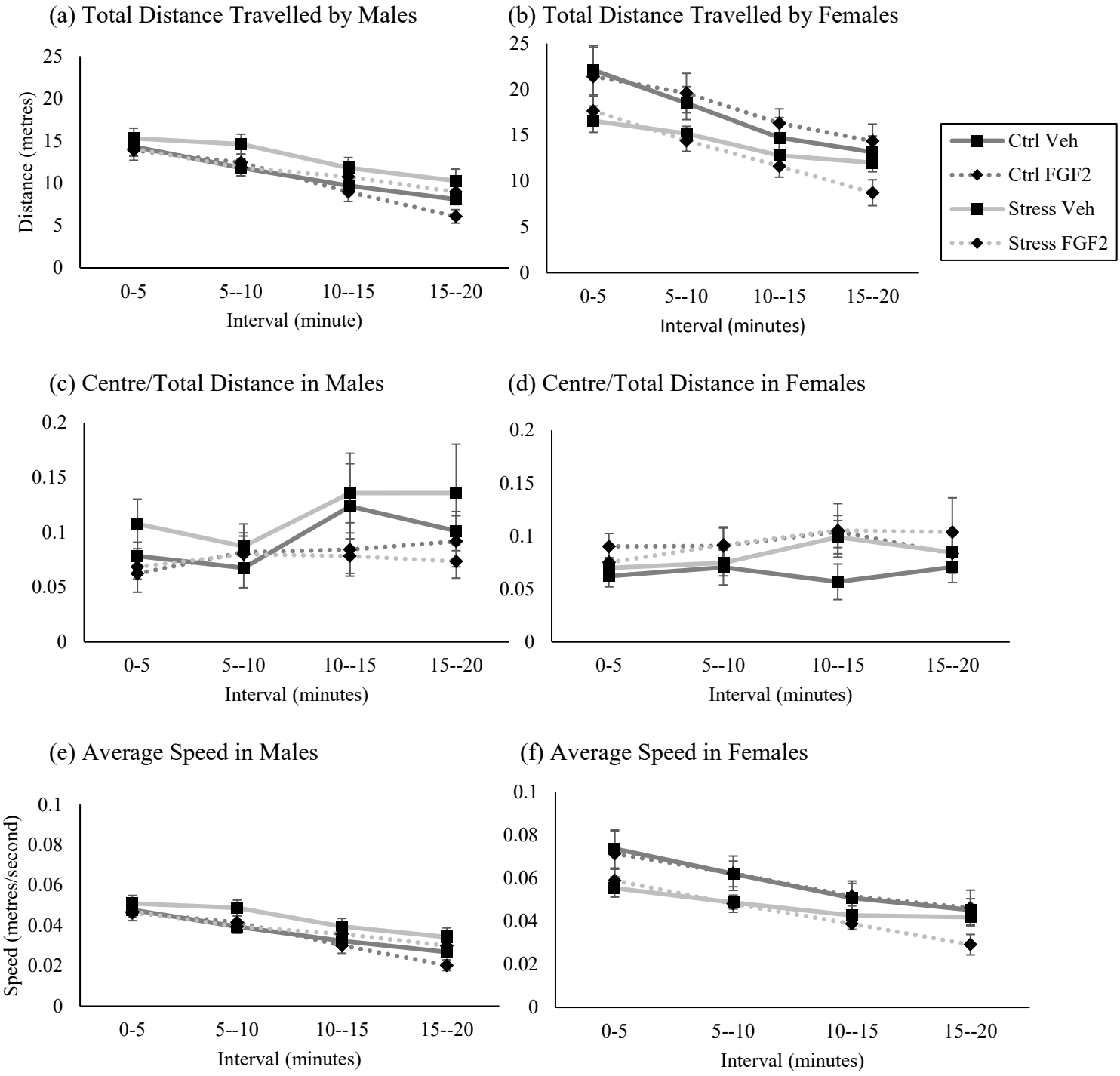


Figure 8. Locomotor measures over 4x5-minute intervals on the OF. (a/b) There was a significant main effect of time interval, such that males and females were found to travel significantly less distance during interval 15-20 minutes than any other interval. (c/d) There were no significant interactions or main effects on centre/total distance travelled. (e/f) There was a significant main effect of time interval on average speed, such that males and females were found to have a slower average speed during interval 15-20 minutes than any other interval. (M/Veh/Ctrl, n=12; M/Veh/Stress, n=14) Data expressed as mean ± SEM.

Elevated Plus Maze

A three-way ANOVA was completed to determine the effects of early life stress (control vs. stress), treatment (vehicle vs. FGF2), and sex (male vs. female) on anxiety-like behaviour from the 5-minute elevated plus maze. There were no significant three-way interactions found for any measure; time in closed arms ($F(1,95)=1.275$, $p=0.262$), time in open arms ($F(1,95)=0.633$, $p=0.428$), time in centre zone ($F(1,95)=0.172$, $p=0.679$), time in open and centre zones ($F(1,95)=0.514$, $p=0.475$) (Figure 9), latency to enter open arms ($F(1,95)=0.251$, $p=0.617$), time freezing in open arms ($F(1,95)=0.054$, $p=0.817$), distance travelled in open arms ($F(1,95)=0.405$, $p=0.526$), and total distance travelled ($F(1,95)=0.714$, $p=0.400$) (Figure 10). There were also no significant two-way interactions or main effects of stress or treatment for any measure, but there was a main effect of sex for time spent in the open arms ($F(1,95)=4.668$, $p=0.033$), distance travelled in the open arms ($F(1,95)=4.023$, $p=0.048$), and total distance travelled ($F(1,95)=18.322$, $p=0.000$), such that females spent more time in the open arms, and travelled more both overall and in the open arms, than males.

Males and females were then analyzed separately, each with a two-way (early life stress vs. treatment) between-subjects ANOVA. There were no significant two-way interactions found for either sex, and there were also no main effects of either early life stress or treatment on any measure for females. There was a significant main effect of early life stress found in males on the time spent in the open arms ($F(1,53)=4.839$, $p=0.032$) and distance travelled in the open arms ($F(1,53)=4.277$, $p=0.044$), such that stress males spent significantly more time and travelled farther in the open arms than control males.

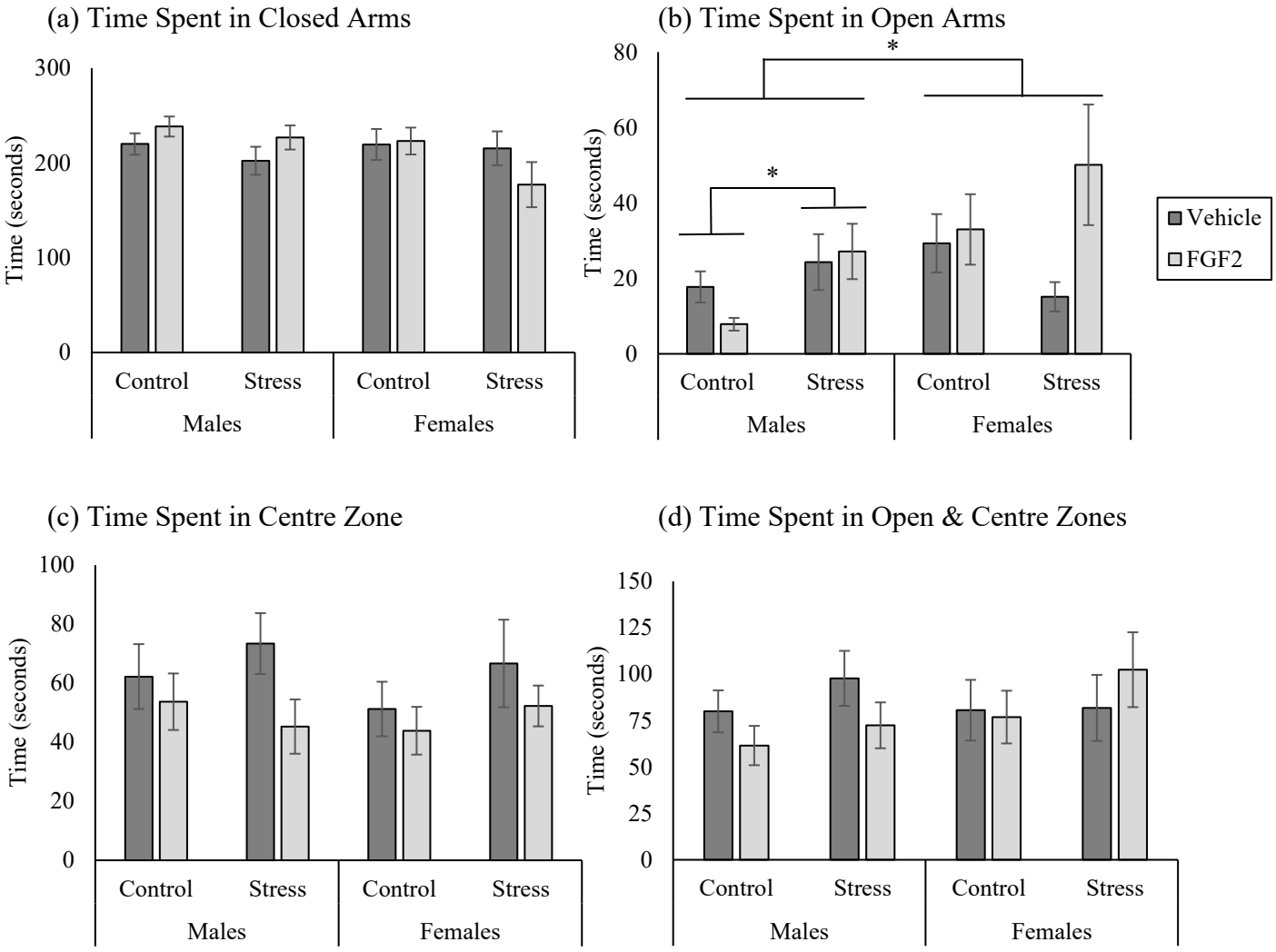


Figure 9. Anxiety measures on the EPM. (a) There were no significant interactions or main effects for the time spent in the closed arms. (b) There was a significant main effect of sex for time spent in the open arms, such that overall, females spent more time in the open than males. There was also a main effect of early life stress in males, such that the stress animals spent more time in the open than control animals. (c) There were no significant interactions or main effects for the time spent in the centre zone. (d) There were no significant interactions or main effects for the time spent in the open and centre zones combined. Data expressed as mean ± SEM. *$p<0.05$

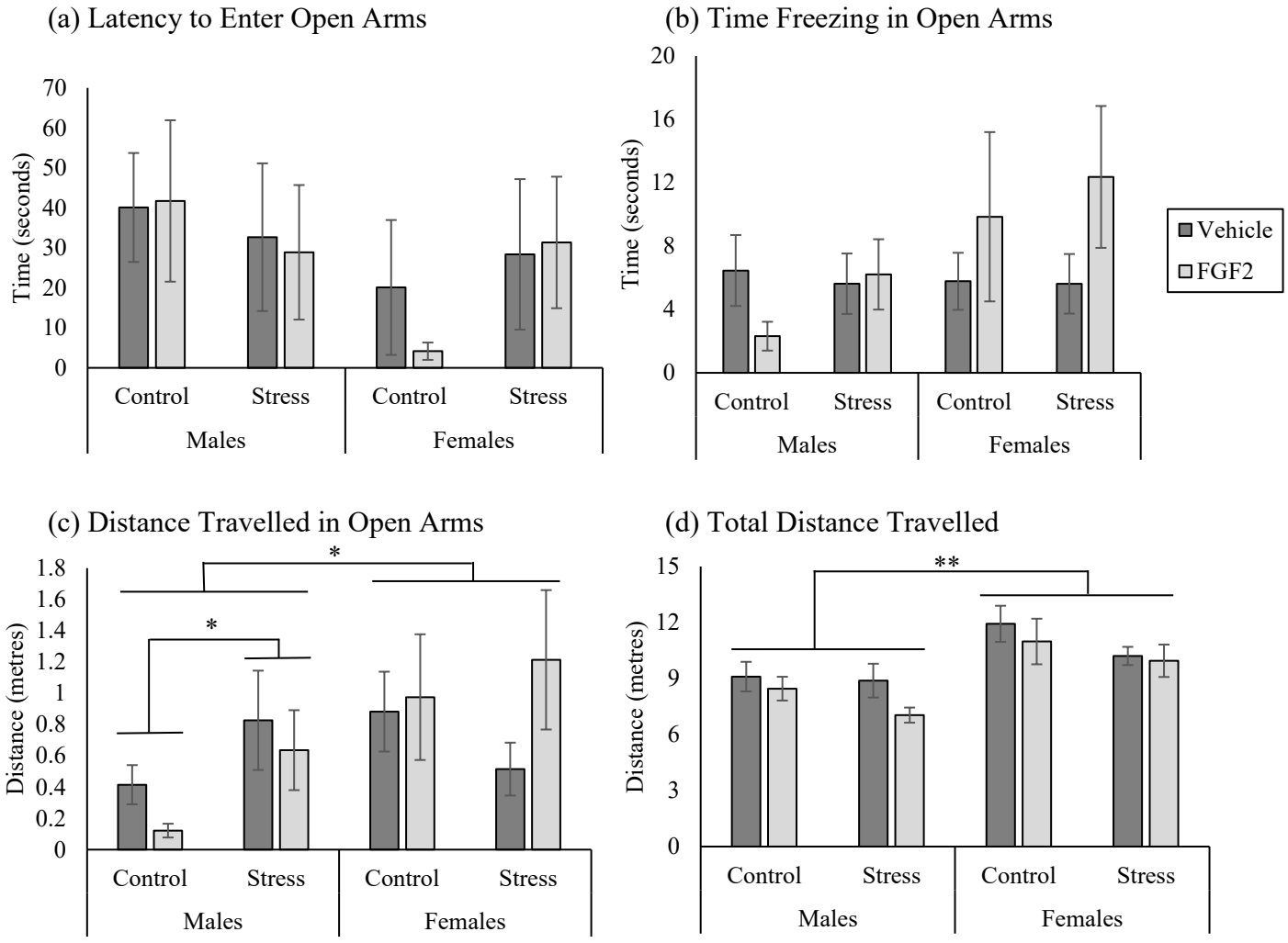


Figure 10. Anxiety and locomotor measures on the EPM. (a) There were no significant interactions or main effects for the latency to enter the open arms. (b) There were no significant interactions or main effects for the time freezing in the open arms. (c) There was a significant main effect of sex for the distance travelled in the open arms, such that overall, females travelled more than males. There was also a significant main effect of early life stress in males, such that the stress animals travelled more in the open arms than control animals. (d) There was a significant main effect of sex for the total distance travelled, such that overall, females travelled more than the males. Data expressed as mean ± SEM. *$p<0.05$, **$p<0.001$

Depressive-Like Behaviour

Sucrose Consumption Test

A three-way ANOVA was completed to determine the effects of early life stress (control vs. stress), treatment (vehicle vs. FGF2), and sex (male vs. female) on anhedonic behaviour from the sucrose consumption test. The amount of sucrose consumed was taken using direct sucrose consumption, sucrose consumption relative to baseline water consumption (SC/WC) to control for consumption due to thirst, and sucrose consumption relative to animal weight (SC/Wt) to control for differences in body weight. There were no significant three-way interactions found for any measure; sucrose consumption ($F(1,81)=0.297$, $p=0.587$), SC/WC ($F(1,81)=0.058$, $p=0.810$), and SC/Wt ($F(1,81)=0.256$, $p=0.614$) (Figure 11). There were also no significant two-way interactions or main effects of stress or treatment for any measure, but there was a main effect of sex for sucrose consumption ($F(1,81)=64.643$, $p=0.000$), and SC/WC ($F(1,81)=5.471$, $p=0.022$), such that males consumed more sucrose overall.

Males and females were then analyzed separately, each with a two-way (early life stress vs. treatment) between-subjects ANOVA. There were no significant two-way interactions found for either males or females, and there were also no main effects of either early life stress or treatment found on any measure for males. There was a significant main effect of early life stress found for females on sucrose consumption ($F(1,36)=5.185$, $p=0.029$), such that stress females consumed more sucrose overall than the control females.

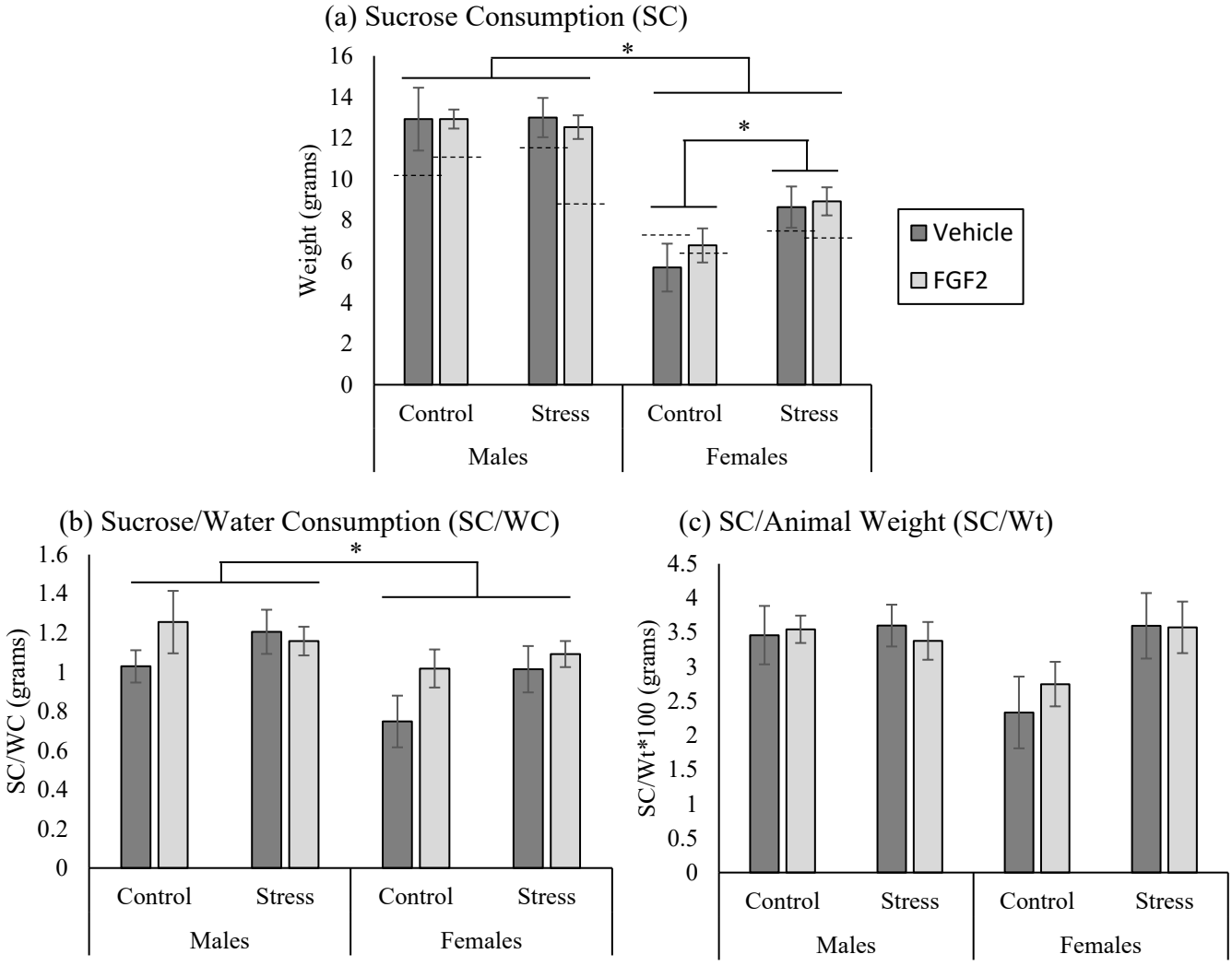


Figure 11. Measures of sucrose consumption on the SCT. (a) There was a significant main effect of sex on sucrose consumption, such that males consumed more sucrose overall than females. There was also a significant main effect of early life stress in females, such that the stress animals consumed more sucrose overall than the control females. The dotted lines denote the water consumption (in grams) for each group for comparison. (b) There was a significant main effect of sex on sucrose consumption relative to water consumption, such that males consumed more sucrose than females. (c) There were no significant interactions or main effects of sucrose consumption relative to animal body weight. Data expressed as mean ± SEM. *$p<0.05$

Forced Swim Test

A three-way ANOVA was completed to determine the effects of early life stress (control vs. stress), treatment (vehicle vs. FGF2), and sex (male vs. female) on learned helplessness on the forced swim test. There were no significant three-way interactions found for the time spent immobile ($F(1,95)=0.265$, $p=0.608$) or latency to immobility ($F(1,95)=0.181$, $p=0.671$) (Figure 12). There were also no significant two-way interactions or main effects for total time spent immobile, but there was a main effect of sex found for latency to immobility ($F(1,95)=4.070$, $p=0.046$), such that males overall had a faster latency to immobility (stopped swimming earlier) than females. Males and females were then analyzed separately, each with a two-way (early life stress vs. treatment) ANOVA. There were no significant two-way interactions or main effects of stress or treatment found for either sex.

The time spent immobile was further analyzed over five 2-minute intervals across the full 10-minute FST using a one-way repeated measures ANOVA with three between-subjects factors (early life stress, treatment, and sex). No significant three-way interaction ($F(4,380)=0.335$, $p=0.855$), two-way interactions, or main effects of any factor were found over time. Males and females were then analyzed separately, using a one-way repeated measures ANOVA with two between-subjects factors (early life stress and treatment). No significant two-way interactions or main effects of either factor were found for immobility over time for both males and females. However, there was an overall main effect of time on immobility for both males ($F(4,212)=164.869$, $p=0.000$) and females ($F(4,168)=126.194$, $p=0.000$), such that both sexes were found to spend significantly more time immobile during intervals 4-6, 6-8, and 8-10 minutes than during intervals 0-2 and 2-4 minutes (Figure 13).

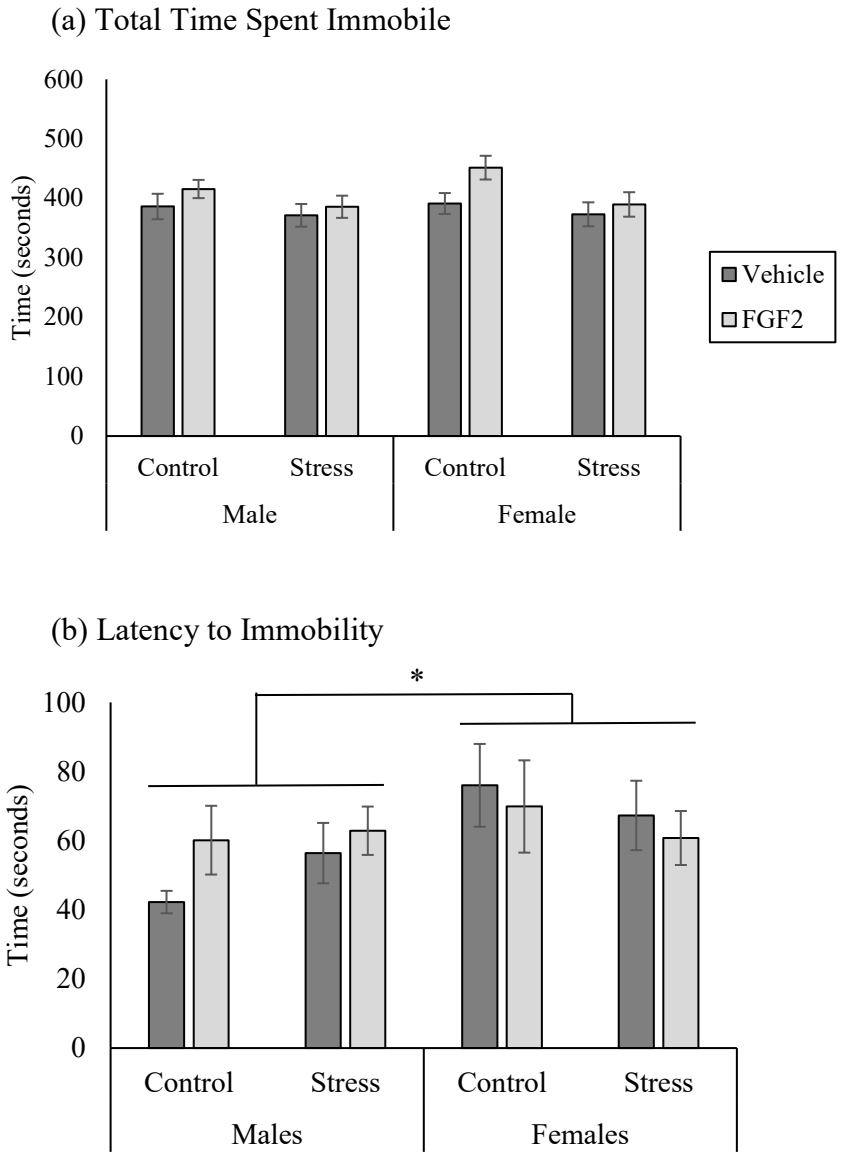


Figure 12. Immobility measures on the FST. (a) There were no significant interactions or main effects of total time spent immobile. (b) There was a significant main effect of sex on latency to immobility, such that males stopped swimming faster than females. Data expressed as mean ± SEM. *$p<0.05$

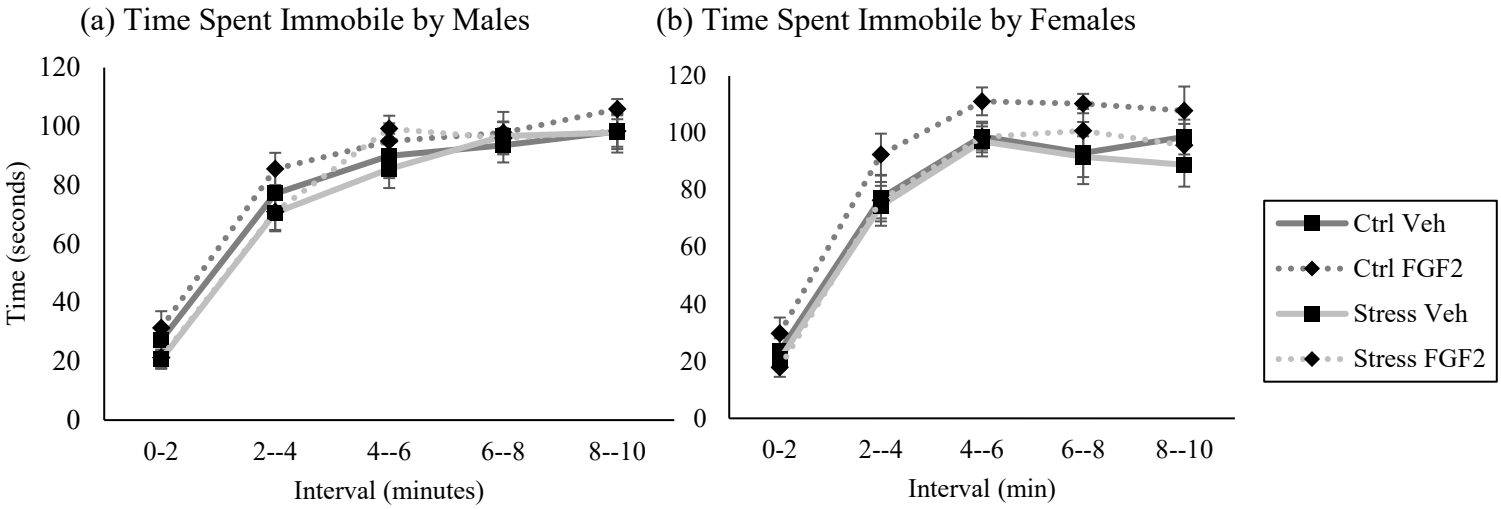


Figure 13. Time immobile over 5x2-minute intervals on the FST. There were no significant interactions or main effects of sex on immobility over time. (a) There were also no significant main effects of early life stress or treatment on immobility in males over time. There was, however, a significant overall main effect of time on immobility, such that animals spent more time immobile during intervals 4-6, 6-8, and 8-10 minutes than during intervals 0-2 and 2-4 minutes. (b) There are no main effects of early life stress or treatment on immobility in females over time, but there is a main effect of time on immobility, such that females spent more time immobile during intervals 4-6, 6-8, and 8-10 minutes than during intervals 0-2 and 2-4 minutes. Data expressed as mean ± SEM.

DISCUSSION

There were two main objectives of the present study: the first was to examine the use of an early life maternal separation stress paradigm to induce the development of an anxious phenotype in adulthood and then examine the potential of fibroblast growth factor 2 (FGF2) to rescue this phenotype, and the second was to examine any sex-specific responses to maternal separation stress and FGF2 treatment. In humans, early life stress, such as parental abandonment, abuse, or traumatic experiences, has been repeatedly identified as a risk factor to developing anxiety disorders later in life (Kessler et al., 1997). The use of a maternal separation stress paradigm aims to mimic this type of stressful early life experience in rodent models, and has previously been shown to cause a persistent anxious phenotype in adulthood (Tata, 2012). It was hypothesized that the MS paradigm used in this study would induce an anxious phenotype in adulthood, but we are unable to make conclusive statements from the current results. Previous research using the MS stress paradigm saw consistent results of increased anxiety and depressive behaviours (Aisa, Tordera, Lasheras, Del Río, & Ramírez, 2007; Huot, Thrivikraman, Meaney, & Plotsky, 2001; Lee et al., 2007; Salzberg et al., 2007), such as decreased time spent in the open arms of the EPM, decreased time spent in the centre of the OF, and increased time spent immobile on the FST. Although we did see some consistent effects of stress on a few behavioural measures, particularly on locomotor behavior in females, these results are not consistent across all measures and behavioural tests, and we therefore cannot conclude that our paradigm of maternal separation was effective at inducing an anxious and depressive phenotype in adulthood. This result makes it difficult to interpret the action of FGF2 in the stress condition. It is known that the FGF system is dysregulated in mood disorders, and that its administration in deficient animals (for example, transgenic knockouts or selectively bred high anxiety animals)

can rescue the anxious phenotype if given both prophylactically in early life (Turner et al., 2011) and therapeutically in adulthood (Salmaso et al., 2016). We hypothesized that FGF2 administration in adulthood in a MS stress model of anxiety would perform similarly. Based on the results of the present study, FGF2 treatment in the stress condition did not appear to rescue the phenotype compared to the vehicle treated controls, and at times, appeared to slightly exacerbate the effect. However, these results must be taken cautiously, since there is no consistent evidence of initial robust stress effects in the data.

While the design and protocol of this study was sufficient to address the research questions, the unfortunate limitation which may have significantly affected our results and masked the effects of early life stress was an unexpected facility relocation mid-way through conduction of this study. This notion is supported in two ways. Firstly, the results of the control groups in the present study showed little exploration and high levels of anxiety-like behaviour when compared to previous studies in similar subjects from our and other laboratories. Control rats in the current study spent less than twenty seconds in the open arms of the elevated plus maze, whereas our similar control rats in previous studies conducted prior to facility relocations spent between one to two minutes exploring the open arms of the elevated plus maze. Additionally, there was a startling difference in stress levels of the control vehicle treated animals between cohorts completed at the initial facility, and at the temporary facility where the majority of the study was conducted (refer to Appendix 2). It appears that the animals at the temporary facility have reached a plateau, such that they are so stressed at baseline, any further perturbations (such as MS stress) or treatments (such as FGF2 administration) will have no effect. This conclusion makes it difficult to interpret the data collected from the temporary facility, and, therefore, the absence of consistent stress and FGF2 effects should not be

considered until replications can be conducted. A full replication of the current study in a stable housing facility environment would be needed to either confirm or refute the current results.

We next examined patterns of FGF2 effects in the control group only (refer to Appendix 3). Previous research has noted that stress but not control animals responded to FGF2 administration, suggesting that FGF2 is only effective in stressed animals (Turner et al., 2011). We did not find any significant effects of FGF2 in either male or female control animals; however, there did appear to be opposing, sex-specific trends in response to FGF2 administration. On measures of anxiety and locomotion, FGF2 treated males tended to perform worse (displayed increased anxiety) compared to their vehicle treated counterparts, but FGF2 treated females tended to perform better (displayed decreased anxiety) than their vehicle treated counterparts. On measures of depressive-like behaviour, there appeared to be differential effects on the two behavioural tests, although these effects are much less pronounced than those seen on measures of anxiety-like behaviour. On the SCT, FGF2 administration tended to not change behaviour in males, but decreased depressive-like behaviour in females. However, on the FST, FGF2 administration tended to decrease depressive-like behaviour in males, but increase it in females.

In summary, it appears that females showed differential responses to FGF2 administration from males, at least under control baseline conditions. This type of sex-specific response to exogenous FGF2 administration in controls has not previously been reported. Based on this result, future studies can further investigate sex differences in response to administration of FGF2. This is especially important if this sex-specific response pattern is seen in models of anxiety and depression, since previous human research has identified females as being more susceptible to developing mood disorders than males (Eaton et al., 2012). Increased

responsiveness to FGF2 under control conditions in females may indicate a similar result under stress conditions, and thus provide a novel avenue for the treatment of mood disorders in females. Future research, therefore, should consider these sex-specific differences when using basal FGF2 levels as a biomarker of mood disorders, specifically that males and females may require different criteria for diagnosis.

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Appendix 1: Injection Preparations

Stock/Vehicle solution (1% BSA):

(I) 0.5g BSA (Bioshop) in 50mL of 1X PBS (MP Biomedicals)

Low FGF2 dose (1µg/kg):

(I) 25µg of FGF2 (Gibco & Novus Biologicals) into 250µL of sterile distilled water

(II) Combine with 25mL of the stock vehicle solution

High FGF2 dose (10µg/kg):

(I) 250µg of FGF2 into 250µL of sterile distilled water

(II) Combine with 25mL of the stock vehicle solution

Appendix 2: Facility Comparison

The data below displays the differences in behaviour of animals tested at Carleton University (initial facility) versus at the University of Ottawa (temporary facility). All data is taken from the control animals that received vehicle injections, with males and females presented together. All data is expressed as mean ± SEM.

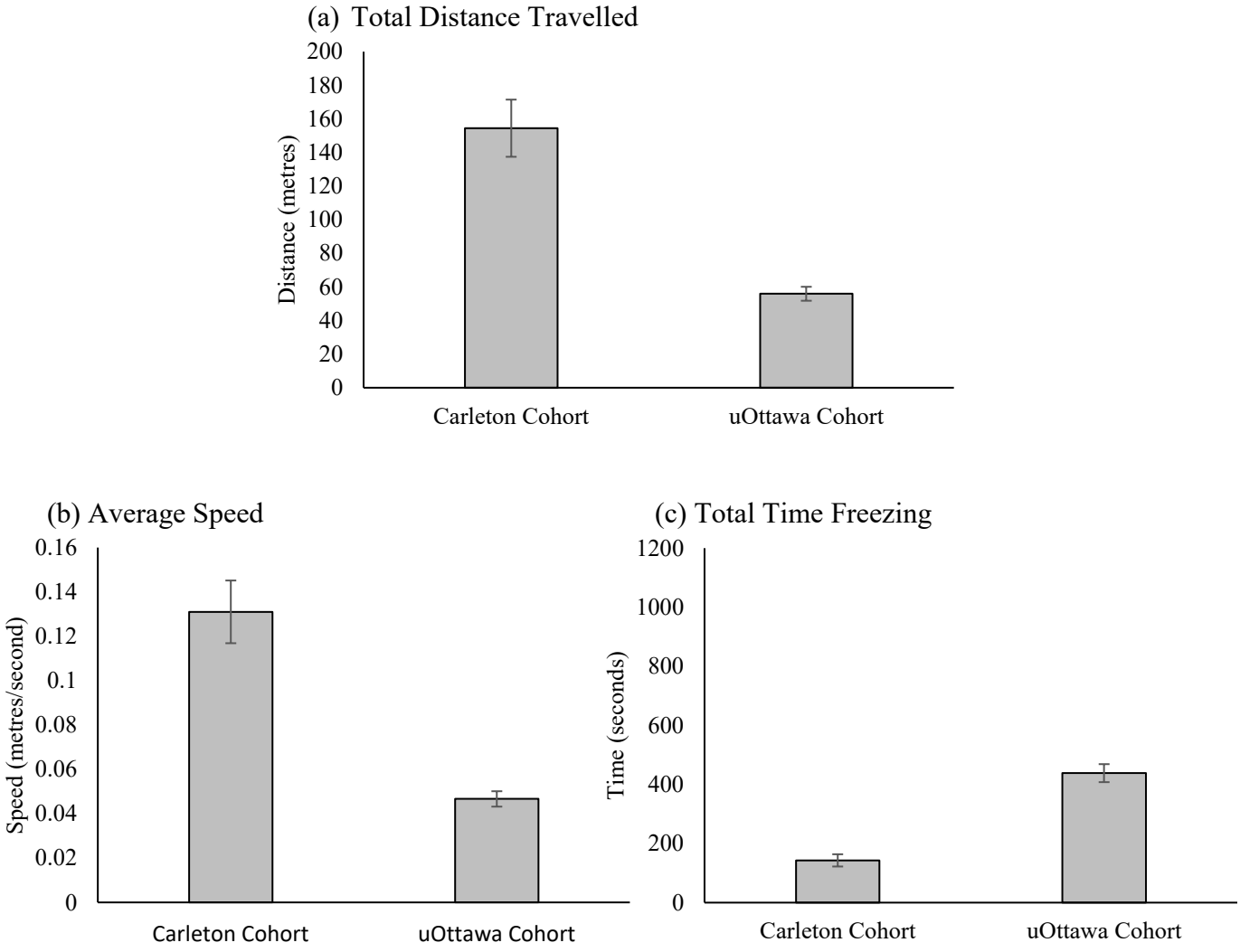

Figure 14. Comparison of anxiety-like and locomotor behaviour on the open field test between facilities.

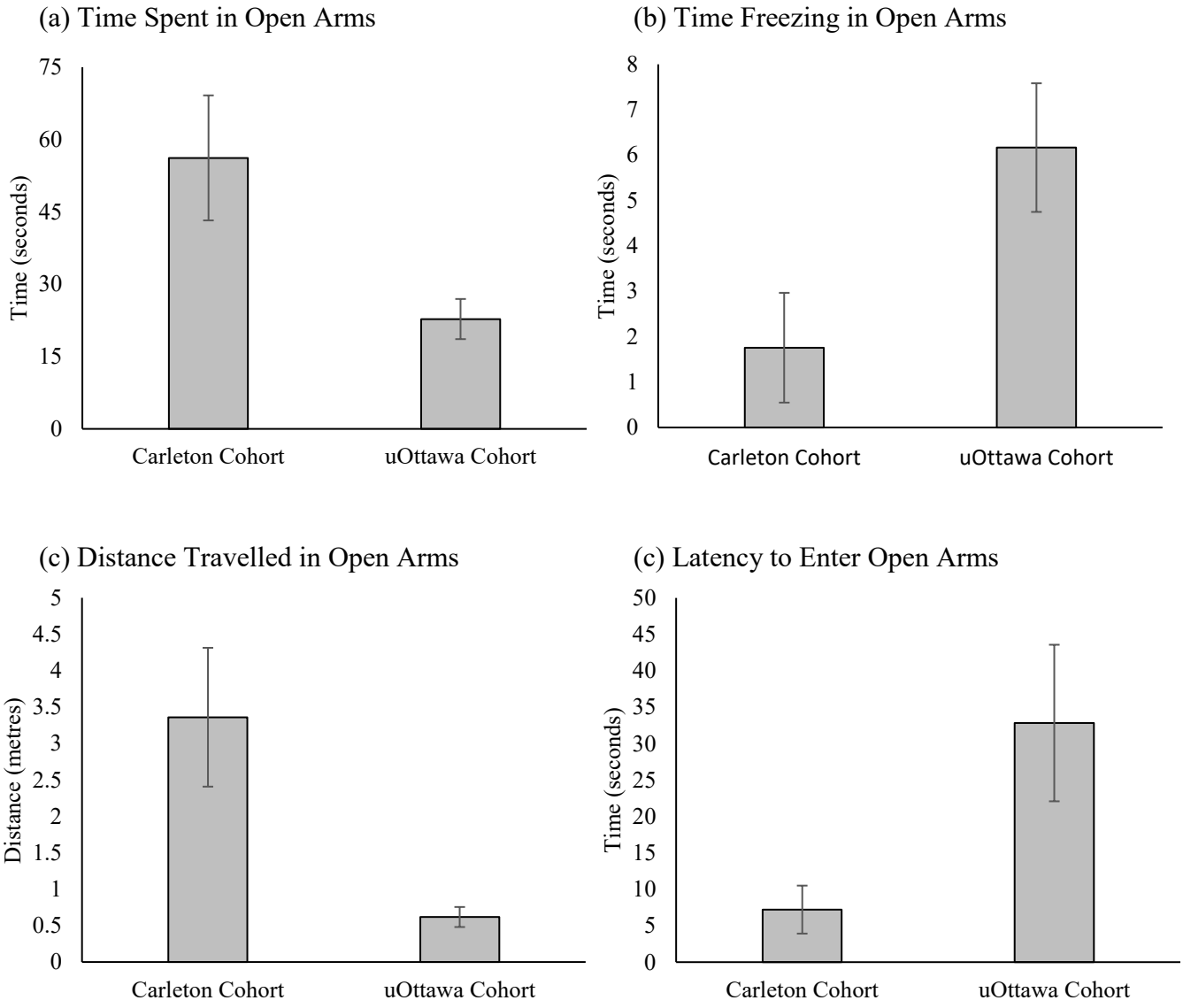

Figure 15. Comparison of anxiety-like behaviour on the elevated plus maze between facilities.

Appendix 3: Data from control animals only

The data below displays only the vehicle and FGF2 animals in the control group on all measures taken. This was done because there were no consistent effects of stress observed, but the sex differences in the action of FGF2 was found to be of interest. All data is expressed as mean ± SEM.

Open Field Test

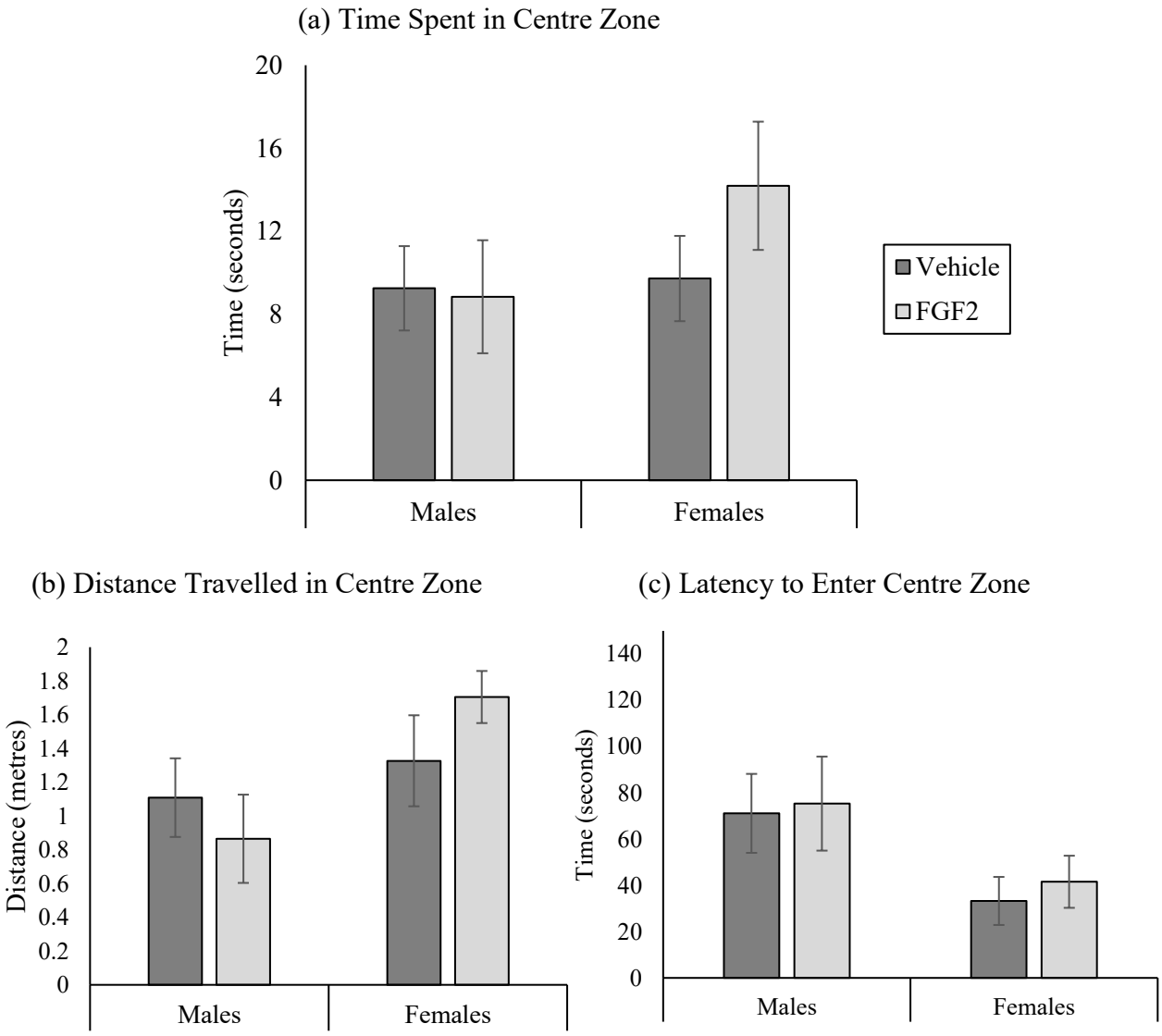

Figure 16. Anxiety measures on the 5-minute OF for control animals.

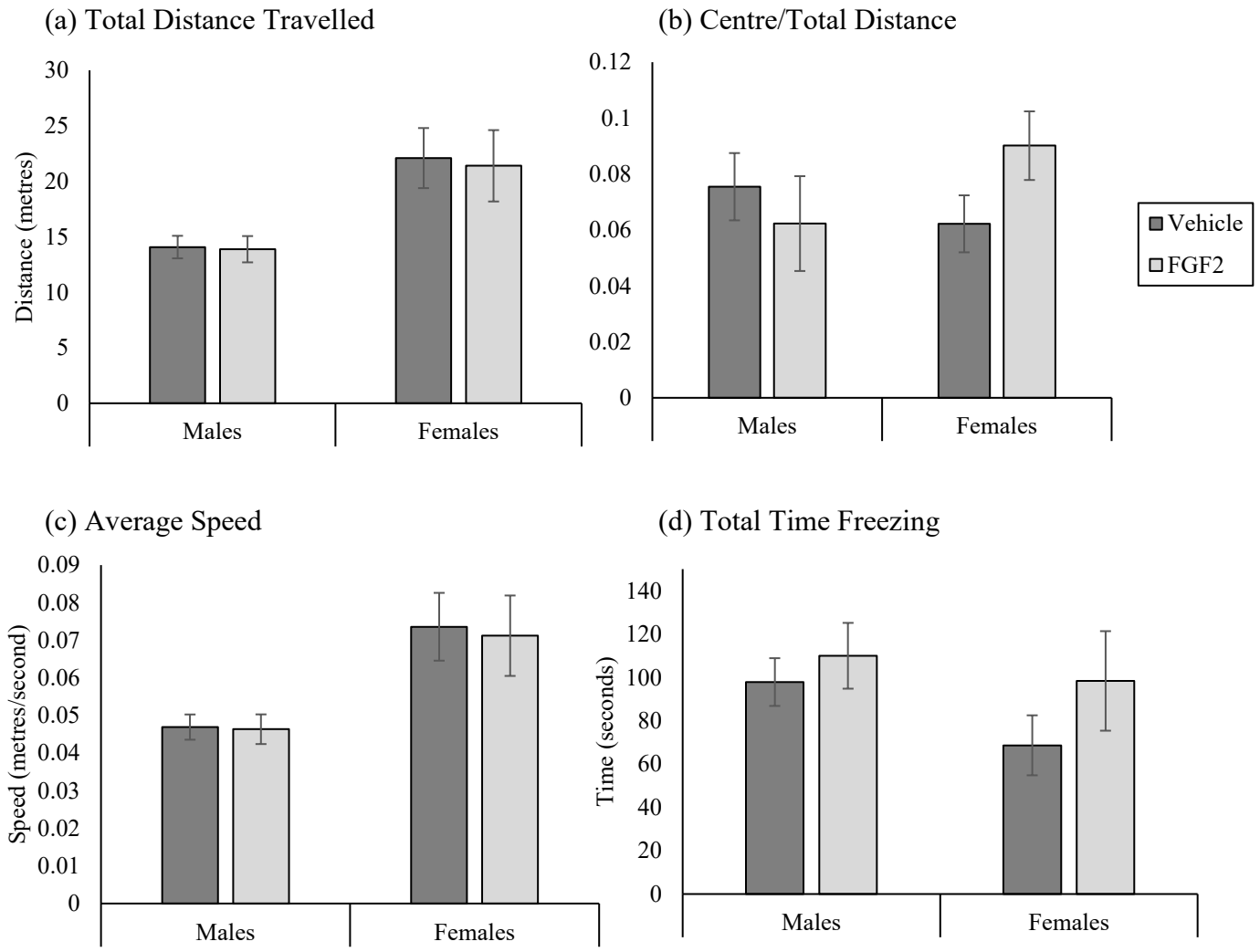

Figure 17. Locomotor measures on the 5-minute OF for control animals.

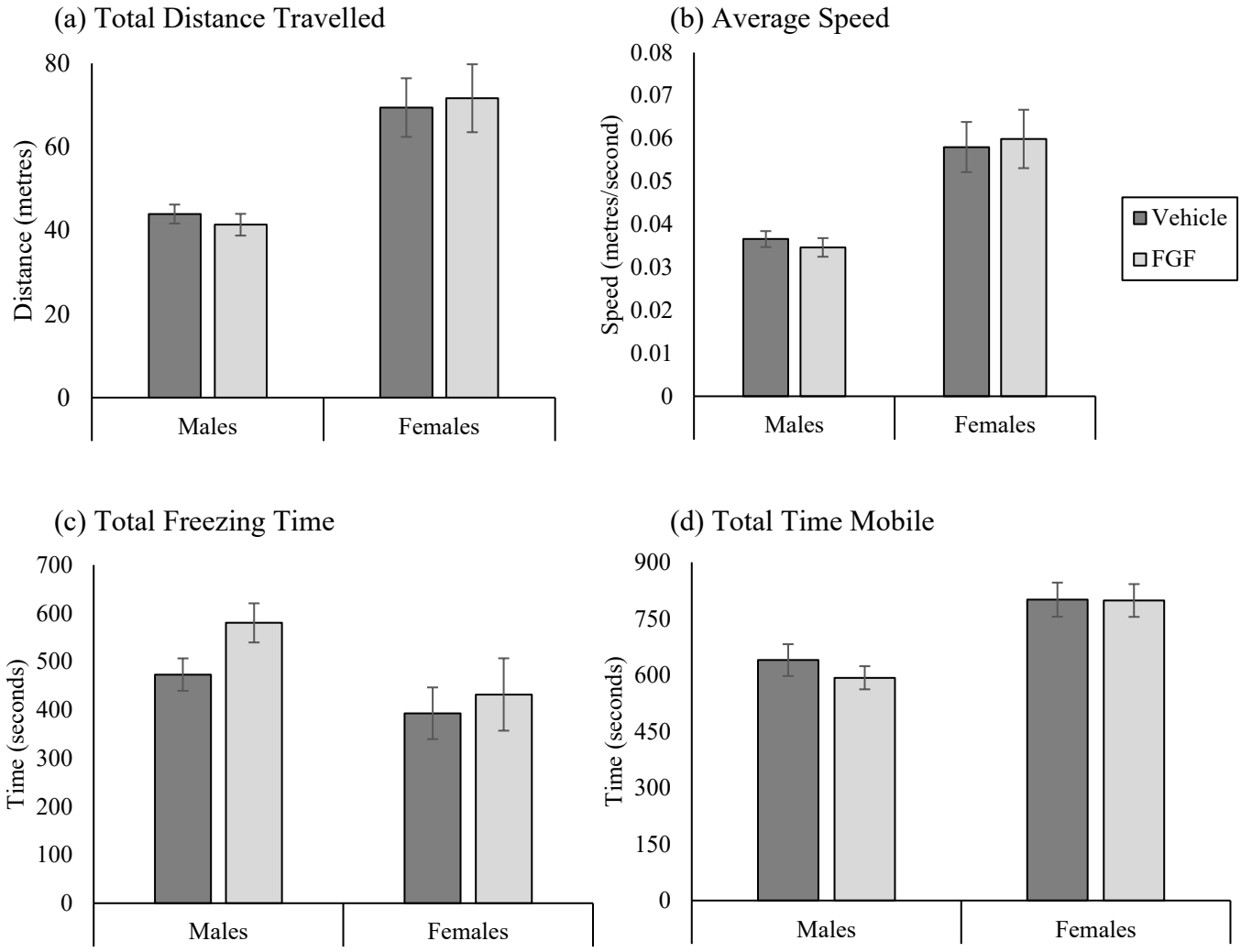

Figure 18. Locomotor measures on the 20-minute OF for control animals.

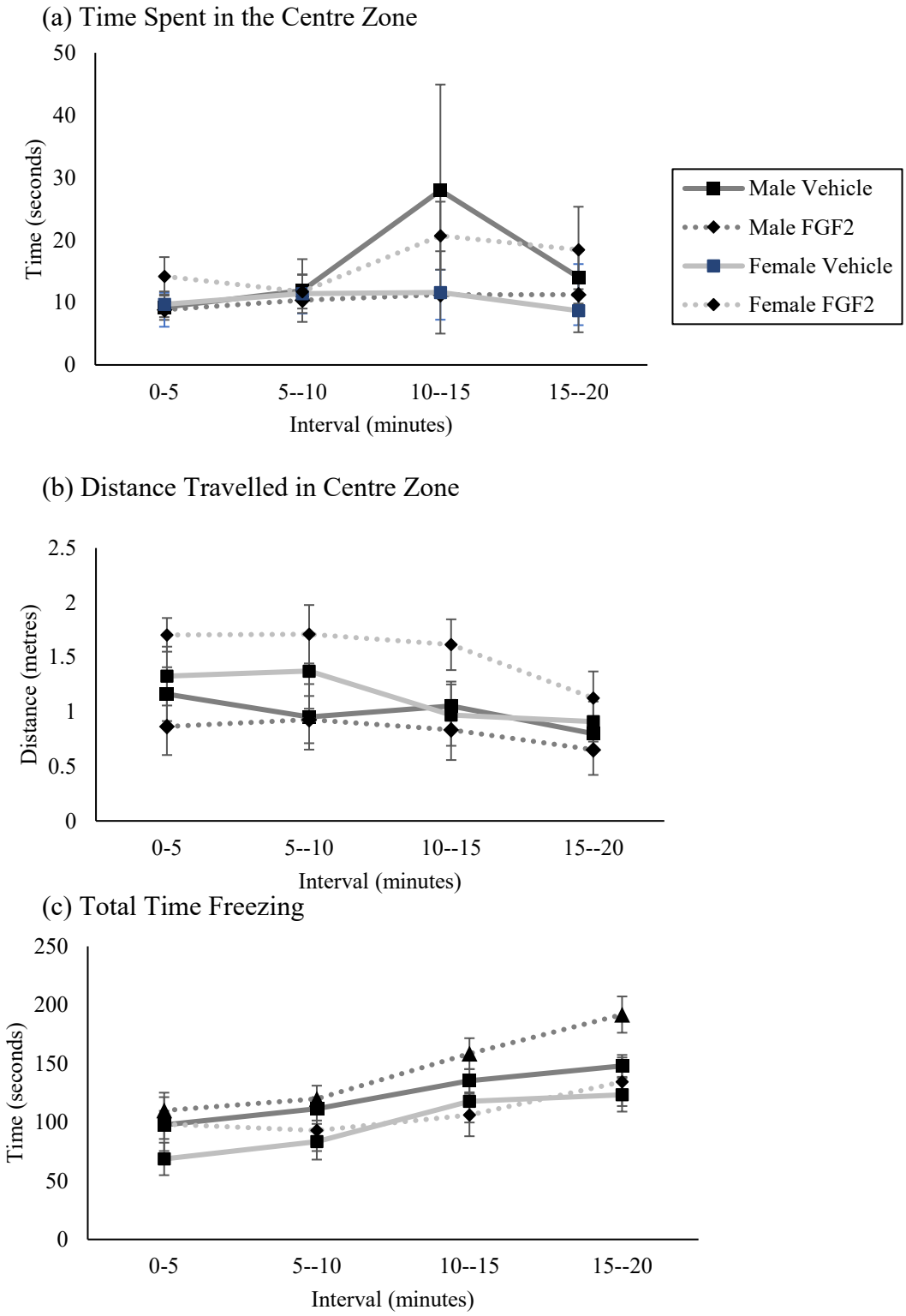

Figure 19. Anxiety and locomotor measures over 4x5-minute intervals in the OF for control animals.

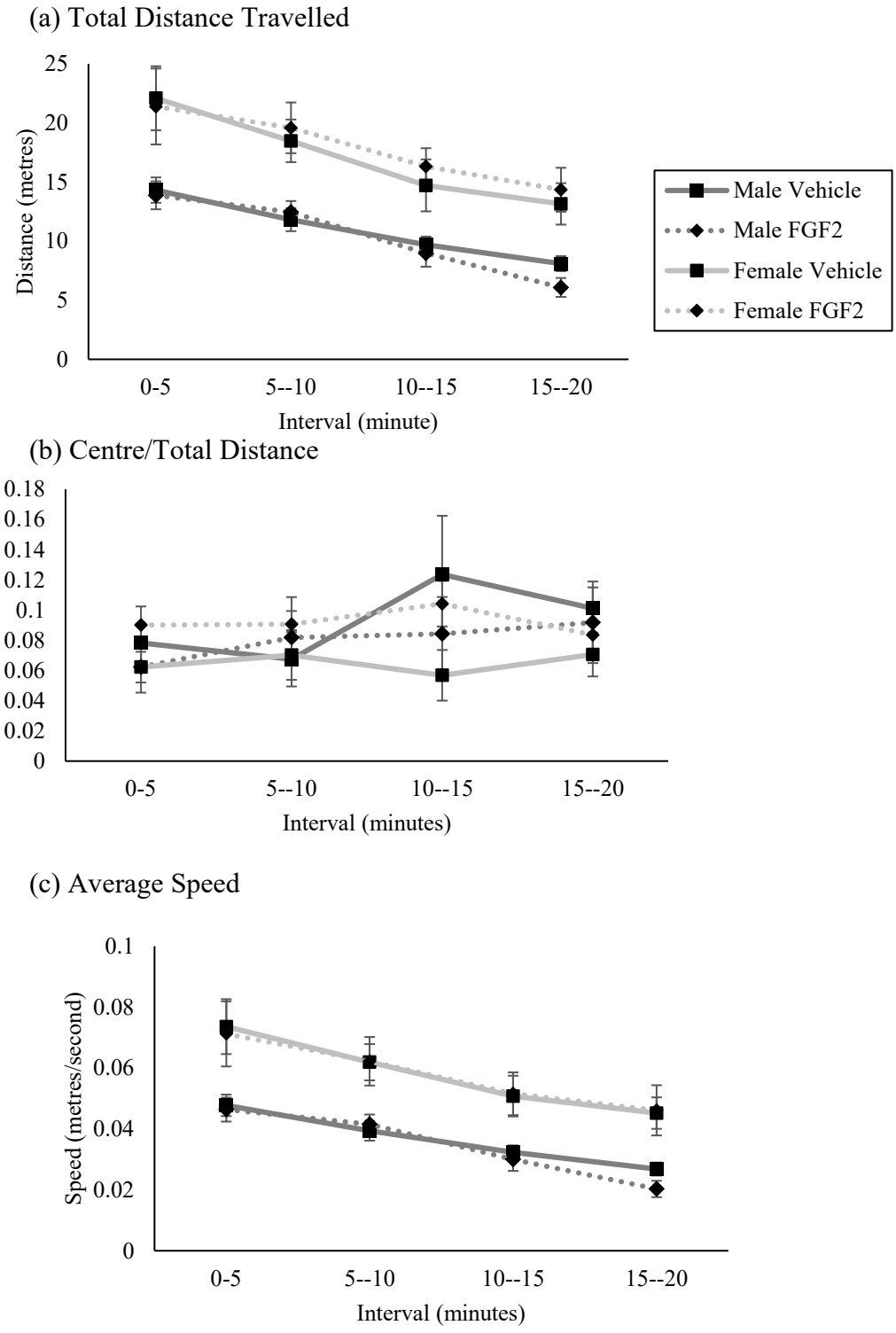

Figure 20. Locomotor measures over 4x5-minute intervals in the OF for control animals.

Elevated Plus Maze

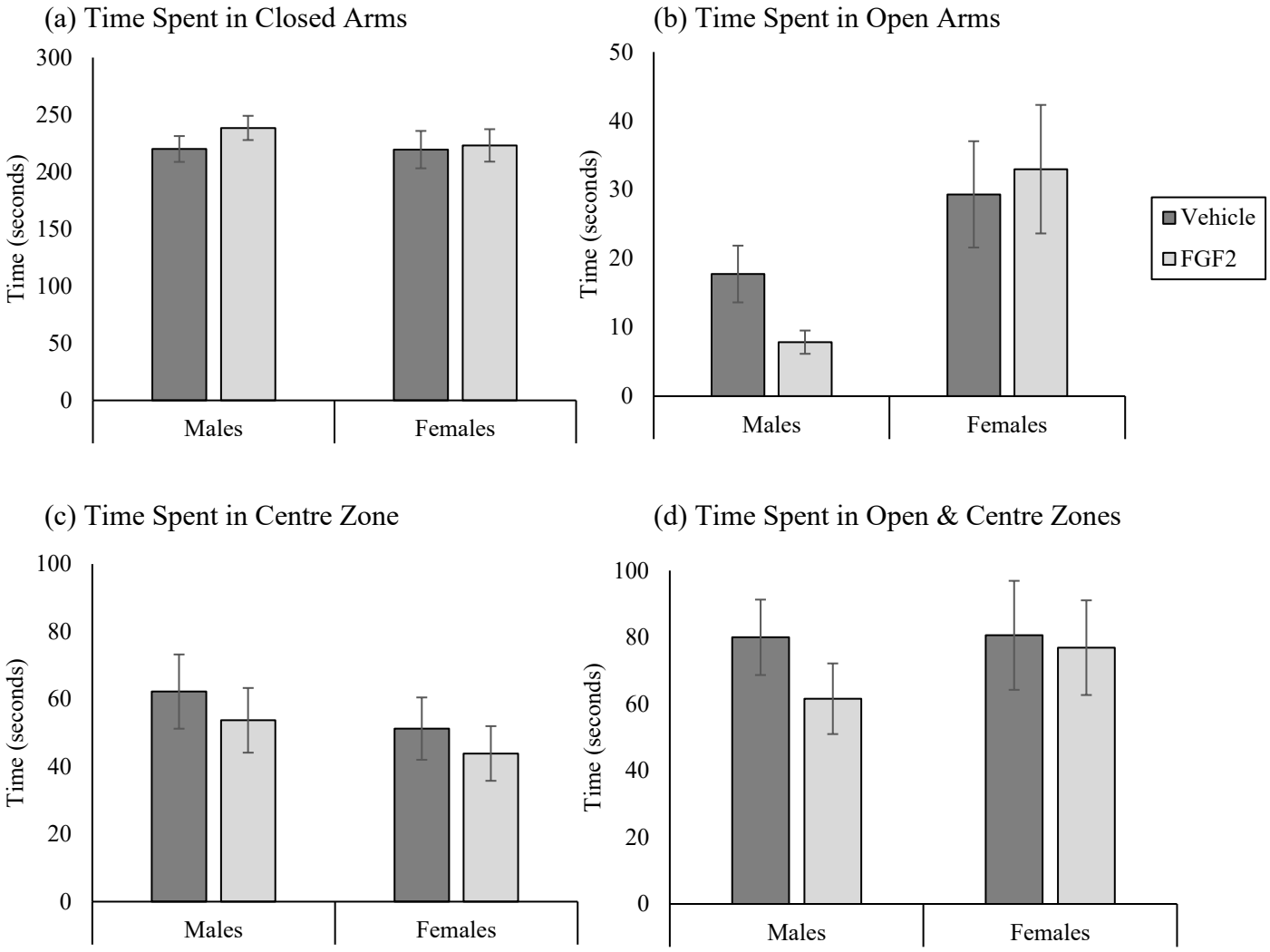

Figure 21. Anxiety measures on the EPM for control animals.

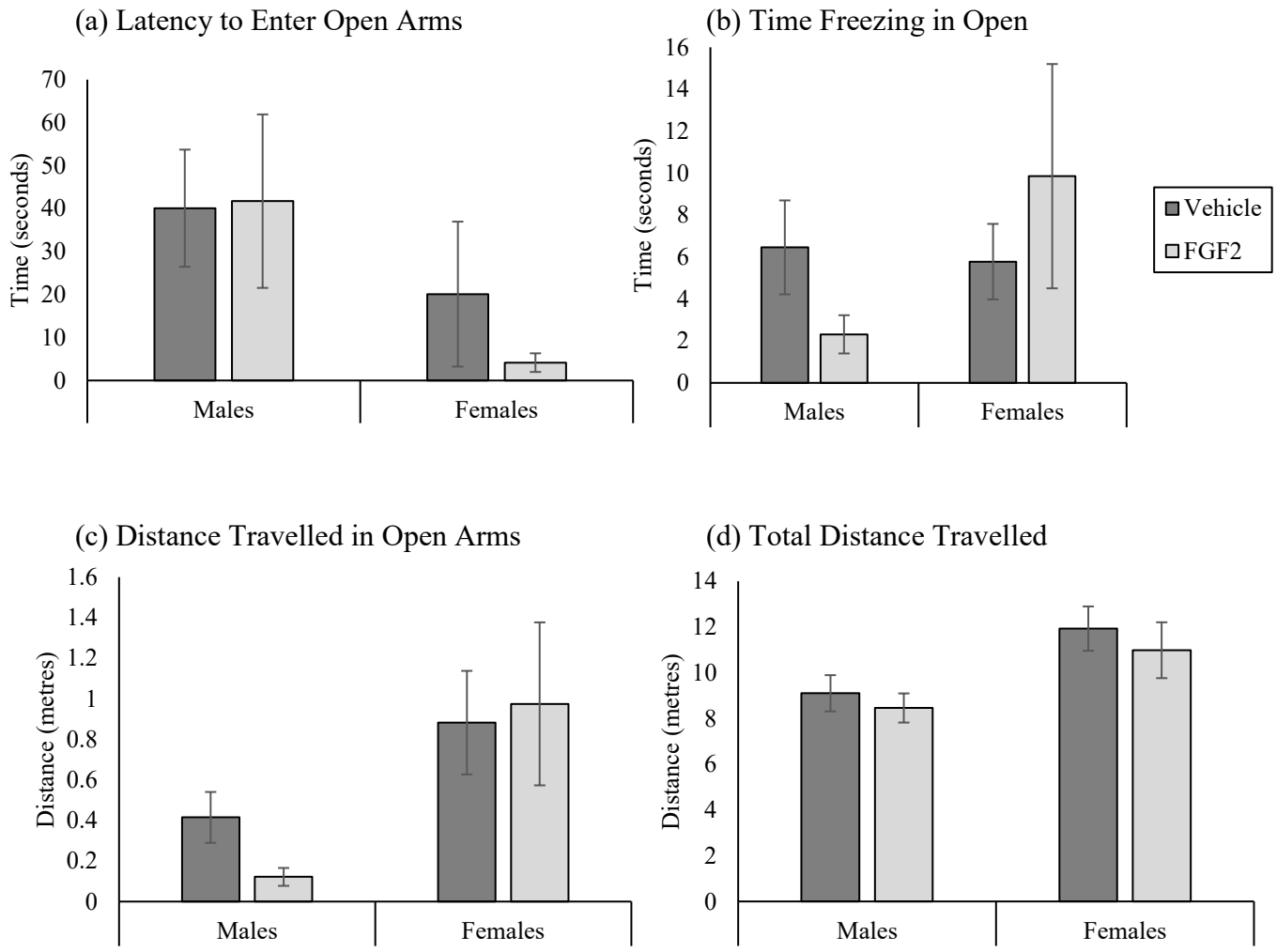

Figure 22. Anxiety and locomotor measures on the EPM for control animals.

Sucrose Consumption Test

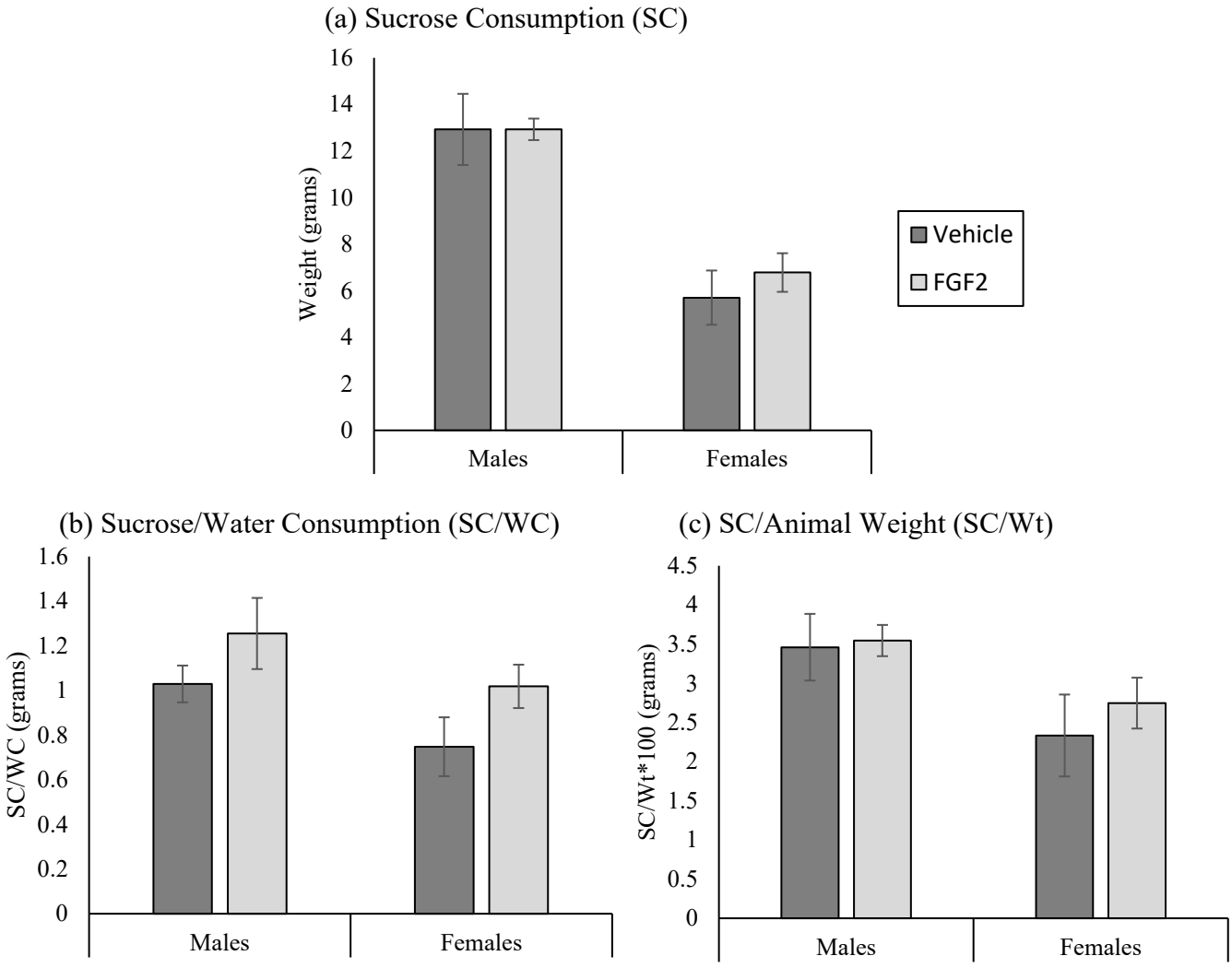

Figure 23. Measures of sucrose consumption on the SCT for control animals.

Forced Swim Test

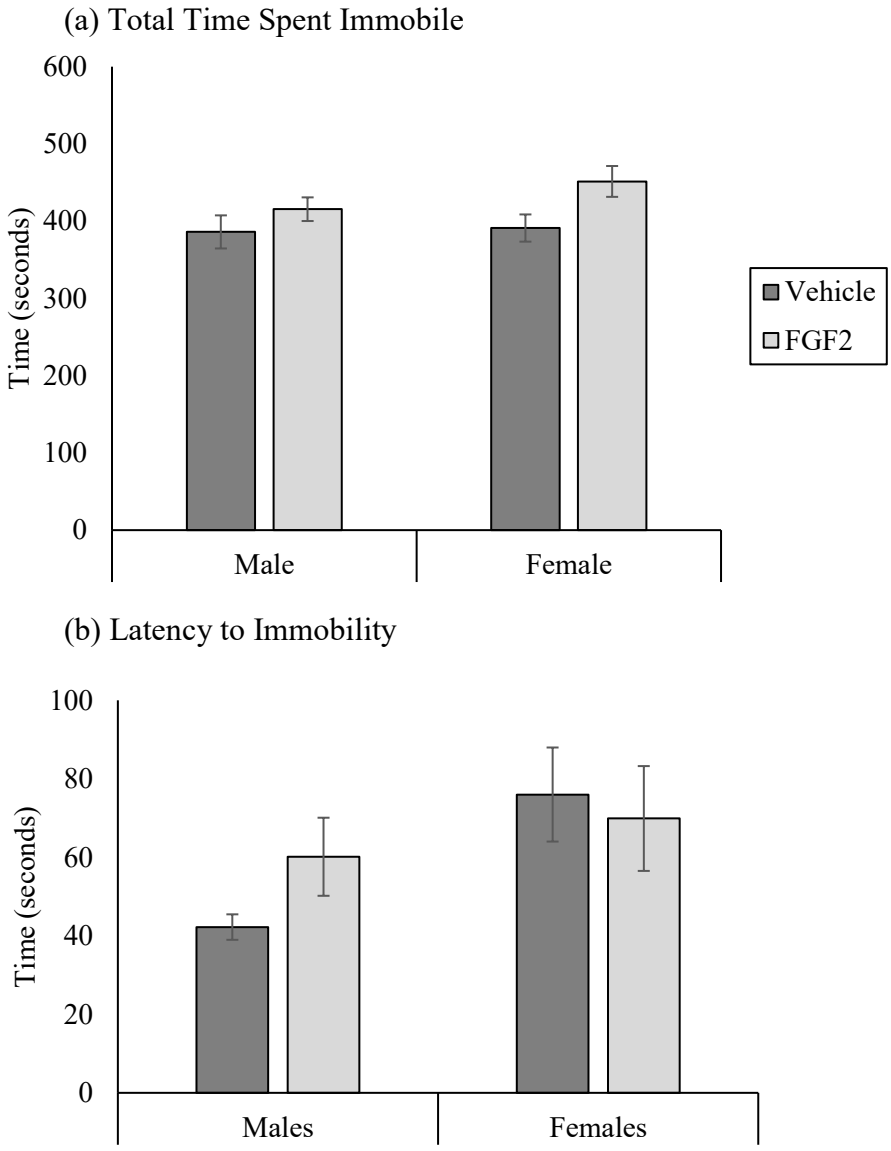

Figure 24. Immobility measures on the FST for control animals.

(a) Time Spent Immobile

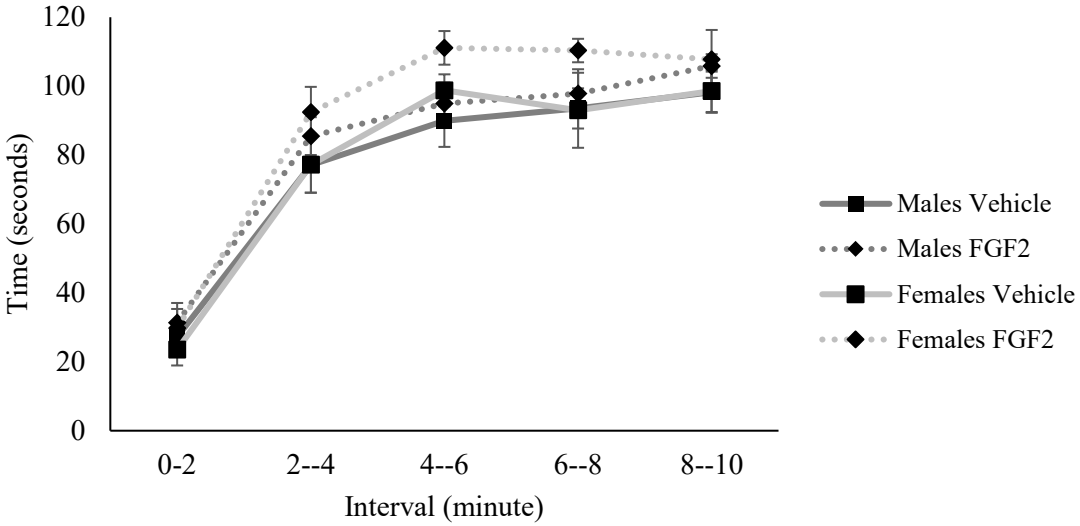

Figure 25. Time spent immobile in controls over 5x2-minute intervals on the FST for control animals.