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We hereby approve the Dissertation

of

Mollie R. Sorrell

Candidate for the Degree

DOCTOR OF PHILOSOPHY

_____
Dr. Kathleen Killian, Director

_____
Dr. Lori Isaacson, Reader

_____
Dr. Dawn Blitz, Reader

_____
Dr. Yoshinori Tomoyasu, Reader

_____
Dr. Jennifer Quinn, Graduate School Representative

ABSTRACT

A NOVEL INSECT MODEL TO STUDY THE ROLE OF FRAGILE X MENTAL RETARDATION PROTEIN IN INNATE IMMUNITY AND BEHAVIOR

by

Mollie R. Sorrell

Fragile X syndrome (FXS) is the most common form of inherited mental retardation and is the primary monogenetic cause of autism spectrum disorders. FXS is caused by a mutation in the regulatory region of the Fragile X Mental Retardation 1 (*FMR1*) gene, that ultimately leads to loss of the gene product Fragile X Mental Retardation Protein (FMRP). As FXS is a neurodevelopmental disorder, most studies have focused on the role of FMRP during development. However, a few studies have shown that the loss of FMRP during adulthood can impact learning, cognition, and behavior. The primary goal of this study was to decrease FMRP during adulthood and examine the impacts on immune function and social behavior using the cricket *Acheta domesticus* as a novel insect model. We examined immune function in males and females by assessing several important immune parameters including: the total number of circulating hemocytes in the hemolymph, the total hemolymph protein content, total phenoloxidase enzyme activity, and fat body *lysozyme* expression. We found that males and females exhibited similar changes in these immune parameters as a result of decreased *Fmr1*, but males were less likely to survive an immune challenge with an injection of lipopolysaccharide (LPS) from *Serratia marcescens*. This is the first study to demonstrate that decreased FMRP during adulthood causes sex-specific effects on the immune system. We also examined the effects of decreased *Fmr1* on the agonistic behavior of adult male crickets. We observed that the average time to first physical contact during an agonistic interaction was significantly increased in fights between a control male and *Fmr1* knockdown male and that *Fmr1* knockdown males were less likely to initiate an aggressive interaction than control males. Despite initiating

interactions less often, these males fought just as aggressively and won as often as controls. In this study, we also evaluated the parameters required to generate a successful parental RNAi response in the cricket *Acheta domesticus*. We found that an *Fmr1* knockdown could be passed on to the next generation, allowing the cricket to be used for both adult and developmental studies of FXS. This study demonstrates that a decrease in FMRP can have significant impacts on animal physiology and behavior regardless of whether this decrease occurs during development or adulthood.

A NOVEL INSECT MODEL TO STUDY THE ROLE OF FRAGILE X MENTAL RETARDATION PROTEIN IN INNATE IMMUNITY AND BEHAVIOR

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Mollie R. Sorrell

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DEDICATION

This manuscript is dedicated with love to my dearly departed friend Sara Ann Neff. I would not have ventured down this path if not for her. Despite her physical absence, she has been a constant source of motivation, and has given me the strength that I needed to see it through to the end. We finally did it!

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Chapter 1: General Introduction

Fragile X syndrome (FXS) is the most common form of inherited mental retardation (Crawford et al., 2001), and is the primary monogenetic cause of autism spectrum disorders (ASD), accounting for 30% of all known cases (Bassell and Warren, 2008; Wang et al., 2010). FXS is caused by a mutation in the promoter region of Fragile X Mental Retardation 1 (*FMR1*), which is located on the X chromosome (reviewed in Bhakar et al., 2012). This mutation increases the number of CGG trinucleotide repeats, thereby expanding the gene (reviewed in Bhakar et al., 2012). Expansion of *FMR1* leads to hyper-methylation, transcriptional silencing, and ultimately the loss of the gene product Fragile X Mental Retardation Protein (FMRP) (Fu et al., 1991; Pieretti et al., 1991). The number of CGG repeats in the regulatory region of *FMR1* directly correlates with the amount of FMRP produced, and results in different disease phenotypes (Li and Zhao, 2014). Unaffected individuals have 5-55 CGG trinucleotide repeats and produce normal amounts of FMRP protein. The pre-mutation state, which is not well understood, results when an individual has 55-200 CGG repeats. This expansion causes reduced levels of FMRP and individuals develop disease phenotypes that are distinct from FXS, including Fragile X-associated tremor/ataxia syndrome (FXTAS) or Fragile X-associated primary ovarian insufficiency (FXPOI) (Li and Zhao, 2014). When the number of CGG repeats exceeds 200, there is a complete loss of FMRP which results in the FXS disease state (Li and Zhao, 2014). The most common features seen in patients with FXS include: craniofacial abnormalities, growth abnormalities, deficits in learning and cognition, hyperactivity, anxiety, shyness, social and language deficits, repetitive body movements and behaviors, motor incoordination, and impaired sensory processing (Hagerman, 2002; Baranek et al., 2005; Reiss and Hall, 2007; Bailey et al., 2008; Bhakar et al., 2012).

With the exception of heart and muscle, FMRP is expressed in every cell type, but is most highly expressed in neurons (Antar and Bassell, 2003; reviewed in Drozd et al., 2018). FMRP functions as a mRNA binding protein that inhibits protein synthesis when it is produced at normal levels (Jin and Warren, 2000). It is important for the regulation of mRNAs involved in synapse growth and function, and inhibition of FMRP leads to increased protein synthesis within synapses (Bassell and Warren, 2008; Bhakar et al., 2012). This deficit in FMRP disrupts normal synaptic development and function, thereby reducing plasticity (reviewed in Bhakar et al., 2012; Contractor et al., 2015). Mutations in *Fmr1* can also impact the function of neural stem cells

(NSC), causing decreased adult neurogenesis, which leads to impaired learning and memory (Guo et al., 2011)

As FXS is a neurodevelopmental disorder, most studies have focused on the role of FMRP during development. However, a few studies have shown that the loss of FMRP during adulthood can impact learning, cognition, and behavior (Hamada et al., 2009; Guo et al., 2011). Guo et al. (2011) depleted FMRP in the neural stem cells of adult mice using inducible gene recombination and found that this depletion impaired their ability to learn. Moreover, restoration of FMRP to the adult neural stem cells rescued this phenotype, and knockout (KO) mice performed as well as control littermates in learning assays (Guo et al., 2011). This finding suggests that new neurons that are FMRP-deficient may form defective synapses that could interfere with existing networks, disrupting the ability to learn. A second study in the cricket *Gryllus bimaculatus* used systemic RNA interference (RNAi) to knockdown (KD) *Fmr1* expression during adulthood (Hamada et al., 2009). They found that decreased *Fmr1* led to significant changes in morphology, social behavior, and motor function (Hamada et al., 2009). KD crickets exhibited defects in wing posture, had abnormal calling songs, and experienced loss of circadian locomotor rhythm (Hamada et al., 2009). Taken together, these studies support that a decrease in FMRP can have significant impacts on animal physiology and behavior regardless of whether this decrease occurs during development or adulthood.

Since very little work has focused on the effects of decreased FMRP during adulthood, one of the primary goals of this dissertation was to fill a much needed gap in current knowledge and focus on how this can affect various aspects of physiology and behavior. In order to do that, we used the cricket *Acheta domesticus* as a novel insect model to study the effects of decreased FMRP on immune function (Chapter 2) and social behavior (Chapter 3).

Immune Function

While *FMR1* is primarily associated with neuronal defects, FXS and ASD patients also exhibit symptoms associated with immune defects (Samsam et al., 2014; Estes and McAllister, 2015). Research conducted on Fragile X premutation carriers and patients with ASD has shown that individuals have altered expression levels of immune genes (Careaga et al., 2014; reviewed in Estes and McAllister 2015). Specifically, patients had increased levels of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, IL12p40, and granulocyte-macrophage colony stimulating factor in

the blood, while the levels of anti-inflammatory cytokines such as IL-10, IL-12 and transforming growth factor-β (TGFβ) were decreased (Careaga et al., 2014; reviewed in Estes and McAllister, 2015). In addition to altered cytokine levels, individuals also had dysfunctional immune cells and exhibited impaired responses to immune challenges (Warren et al., 1986; Warren et al., 1987; Enstrom et al., 2010; Ashwood et al., 2011). Consistent with these findings in human, a recent study in *Drosophila* used tissue-specific RNA interference (RNAi) to knockdown the expression of *Fmr1* in hemocytes, important immune cells that mediate phagocytosis in insects (O'Connor et al., 2017). These knockdown flies were highly sensitive to bacterial infections due to decreased phagocytosis by hemocytes (O'Connor et al., 2017). While it is clear that the immune system is affected by decreased FMRP, these are the only studies to date to examine this. In Chapter 2 of this dissertation, we examined the effects of decreased *Fmr1* expression during adulthood on the innate immune system of a novel insect model of Fragile X. We used RNA interference (RNAi) to suppress *Fmr1* expression in adult male and female *Acheta domesticus* crickets, and then examined the impact on multiple measures of immune function.

Social Behavior

As a result of the neural deficits caused by decreased FMRP, patients with FXS and ASD exhibit impaired social behaviors which typically manifest as social anxiety, withdrawal, and aloofness (reviewed in Bhakar et al., 2012). While these behaviors are common in most patients, the intensity and frequency is different for each individual. Studies in animal models of FXS have also shown that deficiencies in *Fmr1* lead to a wide variety of impaired social behaviors in rats (Hamilton et al., 2014), mice (Spencer et al., 2005), zebrafish (Wu et al., 2017), tadpoles (Truszkowski et al., 2016), fruit flies (Bolduc et al., 2010), and crickets (Hamada et al., 2009). Different aspects of social behavior were examined in these studies, but overall, *Fmr1* animals showed increased social anxiety, and displayed deficits in social communication and interactions. For example, studies in tadpoles (Truszkowski et al., 2016) and zebrafish (Wu et al., 2017) examined the effects of decreased FMRP on schooling behavior. Coordinated group swimming is a social behavior that has been well characterized in both of these species. Upon FMRP knockdown, the normal schooling pattern of unidirectional and parallel swimming was lost. Instead, FMRP deficient animals displayed less aggregated and more disperse swim patterns (Truszkowski et al., 2016; Wu et al., 2017). In another study, *Fmr1* KO rats exhibited fewer

juvenile play behaviors like ear and tail tugging, wrestling, and pinning than their wildtype littermates in direct social interaction tests (Hamilton et al., 2014). These juvenile rats also exhibited increased levels of anxiety by chewing on wooden blocks more often than wildtype rats. In Chapter 3 of this dissertation we used the cricket *Acheta domesticus* to examine the effects of decreased *Fmr1* on male social behavior. The cricket lends itself to this type of study because agonistic behavior has been well characterized in the cricket (Adamo and Hoy, 1995; Stevenson et al., 2000; Hofmann and Schildberger, 2001), and follows a highly stereotyped sequence of increasingly aggressive interactions. We used RNAi to knockdown *Fmr1* expression on the first day of adulthood and assessed whether decreased *Fmr1* outside of development was sufficient to impair agonistic behavior in males.

Another major goal of this study was to demonstrate the utility of the cricket as an animal model for the study of FXS and FX-related disorders. In Chapters 2 and 3 we utilized RNA interference to create a loss-of-function phenotype in the cricket during adulthood. In Chapter 4 of this dissertation we explored the possibility of using parental RNAi in *Acheta domesticus*. This would allow us to knockdown *Fmr1* expression during development and be able to compare these results with the results obtained from our experimental knockdown of adult FMRP.

Parental RNA interference (pRNAi)

Insects are particularly suitable for RNAi studies because a single application of dsRNA initiates silencing of a target gene, which can have long-lasting effects (reviewed in Belles, 2010; Cooper et al., 2018). For example, RNAi can persist for up to 7 months in the bug *Rhodnius prolixus* (Paim et al., 2013), more than 2 months in the red flour beetle *Tribolium castaneum,* 2 months in the cricket *Gryllus bimaculatus* (Moriyama et al., 2008), 1 month in the firebrat *Thermobia domestica* (Uryu et al., 2013), and more than 21 days in the honeybee *Apis mellifera* (Amdam et al., 2003). In some insects, systemic RNAi is observed where transcriptional suppression of a target is observed in all tissues of the body due to the spread of the RNAi signal (reviewed in Cooper et al., 2018). The systemic properties of RNAi allow dsRNA to spread to the germline cells, where gene knockdown (KD) effects can be transmitted to the next generation (Matsumoto and Hatori, 2016). This phenomenon, termed parental RNAi (pRNAi), has been observed in numerous insects (see Bucher et al., 2002; Paim et al., 2013; Coleman et al., 2015 for examples) and can be induced by injection or oral delivery of dsRNA to a female. In Chapter 4 of this

dissertation we evaluated the parameters required to generate a successful pRNAi response in the cricket *Acheta domesticus.* Double-stranded RNA was injected into females on the first day of adulthood, and the efficacy of RNAi was progressively evaluated along multiple stages of the insect's life. We also examined relevant parameters such as concentration response, timing of the dsRNA exposure, effects on unfertilized eggs in female ovaries, the number of offspring hatched from oviposited eggs, the temporal endurance of the RNAi effect in offspring, and the functional effects of RNAi in offspring. Transmitting the KD effects of *Fmr1* to offspring through pRNAi would allow the next generation to be exposed to a decrease in FMRP during development, rather than just adulthood, as normally occurs in patients with FXS. Being able to utilize adult RNAi and pRNAi techniques in the cricket would allow for comparisons between developmental and adult decreases in FMRP, making it an invaluable model for the study of FXS.

This dissertation served two primary purposes, the first of which was to decrease FMRP during adulthood and examine the impacts on immune function and social behavior. The second purpose was to demonstrate the use of *Acheta domesticus* as an insect model for the continued study of FXS. The objective of Chapter 2 was to decrease *Fmr1* expression in adult males and females and examine the impact on multiple measures of immune function. To date, very few studies have directly investigated the effect of decreased *Fmr1* on immune parameters in an animal model of FXS. Furthermore, this is the first study to explore the idea that changes in *Fmr1* expression may have differential effects on the immune system based upon sex. This study reveals that changes in decreased *Fmr1* expression during adulthood have a significant impact on the cellular and humoral defense mechanisms of the insect innate immune system. Additionally, male and female crickets exhibit similar changes in immune parameters, but respond to immune challenges differently. The objective of Chapter 3 was to decrease *Fmr1* expression in adult males and determine the impact on a well characterized social behavior. This second study reveals that the agonistic behavior of adult males is significantly impacted in response to modest decreases in *Fmr1*. Both Chapter 2 and 3 demonstrated the usefulness of the cricket *Acheta domesticus* as a model for the study of FXS. In Chapter 4, the objective was to demonstrate that the effects of RNAi are transmissible to the next generation so that the cricket could be used for both adult and developmental studies of FXS. This study reveals that an Fmr1 knockdown can be passed on to the next generation, supporting that the cricket *Acheta domesticus* is an excellent model for the continued study of FXS.

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Chapter 2: A novel insect model to study the role of *Fragile X Mental Retardation 1* gene on innate immunity

1. Introduction

Fragile X syndrome (FXS) is the most common form of inherited mental retardation (Crawford et al., 2001), and is a leading cause of autism spectrum disorders (Bassell and Warren, 2008). FXS is caused by a mutation in the regulatory region of Fragile X Mental Retardation 1 (*FMR1*), which increases the number of CGG trinucleotide repeats, thereby expanding the gene (reviewed in Bhakar et al., 2012). This expansion leads to hyper-methylation, transcriptional silencing, and ultimately the loss of the gene product Fragile X Mental Retardation Protein, also referred to as FMRP (Fu et al., 1991; Pieretti et al., 1991). The number of CGG repeats in the regulatory region of *FMR1* directly correlates with the amount of FMRP produced, and results in several different disease phenotypes (Li and Zhao, 2014). When an individual has more than 200 CGG repeats there is a complete loss of FMRP, which results in the FXS disease state (Li and Zhao, 2014). Patients with FXS experience a number of symptoms including: deficits in learning and cognition, hyperactivity, attention deficit, shyness, anxiety, repetitive body movements and behaviors such as hand/arm flapping and echolalia, deficits in motor coordination, and impaired sensory processing (Hagerman, 2002; Baranek et al., 2005; Reiss and Hall, 2007; Bailey et al., 2008).

While *FMR1* is primarily associated with neuronal defects, several studies suggest that FXS may also be associated with defects of the immune system (Samsam et al., 2014; Estes and McAllister, 2015; O'Connor et al., 2017). Research conducted on patients with autism spectrum disorders (ASD) has shown that these individuals have altered expression levels of immune genes (reviewed in Estes and McAllister, 2015). Specifically, patients had increased levels of pro-inflammatory cytokines in the blood, while the levels of anti-inflammatory cytokines were decreased (reviewed in Estes and McAllister, 2015). In addition to altered cytokine levels, individuals also had dysfunctional immune cells and exhibited impaired responses to immune challenges (Warren et al., 1986; Warren et al., 1987; Enstrom et al., 2010; Ashwood et al., 2011). Consistent with these findings in human, a recent study in *Drosophila* used tissue-specific RNA interference (RNAi) to knockdown the expression of *Fmr1* in hemocytes, important immune cells that mediate phagocytosis in insects (O'Connor et al., 2017). These knockdown flies were highly sensitive to bacterial infections due to decreased phagocytosis by hemocytes (O'Connor et

al., 2017). Taken together, these studies suggest that the immune system can be affected by decreased FMRP levels.

Since the development of the first animal model of FXS in the early 1990's (reviewed in Drozd et al., 2018), our understanding of the disease has progressed rapidly. However, the idea that it may be associated with defects in the immune system has not been well studied in any model of FXS. While much FXS research has relied on vertebrate models such as the mouse, rat, frog, and zebrafish, the fruit fly *Drosophila melanogaster* has proved to be an invaluable model for the study of FXS. The *Fmr1* homolog was identified in *Drosophila* in 2000 (Wan et al., 2000) and since then, numerous studies have used this insect model to examine the impact of FXS on neural physiology and social behavior (reviewed in Drozd et al., 2018).

In the present study, we used the cricket *Acheta domesticus* as a novel insect model to examine the effects of decreased FMRP on the immune system. The cricket lends itself to this type of study because crickets have a defense system designed to protect them from invading pathogens. The first line of defense is provided by physical barriers such as the external cuticular integument (reviewed in Gillespie et al., 1997; Rosales, 2017). When physical barriers fail, insects rely on an innate immune system that consists of both cellular and humoral defense mechanisms (reviewed in Gillespie et al., 1997; Strand, 2008; Rosales, 2017).

Cellular immunity is mediated by circulating hemocytes, which recognize invading pathogens as foreign (reviewed in Gillespie et al., 1997). Depending upon the size of the pathogen, hemocytes will then phagocytize, encapsulate, or nodulate the invader to prevent its growth and reproduction (reviewed in Gillespie et al., 1997). Antimicrobial peptides are an important component of the insect humoral defense system. These proteins are primarily synthesized by the fat body, an organ that functions similarly to the vertebrate liver (reviewed in Gillespie et al., 1997; Arrese and Soulages, 2010). When the insect immune system detects foreign molecules, such as bacteria, the fat body secretes antimicrobial peptides, such as lysozyme, into the surrounding hemolymph where they can effectively counteract the bacteria (reviewed in Gillespie et al., 1997; Roma et al., 2010).

The current study also assessed immune function in males and females with decreased FMRP, to determine if the effects of FMRP are sex specific. FXS is more common in males than females, affecting 1:2500 males (Hagerman, 2008) and 1:8000 females (Rinehart et al., 2010). Females with FXS also tend to exhibit milder symptoms than affected males because it is an X-linked dominant disorder (Hagerman, 2008). As a result, a majority of research has focused on male patients. While inheritance patterns contribute to symptom severity, the cognitive profiles that characterize male and female FXS patients are different. It is unclear why these differences exist, but sex hormones are thought to play an important role (Rinehart et al., 2010). Ultimately, more studies focusing on how males and females may differentially respond to altered FMRP levels will further our understanding of FXS.

In this study, we examined immune system function in males and females with decreased FMRP, using a novel insect model. We identified the cricket *Fmr1* ortholog and found that it is highly conserved across all current animal models of FXS. Using RNAi-mediated knockdown (KD), we show that a single injection of dsRNA significantly reduces *Fmr1* expression to similar levels in the hemolymph and fat body of both males and females. Despite having a similar KD, females have significantly more circulating hemocytes than males. We also found that *Fmr1* KD causes decreased PO activity and increased *Lysozyme* expression in the hemolymph of males and females. Furthermore, we demonstrate that KD males are unable to survive an LPS immune challenge, while KD females survive as well as controls and never see a significant decrease in survival. These results suggest that decreased *Fmr1* expression has a significant impact on the immune system, and that the effects of FMRP are sex specific.

2. Methods

2.1 Insects

Acheta domesticus crickets were raised in house and maintained in large plastic containers in groups of approximately 50. Once they reached adulthood, undamaged day 1 (D1) males and females were isolated from the group and placed into individual round plastic containers measuring 10cm wide by 8cm high. Isolated adults were given *ad libitum* access to water and

laboratory rat chow while being maintained at a constant temperature of 29°C. Isolated adults were stored in an incubator separate from the rest of the colony, in a 12h light:12h dark cycle.

2.2 Identification of Genes in Acheta domesticus

Total RNA was isolated from the whole body of an individual D1 adult male, D1 adult female, D9 adult male, and D9 adult female using a Maxwell 16 LEV simplyRNA Tissue Kit (Promega; Madison, WI) in conjunction with an automated Maxwell 16 RNA Isolation system (Promega). After extraction, the concentration and quality of the RNA were initially determined by a Nanodrop 2000 (Fisher Thermo Science; Pittsburgh, PA). An Agilent 2100 Bioanalyzer (Applied Biosystems; Waltham, MA) was used to further assess the sample quality. Samples were stored at -80°C until shipping. RNA samples were shipped to Novogene (Sacramento, CA) for library construction, Illumina sequencing, transcriptome assembly, and differential gene expression analysis. Briefly, 150bp pair-end sequencing was performed using the Illumina HiSeq platform and the transcriptome was assembled *de novo* using Trinity (Grabherr et al., 2011). CORSET was used to remove redundancies from the Trinity results and then RSEM software was used for gene expression analysis. The readcount values from the gene expression analysis were normalized and then DESeq was used for differential expression analysis.

Cricket orthologs for Fragile X Mental Retardation 1 (Fmr1) and Lysozyme were identified by performing a BLAST of the *Acheta domesticus* transcriptome using CLC-bio Genomics Workbench 12 software. We used a *D. melanogaster* sequence as a query to identify the ortholog for *A. domesticus* Fmr1 (DmFmr1, Fly Base ID: FBgn0028734) and Lysozyme (DmLysozyme, Fly Base ID: FBgn0004425). After identifying both of these sequences in *A. domesticus* we performed a reciprocal BLAST on the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) database to confirm their sequence orthology to the corresponding *D. melanogaster* genes.

2.3 *Acheta domesticus Fmr1 Sequence Similarity*

After identifying the ortholog for *A. domesticus Fmr1*, we determined the sequence similarity to human *Fmr1* and other current animal models of FXS. We used the BLASTX tool on the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) database to perform species-specific protein alignments against *Homo sapiens*, *Mus musculus*, *Rattus norvegicus, Danio rerio, Xenopus laevis, Drosophila melanogaster, and Gryllus bimaculatus.*

2.4 Cloning of Acheta domesticus Orthologs

In order to clone *A. domesticus Fmr1* and *Lysozyme*, cDNA was synthesized from pooled RNA isolated from four adult cricket brains, using a Bio-Rad iScript Reverse Transcription Kit (Hercules, CA). Cloning primers were designed using CLC-bio Genomics Workbench software to amplify a 200-800 base pair region of the genes (Table 1). These primers were used along with cDNA in a polymerase chain reaction (PCR) to generate a DNA template for cloning. The PCR conditions were as follows: 4 min at 95°C; 35 cycles of 30 sec at 95°C, 30 sec at 58°C, and 40 sec at 72°C; 5 min at 72°C; and a hold at 4°C. The annealing temperature and the extension time were modified for each of the genes to account for the melting temperature of the primers and length of the PCR product. The PCR products were purified from an agarose gel with Freeze 'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad) and then cloned into the PCR4-TOPO vector (PCR4-TOPO-TA Cloning Kit, Invitrogen; Carlsbad, CA). Colony PCR was performed on the transformants and then plasmid DNA was purified using a QIAprep Spin Miniprep Kit (Qiagen; Germantown, MD). Plasmid DNA was sent to Macrogen, Inc. (Rockville, MD) for sequencing. Vector NTI software was used to confirm that the sequences contained the appropriate region for each of the genes before being used in downstream applications.

2.5 Double-Stranded RNA Synthesis

In order to generate double-stranded RNA (dsRNA) for gene-specific knockdown of the *Fmr1* gene, plasmid DNA was purified using a QIAquick PCR Purification Kit (Qiagen). dsRNA was synthesized from the DNA template using a MEGAScript T7 Transcription Kit (Invitrogen) and

then purified using a MEGAclear Transcription Clean-Up Kit (Invitrogen). dsRNA for the gene-specific knockdown of DsRed2 was synthesized from *Discosoma* sp. red fluorescent protein 2 and was a gift from Yoshinori Tomoyasu, Miami University (see Miller et al., 2012). In all experiments, DsRed2 dsRNA was used as the control.

2.6 RNA Interference

Isolated Day 1 adult male and female crickets were weighed immediately prior to injection with dsRNA. Only crickets that fell within the weight range of 0.25-0.35 grams were used. Crickets were anesthetized briefly on ice before receiving a 2μL intra-abdominal injection of 2.5μg Fmr1 (experimental) or DsRed2 (control) dsRNA with a sterile 10μL Hamilton syringe. After injection, crickets were returned to their individual containers and placed into the incubator until their use in downstream assays. In this study, downstream experiments were performed at two time points, 8 days post injection (8dpi; D9 of adulthood) and 20 days post injection (20dpi; D21 of adulthood). Different sets of crickets were used for these two time points in order to avoid serial blood sampling since such sampling has been shown to significantly affect immune parameters (Charles and Killian, 2015). All assays were performed between 12:00h – 18:00h.

2.7 Quantitative Real Time PCR (qPCR)

At 8dpi or 20dpi each cricket injected with dsRNA on D1of adulthood was briefly anesthetized on ice prior to hemolymph and fat body removal. A sterile 25-gauge needle was used to puncture the soft tissue between the left pro- and meso-thoracic legs. A 5μL Drummond microcapillary tube was used to remove between 5-10μL of hemolymph from the puncture site. Hemolymph was dispensed into 400μL of anticoagulant buffer (98mM NaOH, 146mM NaCl, 10mM EDTA, 41mM citric acid, pH 6.5; da Silva et al., 2000) and immediately placed on ice. The hemolymph from 5-8 individual crickets was pooled for every treatment group, so that a large enough concentration of RNA could be isolated for cDNA synthesis.

Immediately after hemolymph collection, the cricket's head was cut off and pinned into a dissecting dish. A small window was cut into the head capsule, and the fat body surrounding the

brain was dissected free and placed into 200μL of cold homogenization buffer (Promega). The fat body from 4-6 individual crickets was pooled for every treatment group. Pooled fat body was homogenized with a disposable pestle and then stored at -80°C until RNA isolation. Pooled hemolymph samples were spun for 10 min at 3,000g in a 4°C centrifuge. The supernatant was removed and 1000μL of cell suspension media (0.9μL 1X PBS/10% BSA, 100μL 0.5M EDTA pH 8) was added to the remaining pellet. Hemolymph samples were centrifuged a second time for 5 min at 3,000g at 4°C. The supernatant was removed and 200μL of cold homogenization buffer (Promega) was added to the pellet. Tubes were vortexed to homogenize the pellet and then stored at -80°C until RNA isolation.

Total RNA was extracted from hemolymph and fat body tissues using a Maxwell 16 LEV simplyRNA Tissue Kit (Promega) in conjunction with an automated Maxwell 16 RNA Isolation system (Promega). After extraction, the concentration and quality of the RNA was determined using a Nanodrop 2000 (Fisher Thermo Science). cDNA was synthesized from a 1μg template (hemolymph) or a 0.8μg template (fat body) using an iScript Reverse Transcription Kit (Bio-Rad) according to the manufacturer's protocol. Forward and reverse primer sets were designed using Integrated DNA Technologies (IDT) software, for Fmr1, Lysozyme, 16s rRNA, and 18s rRNA (Table 2). 16s rRNA (AF514462) and 18s rRNA (AF514506) were used as internal controls for all qPCR assays because they maintain uniform expression throughout development. qPCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) with a CFX Connect Real-Time PCR Detection System (Bio-Rad). The PCR conditions were as follows: 3 min at 95°C; 40 cycles of 15 sec at 95°C, and 10 sec at 60°C; 10 sec at 95°C; 5 sec at 65°C; and a hold at 95°C. The amplification efficiency of each primer set was determined using serially diluted cDNA samples (Table 2). The comparative Ct method was used to determine the relative expression of target genes using CFX Manager software. Each experiment was run in triplicate (three biological replicates, each of which consisted of three technical replicates) and the relative expression for each target gene was determined by taking the mean expression of three independent runs. To determine the variability within a sample set (indicated as a single bar in each histogram), we calculated the standard deviation for all biological and technical replicates within that sample set.

2.8 Hemocyte Counts

At 8dpi or 20dpi crickets injected with dsRNA on D1of adulthood were briefly anesthetized on ice prior to hemolymph removal. A sterile 25-gauge needle was used to puncture the soft cuticle between the left pro- and meso-thoracic legs. A 1μL Drummond microcapillary tube was used to remove exactly 1μL of hemolymph from the puncture site. Hemolymph was dispensed into 12.5μL of anticoagulant buffer, gently mixed, and placed on ice. 10μL of the mixture was pipetted into the counting chamber of a Neubauer hemocytometer and an Olympus U-CA compound microscope was used to count the number of circulating hemocytes for each cricket as previously described (Piñera et al., 2013). All hemocyte counts were performed blindly within 10 min of blood removal.

2.9 Hemolymph Phenoloxidase Enzyme Activity and Protein Content

On 8dpi or 20dpi, crickets injected with dsRNA on D1 of adulthood were anesthetized on ice for 5-7 min, prior to hemolymph removal. A sterile 25-gauge needle was used to puncture the soft cuticle between the left pro- and meso-thoracic legs. A 2μL hemolymph sample was removed by placing a 2μL Drummond microcapillary tube to the puncture site. Hemolymph was dispensed into 98μL of phosphate-buffered saline (PBS, pH 7.4; Sigma; St. Louis, MO), vortexed briefly, and placed onto ice. 50μL of the 100μL hemolymph-PBS mixture was immediately used to determine the total phenoloxidase (PO) enzyme activity in the hemolymph, and the remaining 50μL was stored at -20°C. This 50μL sample was used at a later date to determine the hemolymph protein content.

Total PO enzyme activity in the hemolymph was determined using a previously described protocol (Piñera et al., 2013). Each 50μL sample was transferred into a labeled 1.5mL cuvette and then 70μL of bovine pancreas α-chymotrypsin (1.3mg/mL; Sigma) was added. Samples were vortexed for ~10 sec and then incubated at room temperature for 20 min and then, 600μL of 0.15M 3,4-dihydroxy-L-phenylalanine (L-DOPA; Sigma) was added to each cuvette. The absorbance at 490nm was measured at 0, 8, 15, 23, 30, 45, and 60 min following the addition of

L-DOPA. Due to the light sensitive nature of L-DOPA, the lights were turned off just prior to its addition and remained off for the duration of the experiment. All PO assays were performed on a Beckmann DU530 Spectrophotometer during the hours of 12:00h-18:00h. The total PO activity for each sample was determined by taking the slope of the absorbance during the linear phase of the reaction, which occurs between 8-30 min, and then multiplying this value by 10^3. The total PO activity for each treatment group was reported as the average absorbance during the linear phase of the reaction.

The total protein content in the hemolymph was determined using a standard Bradford assay. 150μL of PBS was added to each thawed 50μL hemolymph sample which was then vortexed briefly and placed on ice. All samples were run in duplicate. In a 96-well microplate, duplicate standards of 0, 1, 1.5, 2, 2.5, 5, 10, and 15μg were made by diluting bovine serum albumin (BSA; Fisher Scientific) with PBS into a total volume of 20μL. 20μL of each experimental hemolymph sample was loaded onto the microplate in duplicate. Finally, 250μL of Bradford Reagent (Sigma) was added to each well before incubating at room temperature for 30 min. The absorbance at 595nm was measured on a Molecular Devices Filter Max Microplate Reader. The absorbances from each duplicate were averaged and this value was divided by the slope of the line generated by the standard curve to determine the protein content of each sample. We accounted for the dilution factor by multiplying the protein content by 10, which gave us our total protein content for each 1μL hemolymph sample.

2.10 Response to an LPS Immune Challenge

Eight days after dsRNA injection (D9 of adulthood), crickets were injected with LPS from *Serratia marcescens* (L6136; Sigma). Stock was prepared under a sterile filter hood using proper aseptic technique to prevent contamination. LPS-*Sm* was resuspended in 1000μL of filtered Grace's Media (Sigma) and slowly mixed before being aliquoted into volumes of 200μL. Aliquots of LPS-*Sm* were stored at -20°C until the time of injection. Stock was removed from the freezer and sonicated for 20 min to ensure that the LPS was resuspended prior to injection. Crickets were anesthetized briefly on ice, before receiving a 6μL intra-abdominal injection of

150μg LPS-SM with a sterile 10μL Hamilton syringe. After injection, crickets were returned to their individual containers and placed into the incubator.

2.10.1 Survival Assay

Male and female crickets were checked every day for a 21-day period following the injection of LPS-*Sm* to determine how well they could respond to this immune challenge. The day of LPS-*Sm* injection was considered day 0 (D0) and 24 h after injection was designated D1 because crickets had been subject to the LPS immune challenge for 24 h. Each day survival was assessed, and dead crickets discarded. Surviving crickets were weighed every 3 days for the entire 21-day period. Six survival assays were performed in total, consisting of crickets from all four treatment groups including: DsRed2-injected (control) males, Fmr1-injected (experimental) males, DsRed2-injected (control) females, and Fmr1-injected (experimental) females. The total number of crickets for each treatment group at the beginning of the survival assay (D0) were as follows: control males (n = 40), KD males (n = 42), control females (n = 41), and KD females (n = 40).

2.10.2 Nodulation Response

Nodulation occurs when bacteria are too large to be phagocytized by an individual hemocyte or when there is a bacterial aggregation in the hemocoel (reviewed in Gillespie et al., 1997). Hemocytes form a multicellular coat around the bacteria that becomes melanized, trapping and killing the foreign invader (reviewed in Gillespie et al., 1997). LPS alone also leads to hemocyte aggregation (Bedick et al., 2000) and nodule formation (Brookman et al., 1989; Goldsworthy et al., 2003). Initially, the nodulation response was examined in crickets that survived to the end of the 21-day period following LPS-*Sm* injection. Since survival decreased significantly for one of our treatment groups, it was difficult to collect a large enough sample size to compare with the other treatment groups. As a result, we decided to examine the nodulation response in a separate group of crickets that were not a part of the survival assay. These crickets were injected with 2.5μg of DsRed2 dsRNA (negative control) or Fmr1 dsRNA (experimental) on Day 1 of adulthood and then injected with 150μg of LPS-*Sm* on Day 9 of adulthood. The number of

nodules were counted 7 days post-LPS injection, instead of 21 days post-LPS injection. This allowed us to count the number of nodules from a large number of crickets representing each of the treatment groups, before there was a significant decrease in survival. In order to count the number of nodules at either time post-injection, crickets were anesthetized briefly on ice before removing their head, wings and appendages. Then a cut was made down the dorsal midline of the thorax and abdomen so that the body could be pinned open in a dissecting dish. We removed the internal organs so that nodules adhering to the left seven abdominal segments of the body wall could be visualized and counted as previously described (Charles and Killian, 2015). Nodules were counted under an Olympus SZ40 dissecting microscope at a magnification of 500x, with all counts performed blindly.

2.11 Response to a Nylon Filament Immune Challenge

Insertion of a nylon filament into the abdomen is a commonly used immune challenge that can measure the encapsulation response of the immune system (Cotter and Wilson, 2002; Siva-Jothy and Thompson, 2002; Bailey and Zuk, 2008; Ardia et al., 2012; Charles and Killian, 2015). In order to determine the response to this immune challenge, we measured encapsulation ability as previously described (Piñera et al., 2013). Nylon filaments were prepared at least 24 h prior to insertion. A nylon thread with a diameter of 0.3mm was roughened with sandpaper, knotted at one end, and then cut 3mm from the knot on the diagonal. Threads were sterilized in ethanol and dried overnight prior to use. Male and female crickets were injected with 2.5μg Fmr1 (experimental) or DsRed2 (control) dsRNA on D1 of adulthood. On D9 (8dpi) and D21 (20dpi) of adulthood, crickets were anesthetized briefly on ice and then a small puncture was made on the right side between the second and third abdominal sternite with a sterile 25-gauge needle. A 3-mm nylon thread was inserted into the puncture site parallel to the anteroposterior axis until the knot rested against the external cuticle of the cricket. Hemolymph that escaped from the insertion site was wicked away to minimize the number of threads that would become dislodged during the experiment. Crickets were returned to their individual containers and placed into the incubator after thread insertion.

24 hr after insertion, the thread was removed by making a small incision in the abdomen. Threads were placed into open 0.5mL tubes and allowed to dry overnight at room temperature. Any thread that did not remain flush against the external cuticle of the cricket during the 24 h period was excluded from the analysis. Fmr1 KD crickets retained their threads as well as did controls; 93% (28/30) of control males retained their threads while 96% (25/26) of Fmr1 KD males retained their threads after 24 hr. For females, 100% (28/28) of controls retained their threads while 97% of KDs (28/29) retained their threads. Uninserted threads served as controls and were processed in the same manner as experimental threads. After drying, threads were stored at -20°C until photographed as previously described (Charles and Killian, 2015). Three different faces of each thread were photographed at 31.5x using a dissecting microscope equipped with a Canon SZX12 camera. To determine the amount of encapsulation that had occurred, ImagePro Plus 6.3 software was used to average the pixel values within a pre-determined rectangular grid (1500 x 200 pixels). This rectangular grid was placed over every image, exactly 300 pixels from the cut end of the thread. A bitmap analysis was then used to determine the pixel value within the grid, and the pixel values from all three faces of each thread were averaged. Finally, the encapsulation score of each thread was determined by subtracting the experimental average from the average pixel value of an uninserted control thread. Larger encapsulation scores correspond to more melanin coating on the nylon filament.

2.12 Statistical Analyses

Student's *t*-tests were performed to determine if there were significant differences ($p < 0.05$) in relative expression between treatment groups for all qPCR assays. We performed three-way factorial ANOVAs to assess the effects of treatment, sex, and time on hemocyte number, total PO enzyme activity, hemolymph protein content, nodulation response, and encapsulation response. Data sets were tested for normal distribution using a Kolmogorov-Smirnov normality test. Hemocyte number, total PO enzyme activity, and hemolymph protein content data sets were not normally distributed, so data were log-transformed prior to analysis of variance. For the survival assay, survival curve analyses were performed using a Log-Rank to determine if there was a significant effect of treatment on survival. Logistic regression analyses were used to

compare survival at individual time points. All statistical analyses were performed using JMP Pro 14 (SAS Institute Inc., Cary, N.C.) software.

3. Results

3.1 Fmr1 is highly conserved across animal models

We identified the *Acheta domesticus* ortholog of *Fmr1* (*AdFmr1*) using a transcriptome generated by RNA-Seq analysis (see section 2.2). Since the fruit fly *Drosophila melanogaster* and the cricket *Gryllus bimaculatus* have both been used as insect models for the study of FXS, we examined the sequence similarity of *AdFmr1* to both models. *Fmr1* is highly conserved across insect species (Table 3), with 76% sequence similarity to *Drosophila melanogaster* (NP_731443.1) and 100% sequence similarity to *Gryllus bimaculatus* (BAG71783.1). Next, we wanted to determine the sequence similarity of *AdFmr1* to other current animal models of FXS including *Mus Musculus*, *Rattus norvegicus*, *Danio rerio*, and *Xenopus laevis*. We found that *Fmr1* is highly conserved across all FXS animal models (Table 3), with 72% or greater sequence similarity. The *AdFmr1* ortholog shared 75% sequence similarity with *Homo sapiens* (NP_001013456.1). Taken together these results support that *Fmr1* is highly conserved across all animal models, including the cricket *Acheta domesticus.*

3.2 Fmr1 is not differentially expressed in the whole body of male and female crickets

We used our assembled transcriptome to perform a differential expression analysis to determine if *Fmr1* expression was sexually dimorphic. First, we examined the Fragments Per Kilobase of transcript per Million reads mapped (FPKM) for D9 adult male and D9 adult female crickets (Table 4). There was not a significant difference in the expression of *Fmr1* in D9 adults ($p = 1$). We also compared the expression of *Fmr1* in D9 adult vs. D1 adult females and males, to see if a sexually mature (D9) cricket would differ from a sexually immature (D1) cricket. *Fmr1* was not differentially expressed in females ($p = 1$) or males ($p = 1$). This suggests that there is not a sex effect or age effect on *Fmr1* expression in *Acheta domesticus*.

3.3 Fmr1 expression is significantly decreased in immune tissues

The expression of Fragile X has been well characterized in the nervous tissue of several animal models, but has only just begun to be examined in immune tissues. To ensure that RNAi would cause a decrease in *Fmr1* expression in two important immune tissues, we characterized *Fmr1* knockdown in the hemolymph and fat body of male and female crickets 8dpi (D9) and 20dpi (D21). The relative expression of *Fmr1* in the hemolymph was determined for both males and females using qPCR (Fig. 1A). The relative expression in KD males was significantly decreased by 59% when compared to age-matched controls ($p = 0.00006$) 8dpi. Similarly, the relative expression of *Fmr1* in the hemolymph was significantly decreased by 56% in KD females when compared to age-matched control females ($p = 0.002$). We also examined *Fmr1* expression in the hemolymph 20dpi. Unlike the earlier timepoint, there was no difference in *Fmr1* expression in males ($p = 0.419$) or females ($p = 0.051$) when compared to their age-matched controls.

We also examined the expression of *Fmr1* in the brain fat bodies of male and female crickets 8dpi and 20dpi (Fig. 1B). Eight days post-injection, the relative expression of *Fmr1* was significantly reduced by 43% in KD males ($p = 0.009$) and by 45% in KD females ($p = 0.001$) when compared to controls. Twenty days post-injection there was a significant increase in *Fmr1* expression in KD males ($p < 0.0001$) when compared to controls, while there was no difference in *Fmr1* expression in the fat body of females 20dpi ($p = 0.802$).

Collectively, these results indicate that both hemolymph and fat body express *Fmr1* and that a single injection of dsRNA significantly reduced the expression of *Fmr1* in both of these immune tissues 8dpi. Additionally, this 2.5μg injection of dsRNA caused a similar decrease in the expression of *Fmr1* in both males and females in each tissue type.

3.4 Effect of decreased Fmr1 expression on number of circulating hemocytes, PO activity, and total hemolymph protein content

We examined the effects of decreased *Fmr1* expression on the total number of circulating hemocytes, total hemolymph PO activity, and hemolymph protein content. To determine if

decreased *Fmr1* expression affected the number of circulating hemocytes, the primary mediators of cellular immunity in insects, we counted the number of hemocytes at 8dpi and 20dpi (Fig. 2A). A three-way factorial ANOVA was conducted on the influence of three independent variables: treatment; sex; and time, on the number of circulating hemocytes. There was not a statistically significant three-way interaction of treatment, sex, and time on the number of circulating hemocytes (Table 5). Additionally, we did not find any significant two-way interactions for treatment and sex, treatment and time, or sex and time (Table 5). However, there was a main effect of treatment ($F_{1,250} = 6.2693$, $p = 0.0129$) on hemocyte number. Fmr1 KD crickets had significantly more circulating hemocytes than controls. Hemocyte number was also impacted by a main effect of sex ($F_{1,250} = 4.2179$, $p = 0.0410$), with females having more hemocytes than males. Taken together, these results support that decreased *Fmr1* expression significantly impacts the number of circulating hemocytes in crickets.

We also examined the effects of decreased *Fmr1* expression on the activity of the immune enzyme phenoloxidase (PO) (Fig. 2B). PO is synthesized by circulating hemocytes and is activated in response to pathogens that may compromise the immune system of the cricket. This enzyme is involved in the humoral defense pathway and is responsible for melanogenesis (reviewed in González-Santoyo and Córdoba-Aguilar, 2012). There was a significant three-way interaction of treatment, sex, and time on PO activity ($F_{1,323} = 4.4251$, $p = 0.0362$). All of the two-way interactions and main effects were also statistically significant (Table 5). Overall, decreased *Fmr1* expression caused a statistically significant decrease in PO activity. *Fmr1* KD males and females had significantly lower total PO activity than their age-matched controls 20dpi. There was also a significant decrease in PO activity over time, as KD males and females had significantly less PO activity 20dpi than KD males and females 8dpi. We also found a main effect of treatment ($F_{1,323} = 76.6735$, $p < 0.0001$), which indicated that controls had significantly higher PO activity than *Fmr1* KD crickets. Sex also had a significant impact on PO activity ($F_{1,323} = 10.7578$, $p = 0.0012$), as females had less PO activity than males. There was also a main effect of time ($F_{1,323} = 152.6320$, $p < 0.0001$), which showed that crickets had significantly higher PO activity 8dpi than 20dpi. These results support that decreased *Fmr1* expression significantly impacts total PO activity in both sexes, and these effects increase over time.

Finally, we determined if decreased *Fmr1* expression could affect the total protein content in hemolymph, as a measure of overall body condition (Fig. 2C). There was not a statistically significant three-way interaction of treatment, sex, and time (Table 5). Additionally, we did not find any significant two-way interactions for treatment and sex or treatment and time (Table 5). We did find a significant interaction of sex and time ($F_{1,327} = 46.4332$, $p < 0.0001$) on total protein content. Interestingly, females had significantly higher protein content 8dpi than 20dpi, while males had significantly higher protein content 20dpi than 8dpi. We found that females had higher protein content than males 8dpi, while males had higher protein content than females 20dpi. There were no main effects of treatment, sex, or time on protein content (Table 5). *Fmr1* KD did not impact the amount of protein in the hemolymph of either sex, suggesting that the body condition of crickets was not affected by decreased *Fmr1* expression.

3.5 Decreased Fmr1 causes a significant increase in Lysozyme expression

When the insect immune system detects invading bacteria, lysozyme is secreted by the fat body into the surrounding hemolymph where it can effectively counteract the bacteria (reviewed in Gillespie et al., 1997). Lysozyme is highly conserved across animal models. We found that *AdLysozyme* shares 73% sequence identity with *Drosophila melanogaster* (AAY85086.1) and 57% sequence identity with *Homo sapiens* (1GFV_A). We determined the effects of decreased *Fmr1* on *Lysozyme* expression in the fat body of males and females 8dpi and 20dpi using qPCR (Fig. 3A). KD males had a significant two-fold increase in *Lysozyme* expression in the fat body when compared to age-matched controls 8dpi ($p = 0.006$). *Lysozyme* expression was also significantly increased in KD females, as shown by a 50% increase in the relative expression ($p = 0.0001$) when compared to controls.

We also examined the relative expression of *Lysozyme* in the fat body 20dpi. KD males had a significant increase in expression when compared to controls ($p = 0.005$). Similarly, KD females also had significantly higher levels of *Lysozyme* in the fat body when compared to age-matched controls ($p = 0.0001$) 20dpi. These significant and sustained increases in the expression of the antimicrobial peptide *Lysozyme* suggest that decreased FMRP may lead to inflammation in males and females (see discussion).

3.6 Response to an LPS immune challenge

We determined if decreased *Fmr1* changed the cricket's ability to effectively respond to an immune challenge by examining long-term survival, body mass, and nodulation response following LPS-*Sm* injection. Survival was assessed by counting the number of surviving crickets every day after LPS-*Sm* injection for a 21-day period (Fig. 4A). A survival curve analysis determined that there was a significant effect of treatment on survival over the 21-day period (χ^2 = 35.45, *df* = 3, $p < 0.0001$). Fmr1 KD males first experienced a significant decrease in survival 9 days post-LPS injection (χ^2 = 6.01, *df* = 1, $p = 0.0142$). At this time point, only 68% of KD males had survived. This decrease in survival continued for KD males and remained significant for the rest of the 21-day period. By the final day of the survival assay only 23% of KD males had survived. In contrast, there was no significant effect on survival of KD females over the course of the entire 21-day period. Overall, experimental males were unable to effectively counteract an LPS immune challenge, while experimental females did not lose their ability to respond to this immune challenge.

Surviving crickets were weighed every 3 days for the entirety of the 21-day period. The mean body mass of KD males and females varied little over time when compared to their age-matched controls (Fig. 4B), indicating that treatment had no effect on body mass.

Finally, we examined the effects of *Fmr1* KD on the nodulation response of male and female crickets injected with LPS-*Sm*. Nodulation occurs when bacteria are too large to be phagocytized by an individual hemocyte or when there is a bacterial aggregation in the hemocoel (reviewed in Gillespie et al., 1997). Hemocytes form a multicellular coat around the bacteria, trapping and killing it (reviewed in Gillespie et al., 1997). LPS is recognized by the pattern recognition receptors on the surface of hemocytes, and stimulates the synthesis of hemolymph proteins that bind LPS (reviewed in Gillespie et al., 1997; Strand, 2008). Once the hemolymph protein Lectin binds LPS, hemocytes are recruited and adhere to the hemolymph proteins, forming a layer around the LPS (reviewed in Gillespie et al., 1997). Nodules were counted in crickets that survived to the end of the 21-day period (Fig. 4C). In another group of crickets we counted the number of nodules 7 days post-LPS injection (Fig. 4C), before the KD males saw a significant

decrease in survival (Fig. 4A). There was not a statistically significant three-way interaction of treatment, sex, and time on nodulation, and we did not find any significant two-way interactions (Table 6). We did find a main effect of treatment ($F_{1,172} = 13.2983$, $p = 0.0004$), which showed that control crickets had significantly more nodules than *Fmr1* KD crickets, and sex ($F_{1,172} = 6.3006$, $p = 0.0130$), which demonstrated that males had significantly fewer nodules than females. We also found a main effect of time ($F_{1,172} = 10.1456$, $p = 0.0017$) indicating that crickets had significantly more nodules 21 days post-LPS injection when compared to the number of nodules 7 days post-LPS injection. These results support that decreased *Fmr1* expression hinders the innate immune system's ability to produce nodules when faced with an LPS immune challenge.

3.7 Response to a nylon filament immune challenge

Encapsulation of a nylon filament is a nonspecific immune response mediated by hemocytes (reviewed in Strand, 2008). When an organism is too large to be phagocytized by a single hemocyte, several layers of hemocytes will adhere to the invader, creating a multi-layered capsule that is then coated with melanin (reviewed in González-Santoyo and Córdoba-Aguilar, 2012). The encapsulation of a novel object, such as a nylon filament, is thought to mimic encapsulation of larger pathogens or parasites (Rantala and Roff, 2007; Smilanich et al., 2009; Ardia et al., 2012). To determine the effects of decreased *Fmr1* expression on the encapsulation ability of adult males and females, crickets were injected with 2.5μg of DsRed2 dsRNA (negative control) or Fmr1 dsRNA (experimental) on Day 1 of adulthood. Nylon filaments were inserted into two separate groups of crickets, 8dpi and 20dpi, and removed 24 h later (Fig. 5). There were no significant three-way or two-way interactions between any variables, and there were no main effects of treatment, sex, or time on the encapsulation response of males and females (Table 7). *Fmr1* KD crickets were able to encapsulate nylon filaments as well as controls, suggesting that decreased *Fmr1* expression does not affect the cricket's ability to encapsulate a foreign object.

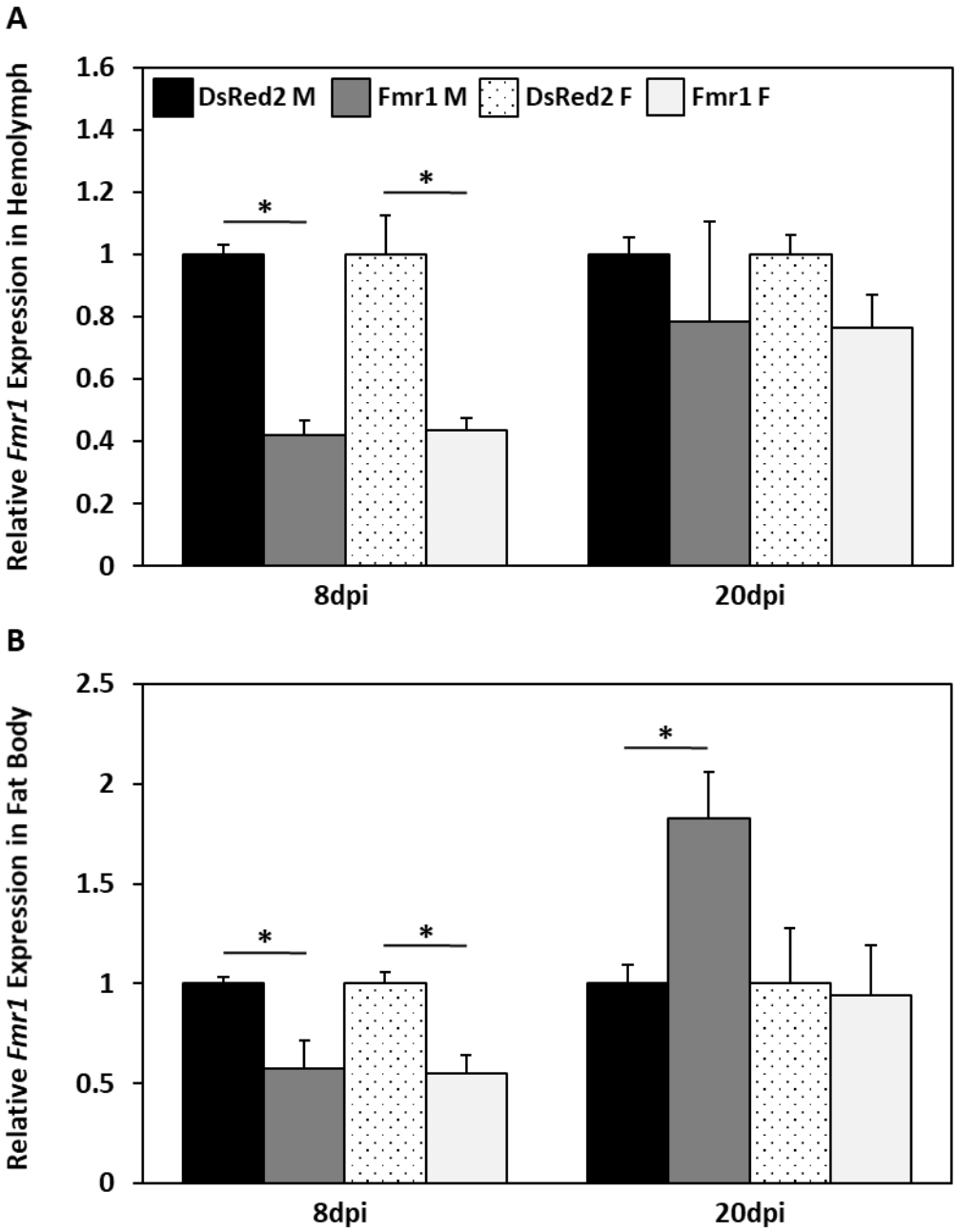
A
Relative Fmr1 Expression in Hemolymph
DsRed2 M
Fmr1 M
DsRed2 F
Fmr1 F
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
8dpi
20dpi
B
Relative Fmr1 Expression in Fat Body
2.5
2
1.5
1
0.5
0
*
*
*
8dpi
20dpi

Figure 1

Figure 1. *Fmr1* expression is significantly reduced in immune tissues. Relative *Fmr1* mRNA expression (mean ± SD) in the hemolymph (A) and fat body (B) of males and females 8 days post injection (8dpi; D9 of adulthood) and 20 days post injection (20dpi; D21 of adulthood) as determined by quantitative Real-Time PCR (qPCR). Crickets were injected with 2.5μg of DsRed2 dsRNA (negative control) or Fmr1 dsRNA (experimental) on Day 1 of adulthood. (A) The relative expression of *Fmr1* in the hemolymph was significantly decreased in both KD males (59%) and females (56%) when compared to age-matched controls 8dpi. There was no significant change in *Fmr1* expression in either sex 20dpi. (B) The relative expression of *Fmr1* in the fat body was reduced in both KD males (43%) and females (45%) when compared to age-matched controls 8dpi. *Fmr1* expression was significantly increased in KD males 20dpi, while females had no change in expression. Student's *t*-test, $p < 0.05$.

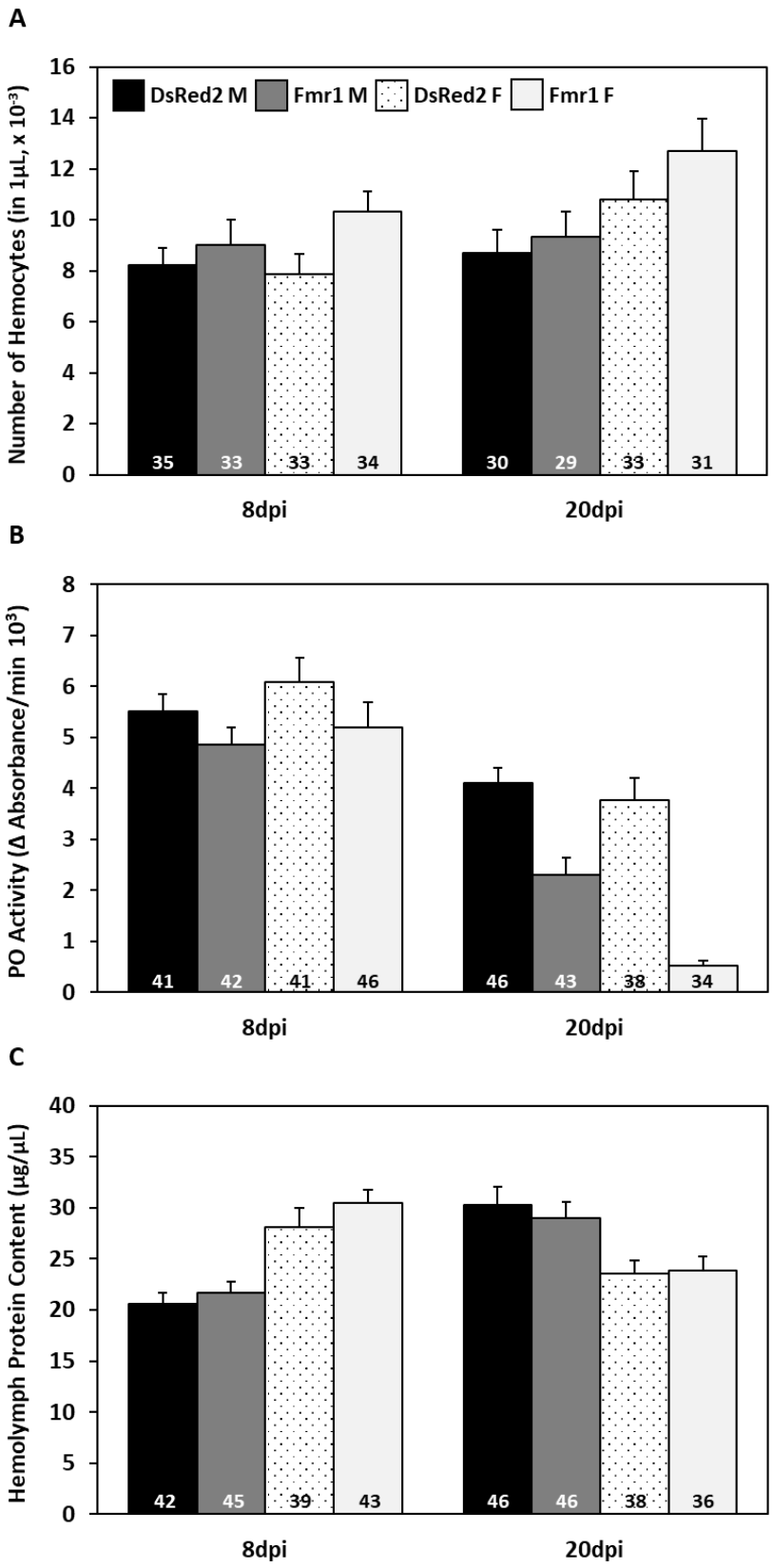
A
DsRed2 M
Fmr1 M
DsRed2 F
Fmr1 F
Number of Hemocytes (in 1µL, x 10⁻³)
16
14
12
10
8
6
4
2
0
35
33
33
34
30
29
33
31
8dpi
20dpi
B
PO Activity (Δ Absorbance/min 10³)
8
7
6
5
4
3
2
1
0
41
42
41
46
46
43
38
34
8dpi
20dpi
C
Hemolymph Protein Content (µg/µL)
40
35
30
25
20
15
10
5
0
42
45
39
43
46
46
38
36
8dpi
20dpi

Figure 2

Figure 2. Decreased *Fmr1* expression impacts immune parameters. Effect of decreased *Fmr1* mRNA expression on the total number of circulating hemocytes (A), total phenoloxidase (PO) activity in the hemolymph (B), and total protein content in the hemolymph (C) in males and females 8dpi and 20dpi. Crickets were injected with 2.5μg of DsRed2 dsRNA (negative control) or Fmr1 dsRNA (experimental) on Day 1 of adulthood. A three-way multifactorial analysis of variance was conducted to assess the effects of treatment, sex, and time on the number of hemocytes, PO activity, and protein content. *P*-values for the significance of main and interactive effects are shown in Table 5. Bars represent the mean ± SEM and the numbers within bars indicate sample sizes for each treatment group.

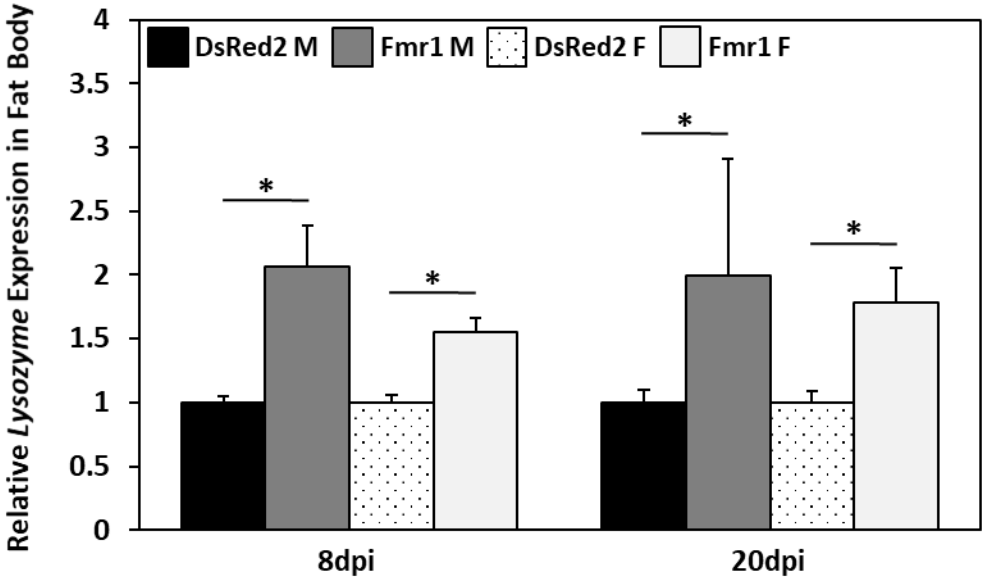
Relative Lysozyme Expression in Fat Body
DsRed2 M
Fmr1 M
DsRed2 F
Fmr1 F
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
*
8dpi
20dpi

Figure 3

Figure 3. *Lysozyme* expression is significantly increased in the fat body. Effect of decreased *Fmr1* on the relative expression (mean ± SD) of *Lysozyme* in the fat body of males and females 8dpi and 20dpi as determined by quantitative Real-Time PCR (qPCR). The relative expression of *Lysozyme* in the fat body was significantly increased following *Fmr1* KD in both males ($p = 0.006$) and females ($p = 0.0001$) when compared to controls 8dpi. There was also a significant increase in *Lysozyme* expression at 20dpi in KD males ($p = 0.005$) and females ($p = 0.0001$). Student's *t*-test, $p < 0.05$.

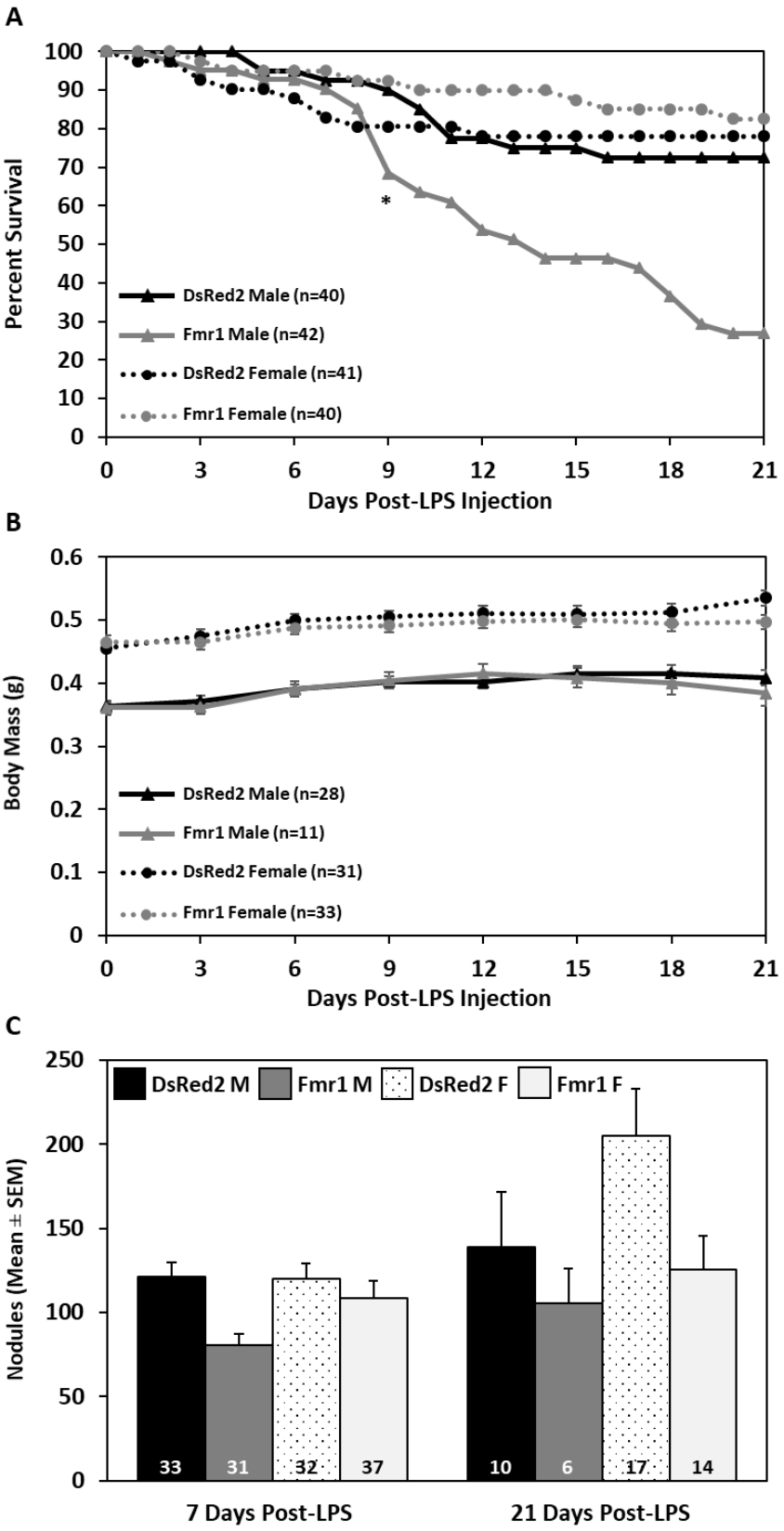
A
Percent Survival
100
90
80
70
60
50
40
30
20
10
0
*
DsRed2 Male (n=40)
Fmr1 Male (n=42)
DsRed2 Female (n=41)
Fmr1 Female (n=40)
0
3
6
9
12
15
18
21
Days Post-LPS Injection
B
Body Mass (g)
0.6
0.5
0.4
0.3
0.2
0.1
0
DsRed2 Male (n=28)
Fmr1 Male (n=11)
DsRed2 Female (n=31)
Fmr1 Female (n=33)
0
3
6
9
12
15
18
21
Days Post-LPS Injection
C
Nodules (Mean ± SEM)
250
200
150
100
50
0
DsRed2 M
Fmr1 M
DsRed2 F
Fmr1 F
33
31
32
37
10
6
17
14
7 Days Post-LPS
21 Days Post-LPS

Figure 4

Figure 4. Response to an LPS immune challenge. Crickets were injected with 2.5μg of DsRed2 dsRNA (negative control) or Fmr1 dsRNA (experimental) on Day 1 of adulthood and then injected with 150μg of LPS-*Sm* on Day 9 of adulthood (A-D). Survival and body mass were measured over a 21-day period (A-B). The number of nodules were counted 7 days and 21 days (C) post LPS-*Sm* injection. (A) Percent of crickets surviving each day over a 21-day period. Fmr1 males first experienced a significant decrease in survival 9 days post LPS-injection (asterisk). This decrease in survival continued for the remainder of the 21-day period. (B) Mean body mass of crickets surviving the entire 21-day period. The body mass of Fmr1 males and females changed little over time when compared to age-matched DsRed2 controls. Initial sample sizes for both A and B are as follows: DsRed2 male (n = 40), Fmr1 male (n = 42), DsRed2 female (n = 41), Fmr1 female (n = 40). Sample sizes of crickets that survived the entire 21-day period are as follows: DsRed2 male (n = 28), Fmr1 male (n = 11), DsRed2 female (n = 31), Fmr1 female (n = 33). (C) Mean number of nodules counted 7 days and 21 days post LPS-SM injection. A three-way multifactorial analysis of variance was conducted to assess the effects of treatment, sex, and time on the number of nodules. *P*-values for the significance of main and interactive effects are shown in Table 6. Bars represent the mean ± SEM and numbers within bars indicate sample sizes for each treatment group.

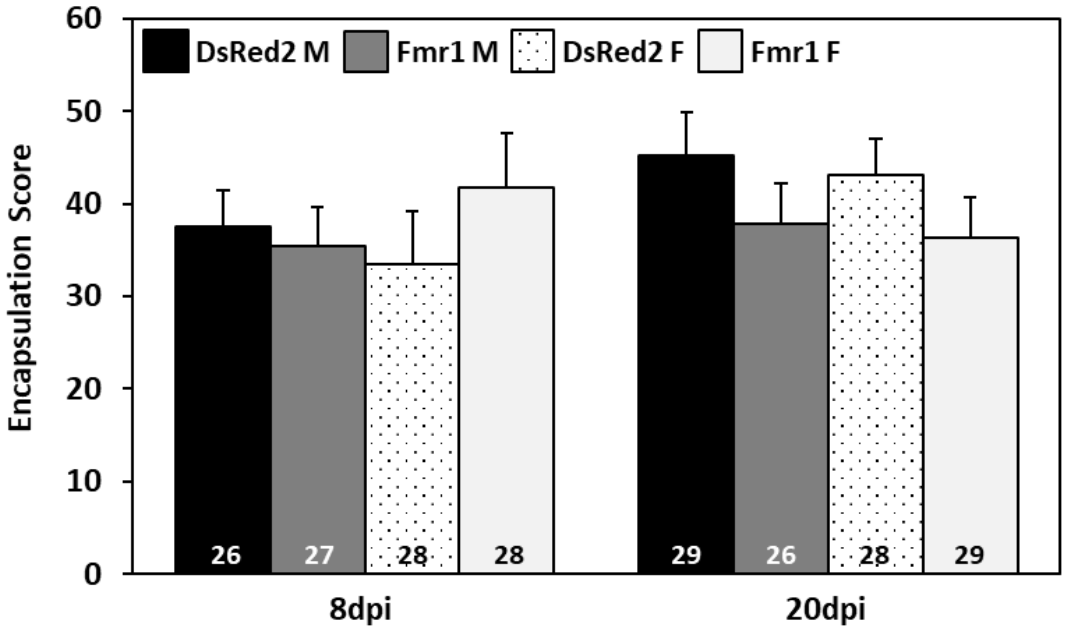
60
50
40
30
20
10
0
Encapsulation Score
DsRed2 M
Fmr1 M
DsRed2 F
Fmr1 F
26
27
28
28
29
26
28
29
8dpi
20dpi

Figure 5

Figure 5. Effect of decreased *Fmr1* on the encapsulation ability of males and females. Crickets were injected with 2.5μg of DsRed2 dsRNA (negative control) or Fmr1 dsRNA (experimental) on Day 1 of adulthood. Nylon filaments were inserted 8dpi and 20dpi and removed 24 h later. A three-way multifactorial analysis of variance was conducted to assess the effects of treatment, sex, and time on encapsulation. Decreased *Fmr1* expression did not affect the encapsulation score of males or females at either time point. *P*-values for the significance of main and interactive effects are shown in Table 7. Each bar represents the mean ± SEM and the numbers within bars indicate sample sizes for each treatment group.

Table 1. Primers used for cloning.

Primer	Sequence	Amplicon
Fmr1 F	TGTGGTGGAATATTTAGGTTGG	716 bp
Fmr1 R	GAGGGATTGTGGGTTGTG	
Lysozyme F	GGTATGGCAGTAAACATAG	315 bp
Lysozyme R	GGCAAAACAAGGACGGAA	

Table 2. Primers used for qPCR analysis.

Primer	Sequence	Amplicon	Efficiency
16s rRNA F	CTTCTCGTCCCCTATTTTCATTTG	150 bp	95.1%
16s rRNA R	GGTATATTGACCGTGCAAAGG		
18s rRNA F	TGTTTTCCTTGCTCGAGAGG	112 bp	96.1%
18s rRNA R	AATGATTCCCGCTTACTAGGC		
Fmr1 F	CTCATGGGTTTGGCAATTGG	96 bp	100.9%
Fmr1 R	GCACGAGTTTTCTTCCAGTTC		
Lysozyme F	TGCTCTTCCAACCATTCCAG	149 bp	88.0%
Lysozyme R	TCGATACTGGTGTGGCATTG		

Table 3. Percent similarity to the amino acid sequence of *Acheta domesticus Fmr1*.

Species	Common Name	Accession Number	Percent Similarity
Homo sapiens	Human	NP_001013456.1	75
Mus Musculus	Mouse	NP_032079.1	75
Rattus norvegicus	Rat	NP_001012179.1	72
Danio rerio	Zebrafish	NP_958458.2	74
Xenopus laevis	Frog	AAC59682.1	72
Drosophila melanogaster	Fruit Fly	NP_731443.1	76
Gryllus bimaculatus	Cricket	BAG71783.1	100

Table 4. Results of differential expression analysis.

Gene ID	Sample	FPKM	Sample	FPKM	Adjusted *p* Value
c115825_g1_i2	D9 Male	326.4342792	D9 Female	354.2456143	1
c115825_g1_i2	D9 Female	18.66283251	D1 Female	24.20779288	1
c115825_g1_i2	D9 Male	14.75945157	D1 Male	21.72254892	1

Table 5. Three-way analysis of variance assessing the effects of treatment, sex, and time on hemocyte number, PO activity, and protein content.

Source of Variation	d.f.	F Ratio	*P* Value
Hemocyte Number			
Treatment	1, 250	6.2693	**0.0129**
Sex	1, 250	4.2179	**0.0410**
Time	1, 250	3.7939	0.0526
Treatment x Sex	1, 250	1.8306	0.1773
Treatment x Time	1, 250	0.6820	0.4097
Sex x Time	1, 250	2.6886	0.1023
Treatment x Time x Sex	1, 250	0.9277	0.3364
PO Activity			
Treatment	1, 323	76.6735	**<.0001**
Sex	1, 323	10.7578	**0.0012**
Time	1, 323	152.6320	**<.0001**
Treatment x Sex	1, 323	5.7896	**0.0167**
Treatment x Time	1, 323	36.2339	**<.0001**
Sex x Time	1, 323	14.7805	**0.0001**
Treatment x Time x Sex	1, 323	4.4251	**0.0362**
Protein Content			
Treatment	1, 327	0.8569	0.3553
Sex	1, 327	1.9333	0.1653
Time	1, 327	1.7406	0.1880
Treatment x Sex	1, 327	0.4058	0.5246
Treatment x Time	1, 327	1.7144	0.1913
Sex x Time	1, 327	46.4332	**<0.0001**
Treatment x Time x Sex	1, 327	0.0442	0.8335

Significant effects ($P < 0.05$) are shown in bold. d.f., degrees of freedom

Table 6. Three-way analysis of variance assessing the effects of treatment, sex, and time on nodule number.

Source of Variation	d.f.	F Ratio	*P* Value
Treatment	1, 172	13.2983	**0.0004**
Sex	1, 172	6.3006	**0.0130**
Time	1, 172	10.1456	**0.0017**
Treatment x Sex	1, 172	0.1361	0.7127
Treatment x Time	1, 172	1.7791	0.1840
Sex x Time	1, 172	1.7577	0.1867
Treatment x Time x Sex	1, 172	2.7905	0.0966

Significant effects ($P < 0.05$) are shown in bold. d.f., degrees of freedom

Table 7. Three-way analysis of variance assessing the effects of treatment, sex, and time on encapsulation ability.

Source of Variation	d.f.	F Ratio	*P* Value
Treatment	1, 213	0.3520	0.5536
Sex	1, 213	0.0111	0.9162
Time	1, 213	1.1758	0.2794
Treatment x Sex	1, 213	0.6832	0.4094
Treatment x Time	1, 213	2.3171	0.1294
Sex x Time	1, 213	0.1972	0.6574
Treatment x Time x Sex	1, 213	0.5148	0.4739

Significant effects ($P < 0.05$) are shown in bold. d.f., degrees of freedom

4. Discussion

In the present study, we have examined the effects of decreased *Fmr1* expression on the innate immune system of a novel insect model of Fragile X. We used RNAi to suppress *Fmr1* expression in adult male and female *Acheta domesticus* crickets, and then examined the impact on multiple measures of immune function. Research conducted on Fragile X premutation carriers and patients with autism spectrum disorders (ASD) has shown that individuals have altered expression levels of immune genes (Careaga et al., 2014; reviewed in Estes and McAllister 2015). Specifically, patients had increased levels of the pro-inflammatory cytokines interleukin 1 β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 12 p40 (IL12p40), and granulocyte-macrophage colony stimulating factor in the blood, while the levels of anti-inflammatory cytokines such as interleukin 10 (IL-10), interleukin 12 (IL-12) and transforming growth factor-β (TGFβ) were decreased (Careaga et al., 2014; reviewed in Estes and McAllister, 2015). In addition to altered cytokine levels, individuals also had dysfunctional immune cells and exhibited impaired responses to immune challenges (Warren et al., 1986; Warren et al., 1987; Enstrom et al., 2010; Ashwood et al., 2011). To date, there has only been a single study to directly investigate the effects of decreased *Fmr1* on immune parameters in an animal model of FXS (see O'Connor et al., 2017). In that study, O'Connor et al. (2017) found that RNAi-mediated knockdown of *Fmr1* in the hemocytes of male *Drosophila* flies caused a defect in the phagocytic activity of hemocytes, which made the flies highly susceptible to bacterial infections. Furthermore, this is the first study to explore the idea that changes in *Fmr1* expression may have differential effects on the immune system based upon sex.

Since the development of the first animal model of FXS (reviewed in Drozd et al., 2018) in the early 1990's, our understanding of the disease has progressed rapidly. While most FXS research has relied on vertebrate models such as the mouse, rat, and zebrafish, the fruit fly *Drosophila melanogaster* has proved to be an invaluable model for the study of FXS. The *Fmr1* homolog was identified in *Drosophila* in 2000 (Wan et al., 2000) and since then, several studies have used this insect FXS model to examine everything from neural physiology to social behavior (reviewed in Drozd et al., 2018). In addition to the fruit fly, the cricket *Gryllus bimaculatus* has been used to study FXS, but its use as a model organism was limited to a single study examining

the impact of decreased *Fmr1* expression on calling song and circadian locomotor rhythm (Hamada et al., 2009). We identified the *Fmr1* ortholog in *Acheta domesticus* and found that it was highly homologous to other insect species. As with insects, *AdFmr1* was also highly homologous to all vertebrate models of FXS including *Mus musculus*, *Rattus norvegicus, Danio rerio*, and *Xenopus laevis*. Finally, we examined the sequence similarity of *AdFmr1* to human *Fmr1* and found that it was also highly conserved, suggesting that the cricket *Acheta domesticus* is a promising new model for the study of FXS.

We used RNAi to decrease *Fmr1* expression in adult male and female crickets. Crickets are particularly suitable for RNAi studies because a single injection of dsRNA initiates systemic silencing of a target gene, which can last for more than two months (Moriyama et al., 2008; Tomioka et al., 2009). To ensure that RNAi would suppress *Fmr1* expression in immune tissues, we characterized the knockdown of *Fmr1* in the hemolymph and fat body of males and females. We examined the hemolymph because circulating hemocytes are the primary mediators of cellular immunity (reviewed in Gillespie et al., 1997). Hemocytes help to recognize invading pathogens as foreign, and then prevent the growth and reproduction of the pathogen by phagocytosis, encapsulation, or nodulation (reviewed in Gillespie et al., 1997). We also examined the fat body because it plays an integral role in insect immunity. The fat body is an organ unique to arthropods, that functions similarly to the vertebrate liver (reviewed in Gillespie et al., 1997; Arrese and Soulages, 2010). It is responsible for the synthesis of antimicrobial peptides, which are secreted into the surrounding hemolymph when the immune system detects foreign molecules (reviewed in Gillespie et al., 1997). Here, we demonstrated that RNAi against *AdFmr1* significantly reduced mRNA levels in the hemolymph and fat body of male and female crickets 8dpi. While this knockdown was effective, it was not long-lasting because *Fmr1* mRNA had returned to control levels by 20dpi. This finding was not surprising since numerous studies have shown that the effects of RNAi are insect specific, tissue specific, and even gene specific (reviewed in Belles, 2010; Cooper et al., 2018; Vogel et al., 2019). For example, in the fruit fly *Drosophila melanogaster* nearly every tissue is insensitive to RNAi except for the hemocytes, which are highly susceptible to RNAi (reviewed in Vogel et al., 2019). Differences in tissue permeability have also been demonstrated in the roundworm *Caenorhabditis elegans*. Kennedy et al. (2004) found that RNAi worked efficiently in most tissues of the roundworm, while the

gonads and neurons were highly refractory to RNAi. It was determined that the gonads and neurons expressed a nuclease that degraded the dsRNA before it could be processed by the RNAi machinery, making these tissues less susceptible to RNAi. Several factors contribute to the variable efficiency of RNAi seen in insects including: stability of dsRNA, cellular uptake of dsRNA, expression of core RNAi machinery components, and the amplification and spreading of RNAi (reviewed in Cooper et al., 2018). It would be interesting to examine more time points between 8dpi and 20dpi to determine exactly how long *Fmr1* KD lasts in the hemolymph and fat body.

Interestingly, we saw a significant increase in *Fmr1* expression in the fat body of KD males 20dpi. Similar to our findings, mRNA levels also increased in the red flour beetle *Tribolium castaneum* following RNAi against genes encoding transcription factors (Wang and Tomoyasu, personal communication). Western blot analysis confirmed that protein levels were decreased, suggesting that feedback mechanisms may be able to counteract decreased mRNA levels with higher rates of transcription. In support of such a mechanism, an RNAi study in the German cockroach *Blattella germanica* showed quick recovery of mRNA levels in the fat body following abdominal dsRNA injection (Ciudad et al., 2007). It would be interesting to examine more time points past 20dpi to see if *Fmr1* expression remains elevated in KD males.

While RNAi significantly decreases *Fmr1* gene expression in the cricket, it does not halt expression altogether, as in a knockout model such as the *Drosophila* Fmr1 mutants used by O'Connor et al. (2017). Therefore, the observed effects on the immune system in this study are the result of moderate suppression of *Fmr1*. Systemic RNAi has been used to examine the effects of decreased *Fmr1* expression in the insect *Gryllus bimaculatus* (Hamada et al., 2009). In that study, RNAi caused a 59% reduction in *Fmr1* expression and led to significant changes in morphology, social behavior, and motor function (Hamada et al., 2009). KD *G. bimaculatus* crickets exhibited defects in wing posture, had abnormal calling songs, and experienced loss of circadian locomotor rhythm (Hamada et al., 2009).

We examined the effects of decreased *Fmr1* expression on innate immunity by counting the number of circulating hemocytes in the blood. As the primary mediators of cellular immunity,

hemocytes help to recognize invading pathogens as foreign (reviewed in Gillespie et al., 1997). Depending upon the size of the pathogen, hemocytes will then phagocytize, nodulate, or encapsulate the invader to prevent its growth and reproduction (reviewed in Gillespie et al., 1997). The number of hemocytes was significantly increased in male and female *Fmr1* KD crickets. There has only been one other study to date to examine the effects of decreased *Fmr1* on the immune system of an animal model of FX. O'Connor et al. (2017) found that RNAi-mediated knockdown of *Fmr1* in the hemocytes of male *Drosophila* flies caused a defect in the phagocytic activity of hemocytes, which made the flies highly susceptible to bacterial infections. Unlike our findings, knockout of *Fmr1* did not alter the number of hemocytes in flies (O'Connor et al., 2017). One explanation for the differences observed could be the method of RNAi used to induce *Fmr1* knockdown. In our study, we used systemic RNAi which potentially knocks down expression in every tissue that takes up dsRNA, while O'Conner et al. (2017) used a GAL-4 driver to target RNAi specifically to hemocytes.

We also examined the effects of decreased *Fmr1* expression on the activity of the immune enzyme phenoloxidase (PO) in the hemolymph of adult crickets. PO is found as the inactive zymogen prophenoloxidase (PPO) in the hemocytes and plasma of insects until it is cleaved into its active form (reviewed in González-Santoyo and Córdoba-Aguilar, 2012; Satyavathi et al., 2014; Dubovskiy et al., 2016). Once activated, PO converts phenols to quinones in a complex cascade of events that results in the production of melanin, which is used to coat the invader, to prevent their growth and reproduction (reviewed in González-Santoyo and Córdoba-Aguilar, 2012; Satyavathi et al., 2014; Dubovskiy et al., 2016). Overall, decreased *Fmr1* expression caused a statistically significant decrease in PO activity in male and female crickets. It was surprising to find a decrease in PO activity since we saw an increase in the number of circulating hemocytes, even though hemocytes are the primary site of proPO synthesis and secretion (reviewed in Gillespie et al., 1997; González-Santoyo and Córdoba-Aguilar, 2012). Similar to our findings, Charles and Killian (2015) found that the number of circulating hemocytes in *Acheta domesticus* crickets were similar between adult females and nymphs, but the PO activity differed between the two groups. There was also no correlation between hemocyte number and PO activity in the honeybee *Apis mellifera* (Rich et al., 2008). These findings suggest that PO activity and hemocyte number are not correlated in insects.

We also assessed the impact of *Fmr1* KD on the expression of *Lysozyme*, an antimicrobial peptide secreted by the fat body when the immune system detects foreign microbes (reviewed in Gillespie et al., 1997). Lysozyme kills bacteria by cleaving the β-(1,4)-glycosidic bonds between the N-acetylmuramic acid and N-acetylglucosamine residues that comprise the peptidoglycan layer of the bacterial cell wall (reviewed in Callewaert and Michiels, 2010). Here, we report a significant increase in *Lysozyme* expression in *Fmr1* KD males and females. In agreement with our findings, significantly increased levels of lysozyme in the blood have been reported in patients with autism spectrum disorders (Montiel et al., 2018). This increase in blood lysozyme levels may be associated with the inflammation and leaky gut that is prevalent in ASD patients (Samsam et al., 2014). Our results suggest that decreased *Fmr1* may lead to inflammation in the cricket. In support of this, a previous study in *Acheta domesticus* also showed that crickets with inflammation had increased lysozyme-like activity accompanied by an increase in the number of hemocytes and decreased PO activity (Charles and Killian, 2015). Although the primary function of lysozyme is antibacterial defense, previous studies in porcine (Lee et al., 2009) and murine (Wang et al., 2017) models have shown that lysozyme plays an important role in reducing intestinal inflammation. Therefore, *Lysozyme* levels may be elevated in the cricket to ameliorate inflammation. In order to test this theory, future experiments will determine if an *Fmr1* KD is sufficient to cause nodule formation in the cricket, which would support that *Fmr1* is inducing inflammation.

After determining that decreased levels of *Fmr1* influenced several key immune parameters, we examined the response of the innate immune system to a challenge with bacterial LPS derived from *Serratia marcescens*. LPS is the primary component of the outer cell membrane of Gram-negative bacteria and is recognized by the pattern recognition receptors located on the surface of hemocytes (reviewed in Gillespie et al., 1997; Strand et al., 2008). Hemocyte recognition initiates the synthesis of hemolymph proteins that bind to LPS and stimulate hemocyte aggregation, activating the proPO cascade (reviewed in Gillespie et al., 1997; Strand et al., 2008). We found that *Fmr1* KD males were not able to survive an LPS-*Sm* immune challenge as well as *Fmr1* KD females. KD males had decreased survival starting 9 days post-LPS injection and continued to die for the remainder of the experiment, while the KD females survived as well as controls, and never saw a significant decrease in survival. What could account for such a

drastic difference in survival between *Fmr1* KD males and females when decreased *Fmr1* expression affected their immune parameters (number of circulating hemocytes, PO activity, *Lysozyme* expression) similarly? Perhaps this difference in survival could be attributed to differences in nodulation.

We examined the effects of *Fmr1 KD* on the nodulation response of male and female crickets injected with LPS-*Sm*. Nodules are multi-cellular hemocytic aggregates that are coated with melanin, following activation of the proPO cascade (reviewed in González-Santoyo and Córdoba-Aguilar, 2012). Our results, and the results of others (Ratcliffe et al., 1991; Bedick et al., 2000; Charles and Killian, 2015) show that LPS injection can induce nodule formation *in vivo*. KD crickets were not able to nodulate as well as controls, suggesting that decreased *Fmr1* expression hinders the cricket's ability to produce nodules when faced with this immune challenge.

While we did not find an interaction between treatment and sex for most of the immune parameters tested, we found an effect of sex on several immune parameters (number of circulating hemocytes, nodulation response). This sex effect may account for the drastic difference in survival between males and females when faced with an LPS-*Sm* challenge. We found that females had significantly more hemocytes than males. In agreement with our findings, females have also been found to have more circulating hemocytes than males in several insect species including monarch butterflies (Lindsey and Altizer, 2009), scorpionflies (Kurtz et al., 2000), damselflies (Robb and Forbes, 2006), and crickets (da Silva et al., 2000). Studies have also shown that the number of circulating hemocytes are positively correlated with immunocompetence (Eslin and Prevost, 1998). We also found that females produced significantly more nodules than males in response to an immune challenge. Many insect studies have demonstrated that females are better able to respond to immune challenges than males. For example, female *Gryllus texensis* crickets had a higher survival rate than males when challenged with an injection of *Serratia marcescens* (Adamo, 2004). Female *Acheta domesticus* crickets were also more resistant than males when infected with *Serratia liquefaciens* (Gray, 1998). When challenged with a nylon filament, female bumblebees (Baer and Schmid-Hempel, 2006) and *Gryllus firmus* crickets (Rantala and Roff, 2006) were able to encapsulate the thread better

than males. This difference in immunocompetence between males and females can be attributed to differing life histories. Females tend to invest more energy in immunity because their fitness is maximized when they increase their life span and rear more offspring (Andersson, 1994; Moore and Wilson, 2002). It has been proposed that the same strategy is not utilized by males, since male fitness can be maximized by increased mating (Andersson, 1994; Moore and Wilson, 2002).

We also inserted a nylon filament into the abdomen of crickets to assess their ability to encapsulate a foreign object. Encapsulation of a nylon filament is a nonspecific immune response mediated by hemocytes (reviewed in Strand, 2008). When an organism is too large to be phagocytized by a single hemocyte, several layers of hemocytes will adhere to the invader, creating a multi-layered capsule that is then coated with melanin (reviewed in González-Santoyo and Córdoba-Aguilar, 2012). The encapsulation of a novel object, such as a nylon filament is thought to mimic encapsulation of larger pathogens or parasites (Rantala and Roff, 2007; Smilanich et al., 2009; Ardia et al., 2012). Surprisingly, experimental males and females were able to encapsulate just as well as controls at both time points examined. Additionally, there was no difference in the encapsulation ability between males and females. Similar to our findings, male and female damselflies of the same age and mass did not differ in their immune response to melanotic encapsulation of a foreign object (Yourth et al., 2002). Interestingly, *Fmr1* KD males were able to encapsulate a foreign object just as well as controls and females, but were not able to survive a challenge with LPS-*Sm*. Why would *Fmr1* KD have a differential effect on these challenges?

Encapsulation of a foreign object and nodulation of LPS-*Sm* both use melanin, however, the proPO cascade is activated differently by these challenges. LPS is recognized by the pattern recognition receptors (PRR) located on the surface of hemocytes (reviewed in Gillespie et al., 1997; Strand et al., 2008). Hemocyte recognition initiates the synthesis of hemolymph proteins that stimulate hemocyte aggregation and bind to LPS, activating the proPO cascade (reviewed in Gillespie et al., 1997; Strand et al., 2008). Unlike LPS, a nylon filament is not recognized by PRR on hemocytes. Instead, the insertion of a nylon filament causes hemocyte lysis, which releases proPO into the hemolymph, activating the PO signaling cascade (reviewed in Gillespie

et al., 1997; Strand et al., 2008). Since nodulation was affected by decreased *Fmr1* while encapsulation was not, *Fmr1* may play a role in one of the initial steps of this process, prior to activation of the proPO cascade.

FMRP is an RNA-binding protein with hundreds of known mRNA targets (Suhl et al., 2014). Dysregulation of these mRNA targets causes the phenotypes that are associated with FXS and ASD. A recent study by Suhl et al. (2014) used datasets from four large-scale studies to elucidate potential FMRP targets. Among these targets was JAK1, a tyrosine kinase protein that is essential for cytokine signaling. Cytokines are essential components of the insect immune system and have been shown to modulate both cellular and humoral responses in *Drosophila* (Oda et al., 2010). It is thus possible that the mis-regulation of this target, or the mis-regulation of multiple mRNA targets may be acting in concert to affect the insect immune response. Additionally, the main substrate of the proPO signaling cascade is tyrosine (reviewed in González-Santoyo and Córdoba-Aguilar, 2012), and the mis-regulation of JAK1 could affect the phosphorylation of tyrosine. Additional research is needed to explore the idea that JAK1 may play an important role in altering the insect immune response following knockdown of *Fmr1*.

In summary, our study has shown that changes in *Fmr1* expression can have a significant impact on the cellular and humoral defense mechanisms of the innate immune system. While males and females exhibited similar changes in immune parameters as a result of decreased *Fmr1*, there were sex-specific differences in their ability to respond to an immune challenge. This novel finding supports the idea that sex is an important factor to consider when diagnosing and treating children with FXS and other FX-related disorders. Moving forward, the cricket *Acheta domesticus* can continue to be an excellent animal model for the study of FMRP and its role in immunity, neurophysiology, and behavior.

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Chapter 3: Decreased *Fmr1* expression during adulthood affects agonistic behavior of male *Acheta domesticus* crickets

1. Introduction

Fragile X syndrome (FXS) is the most common form of inherited mental disability and is the primary monogenetic cause of autism spectrum disorders (ASD), accounting for 30% of all known cases (Bassell and Warren, 2008; Wang et al., 2010). FXS occurs when there is an expansion of CGG trinucleotide repeats in the promoter region of the Fragile X Mental Retardation 1 (*FMR1*) gene (reviewed in Bhakar et al., 2012). When the number of CGG repeats exceeds 200, there is a complete loss of FMRP which results in the FXS disease state (Li and Zhao, 2014). The most common features of FXS include craniofacial abnormalities, growth abnormalities, deficits in learning and cognition, hyperactivity, attention deficit, shyness, social and language deficits, repetitive body movements and behaviors, motor incoordination, and impaired sensory processing (Hagerman, 2002; Baranek et al., 2005; Reiss and Hall, 2007; Bailey et al., 2008; Bhakar et al., 2012).

FMRP is expressed in nearly every cell type, but it is most highly expressed in neurons (Antar and Bassell, 2003; reviewed in Drozd et al., 2018), and functions as a mRNA binding protein that inhibits protein synthesis when it is produced at normal levels (Jin and Warren, 2000). It is important for the regulation of mRNAs involved in synapse growth and function, and inhibition of FMRP leads to increased protein synthesis within synapses (Bassell and Warren, 2008; Bhakar et al., 2012). This deficit in FMRP disrupts normal synaptic development and function, thereby reducing plasticity (reviewed in Bhakar et al., 2012; Contractor et al., 2015). Mutations in *Fmr1* can also impact the function of neural stem cells (NSC), causing decreased adult neurogenesis (Guo et al., 2011), which leads to impaired learning and memory.

As a result of these neural deficits, patients with FXS and ASD exhibit impaired social behaviors (reviewed in Bhakar et al., 2012). Studies in animal models of FXS have also shown that deficiencies in *Fmr1* lead to a wide variety of impaired social behaviors in rats (Hamilton et al., 2014), zebrafish (Wu et al., 2017), tadpoles (Truszkowski et al., 2016), fruit flies (Bolduc et al., 2010), and crickets (Hamada et al., 2009). For example, a recent study by Truszkowski et al. (2016) examined the effects of FMRP knockdown on the schooling behavior of *Xenopus laevis* tadpoles. These animals typically participate in coordinated group swimming behavior that has

been well characterized. Upon FMRP knockdown, the normal schooling pattern of unidirectional and parallel swimming is lost, suggesting that changes in FMRP cause impaired social behavior (Truszkowski et al., 2016).

Most FXS studies have focused on the effects of FMRP when it is suppressed during development. However, we previously determined that decreasing *Fmr1* expression during adulthood can have a significant and sustained effect on the immune system of *Acheta domesticus* crickets (see Chapter 2). Within eight days of decreased *Fmr1* expression we saw an increase in the number of circulating hemocytes, decreased total phenoloxidase activity, and increased *lysozyme* expression. In addition to altered immune parameters, individuals also exhibited impaired responses to immune challenges. Additionally, a study in *Gryllus bimaculatus* used systemic RNA interference (RNAi) to knockdown *Fmr1* expression during the 7th nymphal instar, just one instar prior to the adult molt (Hamada et al., 2009). They found that the average pulse duration of the calling song was significantly shorter in adult knockdown males (Hamada et al., 2009). Taken together, these studies suggest that both the immune system and behavior can be affected by decreased *Fmr1* expression during adulthood.

In the present study, we used the cricket *Acheta domesticus* to examine the effects of decreased *Fmr1* during adulthood on male social behavior. The cricket lends itself to this type of study because agonistic behavior has been well characterized in the cricket (Adamo and Hoy, 1995; Stevenson et al., 2000; Hofmann and Schildberger, 2001), and follows a stereotyped sequence of increasingly aggressive motor acts. Agonistic behavior between conspecific males is triggered by chemosensory antennal contact and will continue until establishment of social rank (Adamo and Hoy, 1995; Stevenson et al., 2000; Hofmann and Schildberger, 2001). After establishment, both crickets will exhibit distinct behaviors that are indicative of their social status. The dominant cricket will produce a rival song, make body jerking movements, and continue to approach the subordinate cricket who quickly flees (Adamo and Hoy, 1995; Stevenson et al., 2000; Hofmann and Schildberger, 2001).

Here, we examined the agonistic behavior of male crickets that underwent RNA interference (RNAi)-mediated knockdown of *Fmr1* on the first day of adulthood. Our goal was to assess

whether decreased *Fmr1* expression outside of development was sufficient to impair this social behavior. We show that the average time to first physical contact during an agonistic interaction is significantly longer between a control and *Fmr1* KD pair than a control and control pair. Furthermore, we demonstrate that *Fmr1* knockdown males are less likely to initiate an aggressive interaction than control males, but these males fight just as aggressively as controls. These results suggest that decreased *Fmr1* expression during adulthood impacts cricket social behavior by impairing a male's motivation to engage in agonistic interactions.

2. Methods

2.1 Insects

Acheta domesticus crickets were raised in house and maintained in groups of approximately fifty in large plastic containers. Containers were checked for newly molted adults every day. Day 1 (D1) adult males with intact body parts were isolated from the colony and placed into individual clear plastic containers measuring 10cm wide by 8cm high. All crickets were housed in incubators at a constant temperature of 29°C and had free access to water and laboratory rat chow. Incubators were kept on a 12h light:12h dark cycle with lights on at 06:00h and lights off at 18:00h.

2.2 Cloning of Acheta domesticus Fmr1 Ortholog

The *A. domesticus* ortholog for Fragile X Mental Retardation 1 (Fmr1) was identified by performing a BLAST of our previously assembled *Acheta domesticus* transcriptome (see Chapter 2) using CLC-bio Genomics Workbench 12 software. We used a *D. melanogaster* sequence as a query to identify the ortholog for *A. domesticus* Fmr1 (DmFmr1, Fly Base ID: FBgn0028734). Then we performed a reciprocal BLAST on the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) database to confirm sequence orthology to the corresponding *D. melanogaster* gene.

In order to clone *A. domesticus Fmr1*, total RNA was extracted from four adult cricket brains using a Maxwell 16 LEV simplyRNA Tissue Kit (Promega) in conjunction with an automated Maxwell 16 RNA Isolation system (Promega). We used an iScript Reverse Transcription Kit (Bio-Rad) to synthesize cDNA from RNA. Cloning primers were designed using CLC-bio Genomics Workbench software, to amplify a 716 base pair region of the *Fmr1* gene. The primer sequences were as follows: Fmr1 Forward; 5' TGTGGTGGAATATTTAG GTTGG - 3', Fmr1 Reverse; 5'GAGGGATTGTGGGT TGTG - 3'. We did a polymerase chain reaction (PCR) with these primers to generate a DNA template for cloning. The PCR conditions were as follows: 4 min at 95°C; 35 cycles of 30 sec at 95°C, 30 sec at 58°C, and 40 sec at 72°C; 5 min at 72°C; and a hold at 4°C. PCR products were run on an agarose gel and then purified using Freeze 'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad). The purified DNA was then cloned into the PCR4-TOPO vector (PCR4-TOPO-TA Cloning Kit, Invitrogen). Colony PCR was performed on the transformants and the products were run on an agarose gel to confirm that fragments of the appropriate size were present. Plasmid DNA was purified from the bacteria using a QIAprep Spin Miniprep Kit (Qiagen). Plasmid DNA was sent to Macrogen, Inc. (Rockville, MD) for sequencing and then Vector NTI software was used to confirm that the sequence contained the 716bp region of the *Fmr1* gene.

2.3 Double-Stranded RNA Synthesis

Double-stranded RNA (dsRNA) for gene-specific knockdown of the *Fmr1* gene was made by purifying plasmid DNA with a QIAquick PCR Purification Kit (Qiagen). We used a MEGAScript T7 Transcription Kit (Invitrogen) to synthesize dsRNA from the DNA template, and then purified the dsRNA with a MEGAclear Transcription Clean-Up Kit (Invitrogen). To determine the quality and concentration of dsRNA, it was run on an agarose gel and then tested on a Nanodrop 2000 (Fisher Thermo Science). We used DsRed2 dsRNA as a control in all of our experiments. DsRed2 was synthesized from Discosoma sp. red fluorescent protein 2 and was a generous gift from Yoshinori Tomoyasu, Miami University (see Miller et al., 2012).

2.4 RNA Interference

On D1 of adulthood, undamaged male crickets were isolated from the colony and placed into individual clear plastic containers. Individuals were weighed, and crickets that fell within the weight range of 0.25-0.35 grams were used for dsRNA injection. Crickets were briefly anesthetized on ice before receiving a 2μL intra-abdominal injection of experimental (Fmr1) or control (DsRed2) dsRNA with a sterile 10μL Hamilton syringe. Crickets were returned to their individual containers after injection and were placed in the incubator until their use in later assays. For adult RNAi studies, crickets were injected with 2.5μg, 3.5μg, or 7μg of dsRNA in order to determine which concentration would cause the most effective knockdown in the brain. The duration of the knockdown was determined by sacrificing experimental and control crickets 2 days post-injection or 2dpi (D3), 8dpi (D9), or 29dpi (D30). For agonistic behavior studies, crickets were injected with 2.5μg of dsRNA on D1 of adulthood and fought 8dpi or 20dpi.

2.5 Quantitative Real Time PCR (qPCR)

Crickets were anesthetized briefly on ice prior to brain dissection. The head was removed and pinned into a dissecting dish, and the brain was dissected from a small window cut into the head. Dissected brains were immediately submerged into 200μL of cold homogenization buffer (Promega). The brains from 3 individual crickets were pooled for every treatment group. Pooled brain tissue was homogenized with a disposable pestle and then stored at -80°C until RNA isolation. Total RNA was extracted from the pooled tissues using a Maxwell 16 LEV simplyRNA Tissue Kit (Promega) and an automated Maxwell 16 RNA Isolation system (Promega). A Nanodrop 2000 (Fisher Thermo Science) was used to determine the concentration and quality of the extracted RNA. cDNA was synthesized from a 0.8μg RNA template with an iScript Reverse Transcription Kit (Bio-Rad) according to the manufacturer's protocol. Forward and reverse primer sets for Fmr1, 16s rRNA, and 18s rRNA (Table 1) were designed using Integrated DNA Technologies (IDT) software. 16s rRNA and 18s rRNA served as internal controls in qPCR experiments because they maintain uniform expression throughout development. qPCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) with a CFX Connect Real-Time PCR Detection System (Bio-Rad) under the following PCR

conditions: 3 min at 95°C; 40 cycles of 15 sec at 95°C, and 10 sec at 60°C; 10 sec at 95°C; 5 sec at 65°C; and a hold at 95°C. We used serially diluted cDNA samples (generated from brain tissue) to determine the amplification efficiency of each primer set (Table 1). The comparative Ct method was used to determine the relative expression of target genes using CFX Manager software. For all experiments each sample was run in triplicate (three technical replicates). The relative expression of each target gene was determined by taking the mean expression of three independent runs (three biological replicates).

2.6 Agonistic Behavior Experiments

We performed agonistic behavior experiments on male crickets injected with 2.5μg of dsRNA on D1 of adulthood. Crickets were weighed 2 days prior to behavioral trials and were assigned a partner with a weight difference of 10% or less. We performed agonistic trials on control pairs (DsRed2 *vs*. DsRed2) and experimental pairs (DsRed2 *vs*. Fmr1) 8dpi or 21dpi. Agonistic pairs were only fought once, so all males used in behavioral trials were naïve. Behavioral trials were conducted in a quiet, dimly lit room in the afternoon between 12:00h – 18:00h. Crickets were placed in the behavior room at least 1 h prior to the start of trials for acclimation. Trials took place in a clear, round Plexiglas arena measuring 15cm wide by 10cm high. Prior to each trial, arenas were cleaned with 70% ethanol and allowed to dry for ~2 min. White paper was placed onto the arena floor prior to every trial to provide traction. Each member of an age and weight-matched trial pair was placed on opposite sides of the arena separated by a dark Plexiglas barrier standing ~10cm high. Crickets spent 15 min acclimating in the arena before the barrier was lifted, initiating the behavioral trial. After establishment of social rank, the pair was allowed to interact for an additional 5 min to confirm social rank. If social rank switched during the 5 min, the pair was given an additional 5 min, and the trial concluded only after social rank was maintained for 5 min. Behavioral trials were recorded from above the arena with a Sony HDR-CX290 camera and were simultaneously projected onto a computer monitor. The arena and barrier were cleaned and dried in between each trial, and a fresh piece of white paper was placed on the floor of the arena to remove any residual pheromones from the previous pair. Each trial was evaluated by a researcher in real time and was re-evaluated at a later date by watching the video recording.

The number of contacts necessary to initiate agonistic behavior were recorded. Males had to physically separate by one body length and then come back together in order for another contact to be scored. We also recorded the identity of the cricket that initiated the agonistic behavior. The initiator was the individual which made the first aggressive action in a trial. This aggressive action always began with one cricket approaching the other cricket, and was followed by antennal touching, if the interaction escalated. The fight level and the identity of the winner and loser of the interaction was also recorded for each trial. Agonistic interactions consisted of seven different levels, characterized by an escalating sequence of aggressive behaviors that were scored according to Stevenson and colleagues (Stevenson et al., 2000). Mutual avoidance (level 0) applied to interactions where both crickets showed no sign of aggression and avoided each other for at least 10 min after the barrier was lifted. This level of interaction was only observed during one behavioral trial (1/181), and was not included in our analysis. All other interactions included pairs reaching fight levels ranging from pre-established dominance (level 1) to tactical combat (level 6). Briefly, the levels of interaction included the following: pre-established dominance (level 1) occurred when one cricket retreated from an approaching aggressor; antennal fencing (level 2) occurred when the antennae of both crickets made contact; unilateral mandible spreading (level 3) occurred when one cricket spread its mandibles while facing the other cricket; bilateral mandible spreading (level 4) occurred when both crickets spread their mandibles while facing one another; mandible engagement (level 5) occurred when the spread mandibles of both crickets were engaged; tactical combat (level 6) occurred when crickets wrestled with each other by tossing, flipping, twisting, and biting. Social rank was established when one cricket retreated (loser/subordinate) and the other cricket produced a rival song accompanied by body jerking movements (winner/dominant), and would chase the subordinate.

2.7 Statistical Analyses

Student's *t*-tests were performed to determine if there were significant differences ($p < 0.05$) in relative expression between treatment groups for qPCR assays. For agonistic behavior, Student's *t*-tests were conducted to determine if there were significant differences in the amount of time to first contact. A χ^2 analysis with Pearson's post-hoc test was used to determine if there were effects of treatment on initiation of agonistic interactions, social rank, and pre-established

dominance. Comparisons of mean maximum fight level scores between groups at the same time point were analyzed with a one-way ANOVA followed by Dunnett's post-hoc test with $\alpha = 0.05$. A two-way ANOVA was used to assess the effects of treatment and time on the mean maximum fight level. All statistical analyses were performed using JMP Pro 14 (SAS Institute Inc., Cary, N.C.) software.

3. Results

3.1 Increasing the dose of dsRNA does not lead to a more effective knockdown in the brains of adult male crickets

Adult RNAi was used to characterize the knockdown (KD) of *Fmr1* in the brain tissue of male crickets injected with 2.5μg, 3.5μg, or 7μg of dsRNA 2dpi (D3), 8dpi (D9) and 20dpi (D21). Crickets are particularly suitable for RNAi studies because a single injection of dsRNA initiates systemic silencing of a target gene, which can last for more than two months in certain species (Moriyama et al., 2008; Tomioka et al., 2009). The relative expression of *Fmr1* in KD males injected with 2.5μg was decreased by 22%, 37%, and 14% when compared to age-matched controls 2dpi, 8dpi, and 20dpi, respectively (Fig. 1A). While expression was reduced in the brain, this decrease was not statistically significant at any of the time points tested. We also examined *Fmr1* expression in males injected with 3.5μg of dsRNA. This caused a 41%, 13%, and 19% decrease in relative expression 2dpi, 8dpi, and 20dpi, respectively (Fig. 1B). Although expression levels were reduced, there was not a significant decrease in *Fmr1* at any of the time points assessed. Finally, we determined the relative expression of *Fmr1* in crickets injected with 7μg of dsRNA. The relative expression of *Fmr1* was significantly reduced in KD males 2dpi ($p = 0.012$) when injected with the highest dose of dsRNA tested (Fig. 1C), however, this concentration did not significantly reduce the expression of *Fmr1* 8dpi or 29dpi. Overall, the effects of adult RNAi were quite variable in the brains of males. We found that increasing the dose of dsRNA increased the strength, but not the duration, of the knockdown. While our knockdown was not statistically significant, moderate decreases in gene expression have been shown to significantly impact cricket wing morphology, social behavior, and motor function (see Moriyama et al., 2008; Hamada et al., 2009 for examples). Moriyama et al. (2008) used systemic

RNAi to examine the effects of decreased *per* expression in the cricket *Gryllus bimaculatus*. In that study, RNAi caused a 48% reduction in *per* expression and led to significant changes during adulthood, whereby crickets completely lost circadian locomotor rhythm for up to 50dpi.

3.2 Agonistic behavior of Fmr1 knockdown males 8 days post-injection

Agonistic behavior was examined in males injected with 2.5μg of dsRNA because we previously determined that decreasing *Fmr1* expression during adulthood can have a significant and sustained effect on the immune system of *Acheta domesticus* crickets (see Chapter 2). Within eight days of decreased *Fmr1* expression we saw an increase in the number of circulating hemocytes, decreased total phenoloxidase activity, and increased *lysozyme* expression. Cricket agonistic behavior follows a stereotyped sequence of events that is triggered by chemosensory antennal contact (Adamo and Hoy, 1995; Stevenson et al., 2000; Hofmann and Schildberger, 2001). The only time that antennal contact does not occur is during mutual avoidance (level 0), where both crickets show no sign of aggressive behavior and avoid one another. We did not observe mutual avoidance during behavioral trials conducted 8dpi, so all 96 pairs engaged in agonistic behavior. We determined the average time to first physical contact for these 96 fighting pairs. The average time for control pairs (DsRed2 *vs.* DsRed2) to make contact was 109 ± 12 s, while the average time for experimental pairs was 90 ± 12 s (Fig. 2A), which was not significantly different ($p = 0.276$). Since a fight does not always ensue after the first physical contact between males, we examined the number of contacts required to initiate a fight. The average number of contacts was 1.13 ± 0.05 for control pairs and 1.18 ± 0.06 for experimental pairs, which was not significantly different ($F_{1,94} = 0.432$, $p = 0.513$). After antennal contact, the interactions followed a stereotyped sequence of increasingly aggressive motor acts which were scored. We determined the number of pairs exhibiting pre-establishment of social rank (level 1) for both groups, and found that control fights were pre-established twice as often as experimental fights (Table 2), but this trend was not statistically significant ($\mathcal{X}^2 = 3.450$, $p = 0.063$). The maximum fight level reached by control and experimental pairs (Fig. 2B) was not significantly different ($F_{1,124} = 0.840$, $p = 0.361$), indicating that *Fmr1* KD males fought just as aggressively as controls. Taken together, these findings suggest that decreased *Fmr1* expression during adulthood does not affect the agonistic behavior of males tested 8dpi.

Next, we determined if *Fmr1* KD could affect a male cricket's motivation to initiate a fight, or its ability to win a fight. The initiator was scored as the first male to approach the other male. We found that *Fmr1* KD males initiated fights as often as controls ($\chi^2 = 0.143$, $p = 0.706$) (Fig. 2C), indicating that decreased *Fmr1* did not affect a male's motivation to fight. Finally, we determined the percentage of fights won by each treatment in an experimental pairing (Fig. 2D). *Fmr1* KD males won agonistic interactions just as often as controls ($\chi^2 = 0.571$, $p = 0.450$), indicating that decreased *Fmr1* does not impact the outcome of a fight.

3.3 Agonistic behavior of Fmr1 knockdown males 20 days post-injection

We previously determined that the impact of decreased *Fmr1* was not always apparent by 8dpi, and could be slow to take effect (see Chapter 2). For example, when *Fmr1* expression was decreased on D1 of adulthood there was no effect on total PO activity 8dpi, but by 20dpi there was a significant decrease in total PO activity. To ensure that we did not miss an effect because we looked prematurely, we also examined agonistic behavior in adult males three weeks after injection of 2.5µg dsRNA (20dpi). We observed mutual avoidance (level 0) in one behavioral trial between a control pair. The remaining 85 pairs engaged in agonistic behavior. We determined the average time to first physical contact for these 85 fighting pairs. Experimental pairs took significantly longer to make physical contact during behavioral trials than control pairs (110 ± 15 s *vs.* 60 ± 11 s, respectively; $p = 0.008$; Fig. 3A). We looked at the amount of time that the control male and *Fmr1* male spent moving in the arena prior to the first physical contact to determine if the locomotion of experimental males was impacted by decreased *Fmr1*. We found that the amount of time that controls (13 ± 2 s) and *Fmr1* (16 ± 3 s) males spent moving did not significantly differ ($p = 0.426$). We also found that the number of contacts required to initiate a fight did not significantly differ between control (1.13 ± 0.05) and experimental (1.10 ± 0.04) pairs ($F_{1,83} = 0.970$, $p = 0.328$). After antennal contact, interactions escalated and were scored for each behavioral pair to determine if control pairs showed different levels of aggression than experimental pairs. We determined the number of pairs exhibiting pre-establishment of social rank (level 1) for both groups (Table 3) and found no difference between control pairs and experimental pairs ($\chi^2 = 0.861$, $p = 0.352$). We also examined the average maximum fight level for each agonistic pair and found that there was no difference between

control and experimental pairs ($F_{1,124} = 0.840$, $p = 0.361$; Fig. 3B). However, we observed an increase in the fight levels of crickets fought 20dpi when compared to 8dpi. A two-way ANOVA confirmed that there was a significant effect of time on the average maximum fight level (Table 4). Fight levels were significantly higher in both groups 20dpi than at 8dpi ($F_{1,258} = 11.399$, $p = 0.0008$), but this effect was independent of treatment which suggests that age can influence aggression. Taken together, these results suggest that decreased *Fmr1* affects a male cricket's motivation to engage in an agonistic interaction, and that this effect is time, or age, dependent.

Next, we examined if *Fmr1* KD male crickets were less likely to initiate fights or attain social dominance. First, we determined the percentage of fights initiated by each treatment in an experimental pairing (Fig. 3C). We found that *Fmr1* KD male crickets initiated fights significantly less often than control males 20 dpi ($\chi^2 = 8.522$, $p = 0.004$). However, despite initiating fights significantly less often, *Fmr1* KD males won agonistic interactions as often as controls ($\chi^2 = 0.696$, $p = 0.404$; Fig. 3D), indicating that decreased *Fmr1* does not impact the outcome of a fight. These results suggest that *Fmr1* KD may affect the motivation to fight, but does not impact the cricket's ability to fight since they were just as aggressive, and likely to become dominant, as control males.

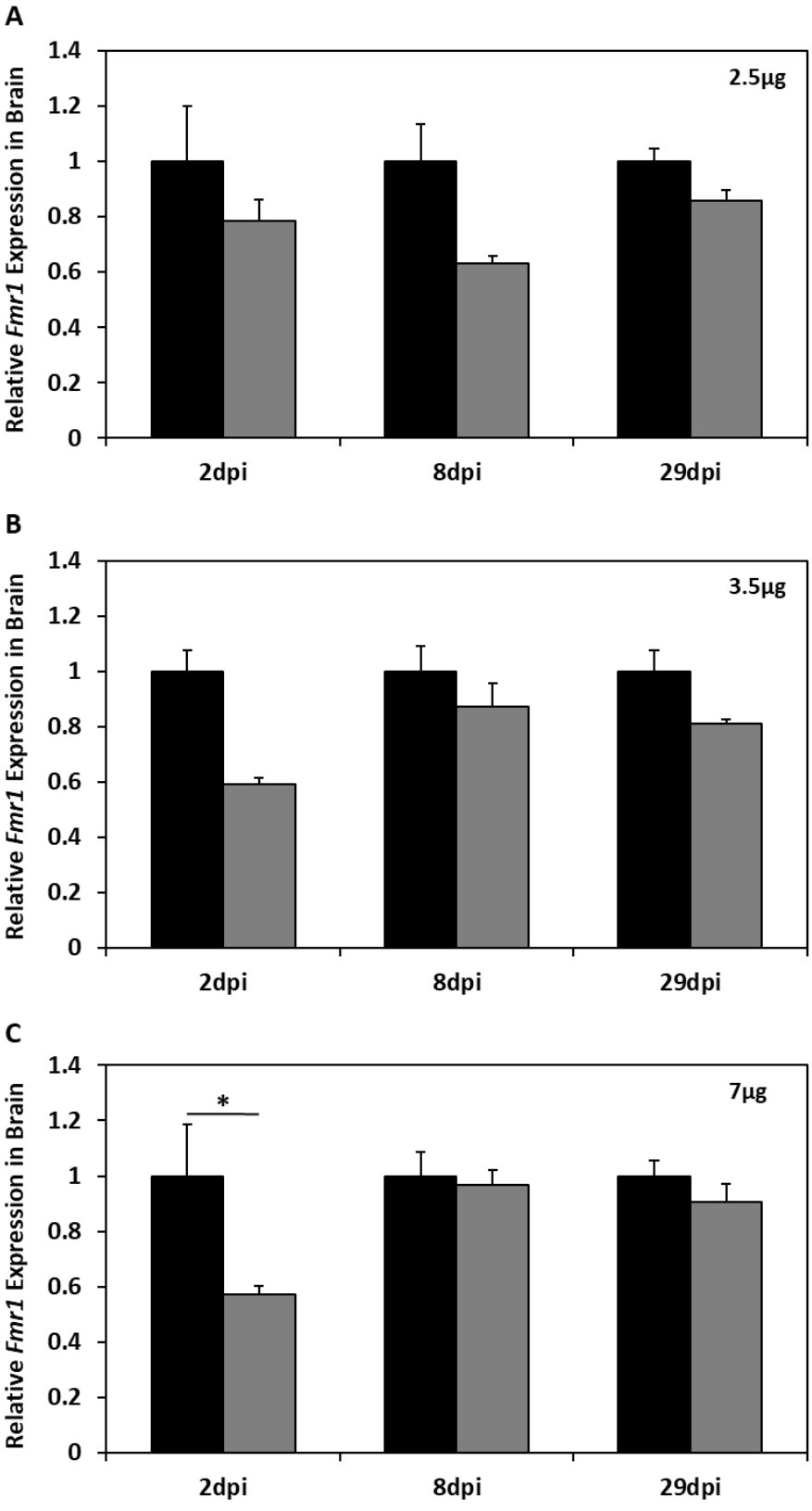
A
Relative Fmr1 Expression in Brain
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2.5µg
2dpi
8dpi
29dpi
B
Relative Fmr1 Expression in Brain
3.5µg
2dpi
8dpi
29dpi
C
Relative Fmr1 Expression in Brain
*
7µg
2dpi
8dpi
29dpi

Figure 1

Figure 1. Increasing the dose of dsRNA does not lead to a more effective knockdown in the brains of male crickets. Relative *Fmr1* mRNA expression in the adult male brain (A-C) as determined by quantitative Real-Time PCR (qPCR). Crickets were injected with 2.5μg (A), 3.5μg (B), or 7μg (C) of DsRed2 dsRNA (negative control) or Fmr1 dsRNA (experimental) on Day 1 of adulthood and the relative expression was determined 2 days post-injection (2dpi), 8dpi, and 29dpi. (A) *Fmr1* expression was not significantly different in experimental males at 2dpi, 8dpi, or 29dpi when compared to age-matched controls. (B) There was no significant change in expression at any timepoint examined with a 3.5μg injection of dsRNA. (C) The relative expression of *Fmr1* in the brain was significantly decreased in experimental males 2dpi ($p = 0.011$) when compared to age-matched controls. Relative mRNA expression is the average of three independent runs (biological triplicates) ± SEM. Significance is denoted by an asterisk, Student's T-test, $p < 0.05$.

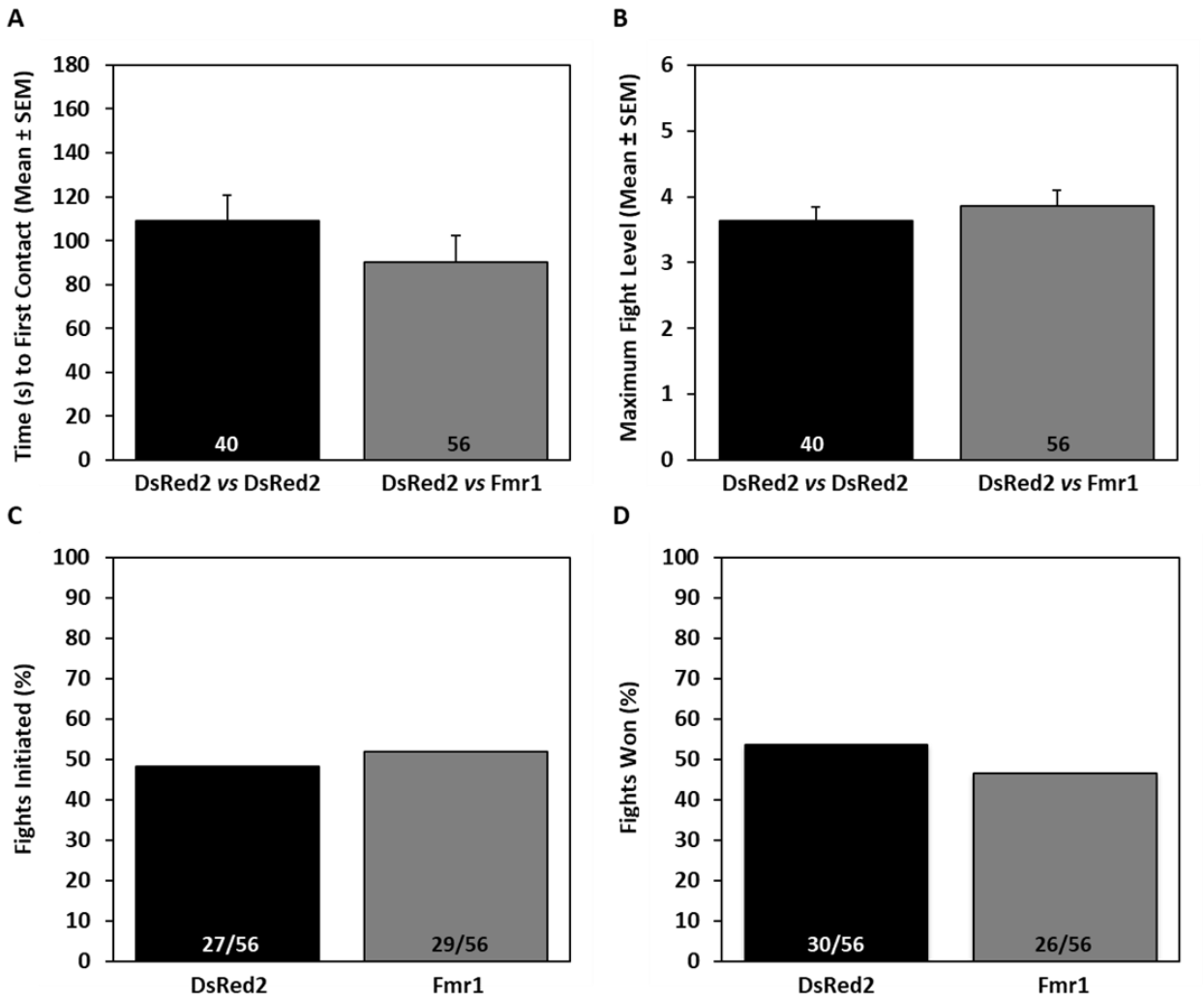
A
Time (s) to First Contact (Mean ± SEM)
180
160
140
120
100
80
60
40
20
0
40
56
DsRed2 vs DsRed2
DsRed2 vs Fmr1
B
Maximum Fight Level (Mean ± SEM)
6
5
4
3
2
1
0
40
56
DsRed2 vs DsRed2
DsRed2 vs Fmr1
C
Fights Initiated (%)
100
90
80
70
60
50
40
30
20
10
0
27/56
29/56
DsRed2
Fmr1
D
Fights Won (%)
100
90
80
70
60
50
40
30
20
10
0
30/56
26/56
DsRed2
Fmr1

Figure 2

Figure 2. Agonistic behavior of *Fmr1* knockdown males 8 days post-injection. Male crickets were injected with 2.5µg dsRNA on D1 of adulthood and behavioral trials were conducted 8dpi. (A) The average time to first contact during an agonistic trial was not significantly different between control (DsRed2 *vs.* DsRed2) and experimental (DsRed2 *vs.* Fmr1) pairs (p = 0.276). (B) The maximum fight level reached by fighting pairs during an agonistic interaction was not significantly different between control and experimental pairs ($F_{1,134}$ = 0.448, p = 0.505). The average fight level included pairs reaching fight levels ranging from pre-established dominance (level 1) to tactical combat (level 6). For A and B: bars represent the mean ± SEM and the numbers within bars indicate sample sizes for each treatment group. (C) The percentage of fights initiated did not differ between controls and *Fmr1* KD males (χ^2 = 0.143, p = 0.706). (D) The percentage of fights won did not differ between controls and *Fmr1* KD males (χ^2 = 0.571, p = 0.450). For C and D: Bars represent the percentage of initiations or wins for the indicated group. The numbers within bars indicate the number of initiations/wins for the indicated group followed by the total number of agonistic trials. Significant differences ($p < 0.05$) between groups denoted by an asterisk *.

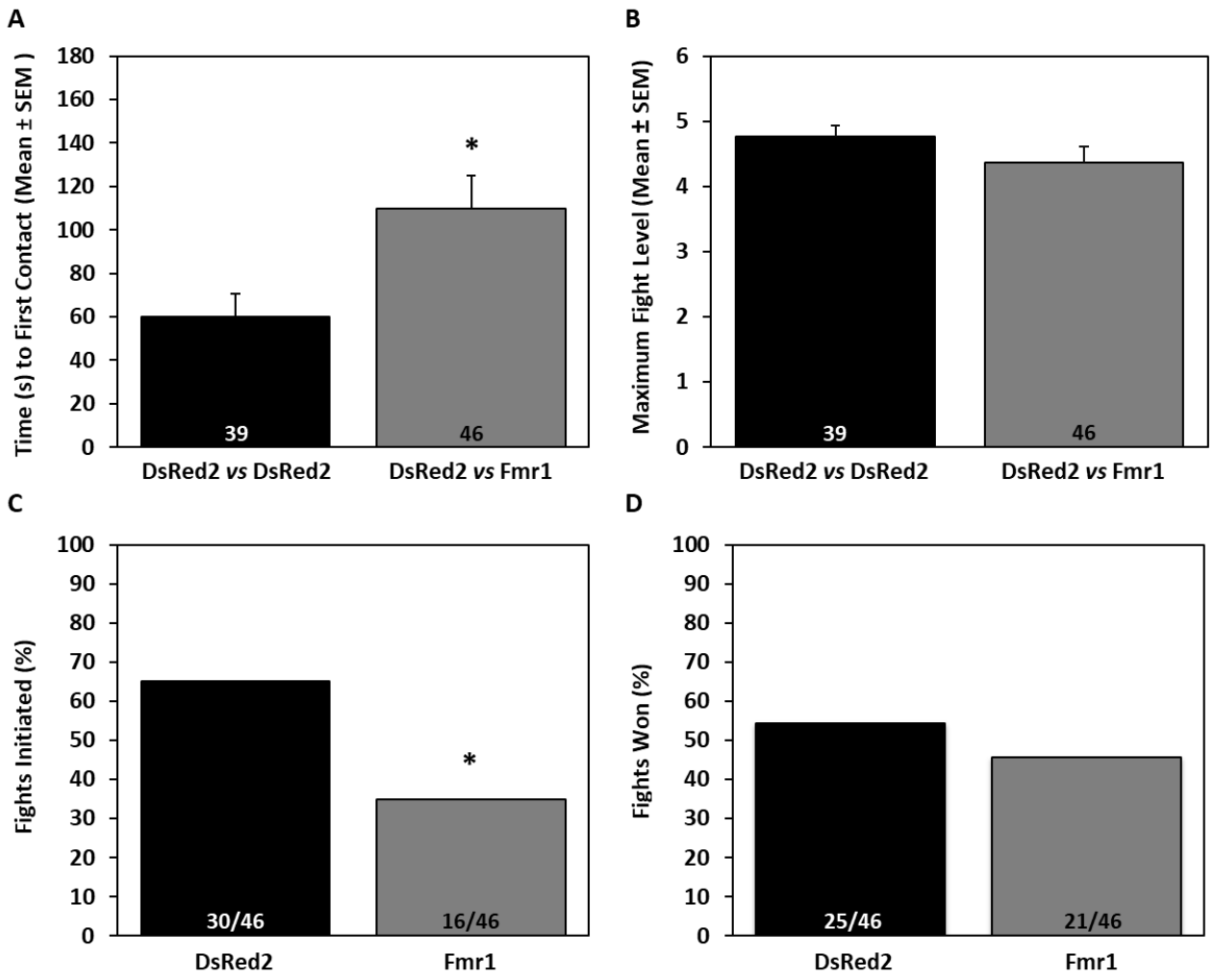
A
Time (s) to First Contact (Mean ± SEM)
180
160
140
120
100
80
60
40
20
0
*
39
46
DsRed2 vs DsRed2
DsRed2 vs Fmr1
B
Maximum Fight Level (Mean ± SEM)
6
5
4
3
2
1
0
39
46
DsRed2 vs DsRed2
DsRed2 vs Fmr1
C
Fights Initiated (%)
100
90
80
70
60
50
40
30
20
10
0
*
30/46
16/46
DsRed2
Fmr1
D
Fights Won (%)
100
90
80
70
60
50
40
30
20
10
0
25/46
21/46
DsRed2
Fmr1

Figure 3

Figure 3. Agonistic behavior of *Fmr1* knockdown males 20 days post-injection. Male crickets were injected with 2.5μg dsRNA on D1 of adulthood and behavioral trials were conducted 20dpi. (A) The average time to first contact during an agonistic trial was significantly increased in experimental pairs (DsRed2 *vs.* Fmr1) when compared to control (DsRed2 *vs.* DsRed2) pairs (p = 0.008). (B) The maximum fight level reached by fighting pairs during an agonistic interaction was not significantly different between control and experimental pairs ($F_{1,124}$ = 0.840, p = 0.361). The average fight level included pairs reaching fight levels ranging from pre-established dominance (level 1) to tactical combat (level 6). For A and B: bars represent the mean ± SEM and the numbers within bars indicate sample sizes for each treatment group. (C) *Fmr1* KD male crickets initiated fights significantly less often than controls (χ^2 = 8.522, p = 0.004). (D) The percentage of fights won did not differ between controls and *Fmr1* KD males (χ^2 = 0.696, p = 0.404). For C and D: bars represent the percentage of initiations or wins for the indicated group. The numbers within bars indicate the number of initiations/wins for the indicated group followed by the total number of agonistic trials. Significant differences ($p < 0.05$) between groups denoted by an asterisk *.

Table 1. Primers used for qPCR analysis.

Primer	Sequence	Amplicon	Efficiency
16s rRNA F	CTTCTCGTCCCCTATTTTCATTTG	150 bp	95.1%
16s rRNA R	GGTATATTGACCGTGCAAAGG		
18s rRNA F	TGTTTTCCTTGCTCGAGAGG	112 bp	96.1%
18s rRNA R	AATGATTCCCGCTTACTAGGC		
Fmr1 F	CTCATGGGTTTGGCAATTGG	96 bp	100.9%
Fmr1 R	GCACGAGTTTTCTTCCAGTTC		

Table 2. Distribution of maximum fight levels for agonistic pairs fought on Day 9.

Fight Level	DsRed2 *vs.* DsRed2	DsRed2 *vs.* Fmr1
Level 1	9/40 (22.5%)	6/56 (10.7%)
Level 2	4/40 (10%)	13/56 (23.2%)
Level 3	7/40 (17.5%)	9/56 (16%)
Level 4	3/40(7.5%)	4/56 (7.1%)
Level 5	7/40 (17.5%)	5/56 (8.9%)
Level 6	10/40 (25%)	19/56 (33.9%)

Table 3. Distribution of maximum fight levels for agonistic pairs fought on Day 21.

Fight Level	DsRed2 *vs.* DsRed2	DsRed2 *vs.* Fmr1
Level 1	1/39 (3%)	3/46 (7%)
Level 2	4/39 (10%)	3/46 (7%)
Level 3	3/39 (8%)	11/46 (24%)
Level 4	5/39 (13%)	5/46 (11%)
Level 5	8/39 (21%)	5/46 (11%)
Level 6	18/39 (45%)	19/46 (41%)

Table 4. Two-way analysis of variance assessing the effects of treatment and time on fight level.

Source of Variation	d.f.	F Ratio	*P* Value
Treatment	1, 258	0.0181	0.8930
Time	1, 258	11.3993	**0.0008**
Treatment x Time	1, 258	1.2259	0.2692

Significant effects ($P < 0.05$) are shown in bold. d.f., degrees of freedom

4. Discussion

In the present study, we examined the effects of decreased *Fmr1* expression during adulthood on the social behavior of male *Acheta domesticus* crickets. We used systemic RNAi to suppress *Fmr1* expression in adult males and then examined the impact on agonistic behavior. Research conducted on numerous animal models of FXS has shown that deficiencies in FMRP lead to a wide variety of impaired social behaviors, including in rats (Hamilton et al., 2014), mice (Spencer et al., 2005), zebrafish (Wu et al., 2017), tadpoles (Truszkowski et al., 2016), fruit flies (Bolduc et al., 2010), and the cricket *Gryllus bimaculatus* (Hamada et al., 2009). Different types of social behaviors were examined in these studies, but overall *Fmr1* animals displayed deficits in social communication and interactions, as well as increased social anxiety. For example, *Fmr1* KO rats exhibited fewer juvenile play behaviors like ear and tail tugging, wrestling, and pinning than wildtype littermates in direct social interaction tests (Hamilton et al., 2014). These juvenile rats also exhibited increased levels of anxiety by chewing on wooden blocks more often than wildtype rats. Additionally, a recent study by Wu et al. (2017) examined the effects of FMRP knockout on the shoaling behavior of zebrafish. Coordinated group swimming is an inherent social behavior seen in several species of fish. FMRP knockout fish displayed abnormal shoaling behavior and had higher anxiety levels during novel tank testing (Wu et al., 2017).

We used systemic RNAi to decrease *Fmr1* expression in adult males. This technique can be used to create a loss-of-function phenotype at the desired stage of development with a single injection of dsRNA (reviewed in Belles, 2010). RNAi is particularly well suited to crickets and other non-model species where transgenic mutants are not feasible (reviewed in Belles, 2010). Previous studies have shown that crickets are highly susceptible to RNAi, and that a single injection can silence a target gene for more than two months (Moriyama et al., 2008; Tomioka et al., 2009). To ensure that RNAi would suppress *Fmr1* expression in brain tissue, we injected each of three different concentrations of dsRNA and used qPCR to measure expression at different times post-injection. Here, we demonstrated that RNAi against *AdFmr1* was variable in the brain. While expression was reduced by as much as 41%, it was not statistically significant at most of the time points tested due to the variability between triplicate runs. Additionally, we did not see an increase in the overall strength or duration of the knockdown after administering a larger dose of

dsRNA. In a previous study, we found that a 2.5µg injection of dsRNA caused a significant decrease in *Fmr1* expression in the hemolymph (59%) and fat body (43%) of male crickets 8dpi (see Chapter 2). This variability seen in brain tissue may be attributed to differences in tissue permeability. Tissue permeability could be affected by the inability to take up dsRNA, the expression of core RNAi machinery components, or the expression of dsRNA-degrading nucleases (reviewed in Belles, 2010; Cooper et al., 2018). It is possible that there were differences in tissue permeability between individual males, and the pooled brains had differing levels of *Fmr1* knockdown.

Similar to our findings, the RNAi conditions for efficient gene silencing in the midgut and hemolymph of the mosquito *Anopheles gambiae*, were not sufficient for silencing gene expression in salivary glands (Boisson et al., 2006). They determined that a significant knockdown could be attained in the salivary glands by increasing the amount of dsRNA, which required 5x more dsRNA than hemocytes and 30x more dsRNA than midgut cells. Differences in tissue permeability have also been demonstrated in the roundworm *Caenorhabditis elegans*. Kennedy et al. (2004) found that RNAi worked efficiently in most tissues of the roundworm, while the gonads and neurons were highly refractory to RNAi. It was determined that the gonads and neurons expressed a nuclease that degraded the dsRNA before it could be processed by the RNAi machinery, making these tissues less susceptible to RNAi. It would be interesting to explore this idea in the cricket and determine if there are nucleases expressed in the brain tissue that degrade the dsRNA.

We examined the effects of decreased *Fmr1* expression on agonistic behavior in adult male crickets 8dpi and found that there was no observable impact on this behavior. Since we did not observe an impact of *Fmr1* KD on agonistic behavior 8dpi, we also examined agonistic behavior three weeks post injection. We found that experimental pairs took significantly more time to make physical contact during a behavioral trial than control pairs. This increase in the amount of time to make contact could be attributed to changes in exploratory behavior or locomotor activity in *Fmr1* KD crickets. Bolduc et al. (2010) found that *Drosophila Fmr1* mutants stopped for longer lengths of time and covered less overall area when placed into a novel test chamber, demonstrating decreased exploratory behavior and locomotor activity. In agreement with these

findings, *Fmr1* KD tadpoles had lower overall swimming speeds when compared to controls (Truszkowski et al., 2016). Additionally, Xu et al. (2004) demonstrated that *Drosophila Fmr1* larvae spent less time crawling than control larvae when placed into vertical tests chambers, indicating that locomotion was impaired in these mutants. We found that the overall locomotor activity of *Fmr1* KD males prior to initial contact did not differ from controls in this study. Future studies will focus on examining the amount of time that each member of the agonistic pair spent moving throughout the rest of the fight, and the speed with which they moved, to determine if their locomotion is impacted at other times throughout the agonistic trials.

In an agonistic interaction, the initiator is the individual that makes the first aggressive act. This aggressive action always begins with one cricket approaching the other cricket, followed by antennal touching. We found that *Fmr1* KD males were less likely to initiate a fight than control males. Bolduc et al. (2010), used *Drosophila* to examine the effects on social behavior in wildtype and *Fmr1* mutant flies by placing two isogenic flies in adjacent chambers separated by mesh. They determined the spatial probability distributions and measured inter-fly distances to determine the likelihood of a social interaction. They found that *Fmr1* mutant flies stayed farther away from the divider and maintained larger inter-fly distances than controls, indicating that *Fmr1* mutant were less likely to initiate a social interaction, or to interact with other flies (Bolduc et al., 2010).

It has been proposed that the individual who initiates the first encounter during a fight is more likely to emerge as the winner of the fight (Chen et al., 2002). Moreover, the higher the intensity level with which the individual started the fight, the greater the chances of winning (Chen et al., 2002). We examined the fight outcome between experimental pairs and found that *Fmr1* KD males won agonistic interactions just as often as controls, even though they initiated fights significantly less often. A previous study by Spencer et al. (2005) examined the effects of *Fmr1* knockout on social dominance in male mice. In this test, one *Fmr1* KO mouse and one wildtype mouse were simultaneously placed onto opposite ends of a long white tube facing one another. The match ended when one mouse retreated from the tube (loser/subordinate), and the other mouse remained in the tube (winner/dominant). They found that *Fmr1* KO mice won significantly fewer fights than control mice, suggesting that their social behavior was impaired.

Unfortunately, they did not report on time to first contact, initiations, or levels of aggression, they just reported dominance status. As this is one of the few reports on dominance status as a measure of social behavior in *Fmr1* animals, it would have been interesting to see if any other fight parameters were affected. We also found that the maximum fight level between control pairs and experimental pairs was similar, suggesting that fights were just as aggressive. Why would decreased *Fmr1* expression affect the likelihood of a male to initiate a fight but not its aggressiveness or ability to attain dominance status?

Agonistic interactions typically take place when there is competition for a limited resource such as food, shelter, territory, or a mate (Stevenson and Rillich, 2012). Before engaging in a competition with another male, crickets will assess the benefits and costs of the encounter using sensory information (Stevenson and Rillich, 2012). Crickets use visual, auditory (Hofman and Schildberger, 2001), and chemical cues (Buena and Walker, 2008) to make decisions about initiating a fight, the level of escalation, and when to flee. Many studies have shown that the ability to integrate multisensory input is impaired in *Fmr1* KD animals (Bolduc et al., 2010; Truszkowski et al., 2016; Wu et al., 2017). For example, schooling is a highly structured group behavior that requires the integration of multiple sensory inputs including auditory, olfactory, and visual cues (Truszkowski et al., 2016). Abnormal schooling behavior has been observed in *Fmr1* knockout zebrafish (Wu et al., 2017) and *Xenopus laevis* tadpoles (Truszkowski et al., 2016) that is more dispersed and less aggregated than the tight and unidirectional swimming displayed by controls. While the inability to process sensory input may help to explain our findings, additional research is necessary to test this hypothesis.

Our findings may also be attributed to changes in brain chemistry. The biogenic amines octopamine, dopamine, and serotonin are known for their neuromodulatory actions in invertebrates; these action can affect both motor output and behavior (Kravitz, 1988). Previous studies have shown that each of these amines plays an important role in aggressive behavior. For example, studies in both lobster and crayfish have shown that serotonin increases the duration of fights by interfering with the decision to withdrawal (Huber et al., 1997; Huber and Delago, 1998). Additionally, a study by Stevenson et al. (2000) showed that pharmacological depletion of octopamine, dopamine, and serotonin made male *Gryllus bimaculatus* crickets reluctant to fight.

Despite their reluctance to initiate aggressive interactions, fights between treated males lasted as long and were just as aggressive as fights between control males (Stevenson et al., 2000). It would be interesting to see if these amines contribute to the behavioral phenotype seen in *Fmr1* KD males by examining the levels of octopamine, dopamine, and serotonin in their brains.

In summary, our study has shown that changes in *Fmr1* expression during adulthood have a significant impact on the agonistic behavior of adult male crickets. We found that experimental pairs took significantly more time to make physical contact during a behavioral trial than control pairs, which may be attributed to decreased exploratory behavior or locomotor activity. We also found that *Fmr1* KD males initiated fights less often than controls, but were just as likely to become dominant. These findings suggest that modest decreases in *Fmr1* expression are sufficient to change the behavioral phenotype of adult crickets. Moving forward, the cricket *Acheta domesticus* can continue to be a model for the study of FMRP and its role in social behavior.

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Chapter 4: Using parental RNA interference to decrease *Fmr1* expression during development in the house cricket, *Acheta domesticus*

1. Introduction

RNA interference (RNAi) is a powerful genetic tool that utilizes double-stranded RNA (dsRNA) for gene-specific silencing (reviewed in Belles, 2010). The process of RNAi is initiated when dsRNA is administered to an organism via injection, feeding, incubation, or electroporation (reviewed in Belles, 2010). Double-stranded RNA is taken up by cells and immediately cleaved into short, small-interfering RNAs (siRNAs) by the enzyme Dicer (Meister and Tuschl, 2004; reviewed in Belles, 2010). The siRNAs unwind and the antisense strand incorporates into the multi-protein RNA-induced silencing complex (RISC), that then shuttles siRNA to its complementary target mRNA (Meister and Tuschl, 2004; reviewed in Belles, 2010). Upon binding, the complementary mRNA target is degraded causing depletion of target transcripts (Meister and Tuschl, 2004; reviewed in Belles, 2010).

Since its first successful demonstration in the nematode *Caenorhabditis elegans* (Fire et al., 1998), RNAi has become a popular reverse genetics tool to study gene function in non-model organisms (Coleman et al., 2015). Over the past twenty years, this technique has been successfully applied to over thirty species of insects, representing the orders Orthoptera (see Moriyama et al., 2008; Tomioka et al., 2009 for examples), Dictyoptera (see Martin et al., 2006; Garbutt et al., 2013 for examples), Isoptera (see Zhou et al., 2008; Wu and Li, 2018 for examples), Hemiptera (see Hughes et al., 2000; Fishilevich et al., 2016 for examples), Coleoptera (see Brown et al., 1999; Bucher et al., 2002 for examples), Hymenoptera (see Guidugli et al., 2005 for example), Lepidoptera (see Eleftherianos et al., 2007 for example), and Diptera (see Kennerdell and Carthew, 1998; Osta et al., 2004 for examples). Insects are particularly suitable for RNAi studies because a single application of dsRNA initiates silencing of a target gene, which can have long-lasting effects (reviewed in Belles, 2010; Cooper et al., 2018). For example, RNAi can persist for up to 7 months in the bug *Rhodnius prolixus* (Paim et al., 2013), more than 2 months in the red flour beetle *Tribolium castaneum*, 1 month in the firebrat *Thermobia domestica* (Uryu et al., 2013), and more than 21 days in the honeybee *Apis mellifera* (Amdam et al., 2003).

In some insects, systemic RNAi is observed where transcriptional suppression of a target is observed in all tissues of the body due to the spread of the RNAi signal (reviewed in Cooper et al., 2018). The systemic properties of RNAi allow dsRNA to spread to the germline cells, where gene knockdown (KD) effects can be transmitted to the next generation (Matsumoto and Hatori, 2016). This phenomenon, termed parental RNAi (pRNAi), has been observed in numerous insects (see Bucher et al., 2002; Paim et al., 2013; Coleman et al., 2015 for examples) and can be induced by injection or oral delivery of dsRNA to a female.

In the present study, we used pRNAi in the cricket *Acheta domesticus* to target the Fragile X Mental Retardation 1 (*Fmr1*) gene. Mutations in the regulatory region of this gene lead to loss of the gene product Fragile X Mental Retardation Protein, also referred to as FMRP (reviewed in Bhakar et al., 2012). A complete loss of FMRP results in the Fragile X syndrome (FXS) disease state (Li and Zhao, 2014). This neurodevelopmental disorder is the most common form of inherited mental retardation (Crawford et al., 2001), and is a leading cause of autism spectrum disorders (Bassell and Warren, 2008). Transmitting the knockdown effects of *Fmr1* to offspring via pRNAi allows the F_1 generation to be affected during development, as they would with FXS, making pRNAi an invaluable research tool.

Despite its many advantages, there is one major drawback to RNAi and that is the difference in species sensitivity (reviewed in Belles, 2010; Cooper et al., 2018). Among the many insects tested thus far, species belonging to the orders Coleoptera, Dictyoptera, and Orthoptera seem to be the most sensitive to RNAi, while species belonging to the orders Lepidoptera and Diptera seem to be the least sensitive (reviewed in Belles, 2010; Cooper et al., 2018). Studies have shown that not only are the effects of RNAi species specific, they are also tissue specific and gene specific (reviewed in Belles, 2010; Cooper et al., 2018; Vogel et al., 2019). For example, in the fruit fly *Drosophila melanogaster* nearly every tissue is insensitive to RNAi except for the hemocytes, which are highly susceptible to RNAi (reviewed in Vogel et al., 2019). Several factors contribute to the variable efficiency of RNAi seen in insects including: stability of dsRNA, cellular uptake of dsRNA, expression of core RNAi machinery components, and the amplification and spreading of RNAi (reviewed in Cooper et al., 2018). Due to the high degree

of variability associated with RNAi, every experiment must be optimized for the organism, tissue, and gene of interest.

In this study, we evaluated parameters required to generate a successful pRNAi response in the cricket *Acheta domesticus*. Double-stranded RNA was injected into females on the first day of adulthood, and the efficacy of RNAi was progressively evaluated along several stages of the insect's life. The parameters tested included a concentration response, timing of the dsRNA exposure, effects on eggs in female ovaries and the number of offspring hatched, the temporal endurance of the RNAi effects in the next generation, and functional effects of RNAi in F_1 offspring. We found that *Fmr1* knockdown could be transmitted to the next generation via pRNAi, but the strength and duration of this effect varied during several developmental stages. Despite this variation, decreased *Fmr1* expression during short windows of time in development was sufficient to impact the social behavior of pRNAi offspring. These results support that pRNAi could be a useful tool for the continued study of FXS in *Acheta domesticus*.

2. Methods

2.1 Insects

Acheta domesticus crickets were raised in house in groups of approximately 50. Crickets were maintained in large plastic containers and given free access to water and laboratory rat chow. Newly molted day 1 (D1) adults with intact body parts were isolated from the group and placed into individual clear round containers measuring 10cm wide by 8cm high. Incubators were kept on a 12h light:12h dark cycle with lights on at 06:00h and lights off at 18:00h and were maintained at a constant temperature of 29°C.

2.2 Cloning of Acheta domesticus Fmr1 Ortholog

The *A. domesticus* ortholog for Fragile X Mental Retardation 1 (Fmr1) was identified by performing a BLAST of our previously assembled *A. domesticus* transcriptome using CLC-bio Genomics Workbench 12 software (see Chapter 2). We used a *D. melanogaster* sequence as a

query to identify the ortholog for *A. domesticus* Fmr1 (DmFmr1, Fly Base ID: FBgn0028734). Then we performed a reciprocal BLAST on the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) database to confirm sequence orthology to the corresponding *D. melanogaster* gene.

In order to clone *A. domesticus Fmr1*, total RNA was extracted from four adult cricket brains using a Maxwell 16 LEV simplyRNA Tissue Kit (Promega). cDNA was synthesized from extracted RNA using an iScript Reverse Transcription Kit (Bio-Rad). CLC-bio Genomics Workbench software was used to design cloning primers to amplify a 716 base pair region of the *Fmr1* gene. The primer sequences were as follows: Fmr1 Forward; 5' TGTGGTGGAATATTT AG GTTGG - 3', Fmr1 Reverse; 5'- GAGGGATTGTGGGTTGTG - 3'. These primers were used in a polymerase chain reaction (PCR) to generate a DNA template for cloning. The PCR conditions were as follows: 4 min at 95°C; 35 cycles of 30 sec at 95°C, 30 sec at 58°C, and 40 sec at 72°C; 5 min at 72°C; and a hold at 4°C. PCR products were purified from an agarose gel using Freeze 'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad). Purified DNA was cloned into the PCR4-TOPO vector (PCR4-TOPO-TA Cloning Kit, Invitrogen), according to the manufacturer's protocol. Colony PCR was performed on the transformants and the products were run on an agarose gel to confirm that fragments of the appropriate size were present. A QIAprep Spin Miniprep Kit (Qiagen) was used to purify plasmid DNA, which was sent to Macrogen, Inc. (Rockville, MD) for sequencing. Vector NTI software was used to confirm that the sequence contained the 716bp region of the *Fmr1* gene.

2.3 Double-Stranded RNA Synthesis

In order to generate double-stranded RNA (dsRNA) for gene-specific knockdown of the *Fmr1* gene, plasmid DNA was purified using a QIAquick PCR Purification Kit (Qiagen). dsRNA was synthesized from purified DNA template using a MEGAScript T7 Transcription Kit (Invitrogen), and purified with a MEGAclear Transcription Clean-Up Kit (Invitrogen). dsRNA was run on an agarose gel and tested on a Nanodrop 2000 (Fisher Thermo Science) to determine the quality and concentration. In all experiments, DsRed2 dsRNA was used as the control. dsRNA for the gene-

specific knockdown of DsRed2 was synthesized from *Discosoma* sp. red fluorescent protein 2 and was a gift from Yoshinori Tomoyasu, Miami University (see Miller et al., 2012).

2.4 RNA Interference Experiments

2.4.1 Adult RNA Interference

On Day 1 (D1) of adulthood, undamaged female crickets were weighed immediately prior to injection with dsRNA. Females that fell within the weight range of 0.25-0.35 grams were used for dsRNA injection. Crickets were anesthetized for 5-7 min on ice before receiving a 2μL intra-abdominal injection of control (DsRed2) or experimental (Fmr1) dsRNA with a sterile 10μL Hamilton syringe. After injection, crickets were returned to individual containers and placed into the incubator. For adult RNAi studies, crickets were injected on D1 with 2.5μg, 3.5μg, or 7μg of dsRNA in order to determine the dose-dependent strength of the knockdown in the brain. The duration of the knockdown was determined by sacrificing control and experimental crickets at three time points, 2 days post-injection or 2dpi (D3), 8dpi (D9), or 29dpi (D30). RNAi was validated using quantitative Real-Time PCR (see section 2.5).

2.4.2 Parental RNA Interference

In order to optimize pRNAi in *Acheta domesticus*, we designed pRNAi experiments so we could determine which concentration of dsRNA caused the most effective knockdown, and what stage of reproductive development (D1 or D5) was optimal for injecting the adult female.

Undamaged D1 adult males and females were isolated from the colony, placed into individual containers, and then weighed as previously described (see section 2.4.1). Only female crickets falling into the correct weight range were injected with 2μL of control (DsRed2) or experimental (Fmr1) dsRNA. Injected females and uninjected, weight-matched males were returned to individual containers and placed into the incubator until their use in downstream assays.

Females were injected with 2.5μg, 3.5μg, or 7μg of control or experimental dsRNA on D1 of adulthood. We also injected an additional group of females with 3.5μg of control or experimental dsRNA on D5 of adulthood. All injected females were mated with uninjected, control males 9dpi (D10). There were 8 mating crosses in total: D1 2.5μg DsRed2 females x uninjected males, D1 2.5μg Fmr1 females x uninjected males, D1 3.5μg DsRed2 females x uninjected males, D1 3.5μg Fmr1 females x uninjected males, D1 7μg DsRed2 females x uninjected males, D1 7μg Fmr1 females x uninjected males, D5 3.5μg DsRed2 females x uninjected males, and D5 3.5μg Fmr1 females x uninjected males. Each mating cross consisted of 6 males and 6 females that were placed into a large plastic container with food and a damp cotton ball. All 6 females in a mating box were able to oviposit into the damp cotton ball for 4 days, and then the cotton ball was removed and placed into a separate round container and stored in the incubator so that the cotton ball could be monitored for hatched nymphs. As soon as the first cotton ball was removed, it was immediately replaced with a second damp cotton ball. Females were able to oviposit into the second cotton ball for 4 additional days. Then, this cotton ball was removed and placed into an individual round plastic container and returned to the incubator so it could be monitored for hatched nymphs.

After the second cotton ball was removed from the mating box, we immediately took these now D18 females and dissected their ovaries and counted the total number of eggs and determined the number of clear versus melanized eggs. Insect eggshells contain phenoloxidase and can become melanized (Li, 1994; Kim et al., 2005). We identified four different degrees of melanization ranging from light tan to black (Fig. 3B). These four primary phenotypes of melanized eggs were all classified as melanized and were assumed to be non-viable based upon previous studies showing that cricket eggs turn black when they are damaged, and do not hatch (Masaki, 1960). Females were anesthetized on ice for 5-7 min and then the head, wings, and appendages were removed. The thorax and abdomen were pinned in a dissecting dish and then cut down the dorsal midline to expose the ovaries. The number of eggs within the two dissected ovaries of each female were counted within 20 min of dissection and counts were performed blindly under a dissecting microscope. Representative ovaries and eggs from D18 females were photographed under a dissecting microscope equipped with a Canon SZX12 camera at a magnification of 31.5x.

After their removal from the mating boxes, cotton balls were assessed every two days to count and record the number of hatched nymphs. Cotton balls continued to be monitored until no nymphs hatched from the cotton ball for three consecutive checks (6 days). After the nymphs hatched and were counted, they were placed into large plastic containers and raised to adulthood. For each treatment group, nymphs were collected at every stage of development (1^{st} instar through 9^{th} instar) to determine how long the effects of pRNAi would last in offspring. Boxes were staged every day and all newly molted nymphs were removed to ensure that collected nymphs had molted within 24 h.

2.5 Quantitative Real Time PCR (qPCR)

For adult RNAi experiments, males and females were injected with 2.5μg, 3.5μg, or 7μg of dsRNA on D1 of adulthood and were sacrificed 2dpi (D3), 8dpi (D9), and 29dpi (D30). Crickets were anesthetized briefly on ice prior to brain dissection. The head was removed and pinned into a dissecting dish, and the brain was dissected from a small window cut into the head capsule. Dissected brains were immediately submerged into 200μL of cold homogenization buffer (Promega). The brains from 3 individual crickets were pooled for every treatment group. Pooled brain tissue was homogenized with a disposable pestle, vortexed for ~30 sec, and then stored at -80°C until RNA isolation.

For pRNAi experiments, nymphs were collected within 24 h of molting. Nymphs were briefly anesthetized on ice prior to dissection. The head was removed and immediately submerged into 200μL of cold homogenization buffer (Promega). For all stages of development, the heads of 4 individual crickets were pooled for every treatment group. Prior to the 7^{th} instar, the sex of nymphs cannot be distinguished. For the 7^{th} – 9^{th} instars, when sex could be determined, we pooled two males and two females for each treatment group. Pooled tissue was homogenized with a disposable pestle and then vortexed for ~30 sec, before being stored at -80°C.

For adult and pRNAi experiments, total RNA was extracted from the pooled tissues using a Maxwell 16 LEV simplyRNA Tissue Kit (Promega) in conjunction with an automated Maxwell 16 RNA Isolation system (Promega). A Nanodrop 2000 (Fisher Thermo Science) was used to

determine the concentration and quality of the extracted RNA. cDNA was synthesized from a 0.8µg RNA template using an iScript Reverse Transcription Kit (Bio-Rad) according to the manufacturer's protocol. Forward and reverse primer sets were designed using Integrated DNA Technologies (IDT) software, for Fmr1, 16s rRNA, and 18s rRNA (Table 1). 16s rRNA and 18s rRNA served as internal controls for all qPCR assays because they maintain uniform expression throughout development. qPCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) and a CFX Connect Real-Time PCR Detection System (Bio-Rad). PCR conditions were as follows: 3 min at 95°C; 40 cycles of 15 sec at 95°C, and 10 sec at 60°C; 10 sec at 95°C; 5 sec at 65°C; and a hold at 95°C. The amplification efficiency of each primer set was determined using serially diluted cDNA samples (Table 1). The comparative Ct method was used to determine the relative expression of target genes using CFX Manager software. For all experiments, every sample was run in triplicate (three technical replicates). For adult RNAi experiments, the relative expression of each target gene was determined by taking the mean expression of three independent runs (three biological replicates). For pRNAi experiments, the relative expression for each target gene was determined by taking the mean expression of two independent runs.

2.6 Double vs. Single dsRNA Injection Experiments

Previous studies have shown that you can prolong the efficacy of RNAi by continuously exposing experimental animals to dsRNA (Araujo et al., 2007; Coleman et al., 2015). However, repeated injections, in general, can have detrimental effects. We designed an experiment to determine the effects of a single injection of dsRNA versus a double injection of dsRNA on egg-laying capacity and nymph survival. For the single injection, D1 adult female crickets were injected with 3.5µg of DsRed2 (control) or Fmr1 (experimental) dsRNA, as previously described. On D10 of adulthood they were placed into a mating box with uninjected, age-matched control males. For the double injection, D1 adult females were injected with 3.5µg of DsRed2 (control) or Fmr1 (experimental) dsRNA. On D9 of adulthood, the last day prior to mating, they received a second injection with 3.5µg of DsRed2 (control) or Fmr1 (experimental) dsRNA. On D10 of adulthood they were placed into mating boxes with uninjected, age-matched males. Mating crosses were designed and carried out in the same manner as described above (see

section 2.4.2). Once cotton balls were removed from the mating boxes, the eggs in each ball were monitored for hatching. The number of hatched nymphs was counted and recorded every two days, until no nymphs hatched from the cotton ball for three consecutive checks (6 days).

2.7 Agonistic Behavior Experiments

We performed agonistic behavior experiments on the male offspring of pRNAi KD females that were injected with either 3.5μg or 7μg of dsRNA (see section 2.4.2). Males were weighed 2 days prior to behavioral trials and were assigned a partner with a weight difference of 10% or less. We performed agonistic trials on control pairs (DsRed2 *vs.* DsRed2) and experimental pairs (DsRed2 *vs.* Fmr1) when they reached D9 of adulthood. All behavioral experiments were performed at room temperature in a quiet, dimly lit room between 12:00h – 18:00h. Crickets were placed in the behavior room at least 1 h prior to the start of trials for acclimation. Trials were conducted in a clear, round Plexiglas arena measuring 15cm wide by 10cm high. Arenas were cleaned with 70% ethanol and allowed to air dry for ~2 min prior to each trial, and then a piece of white paper was placed on the bottom of the arena floor to provide traction. Each member of an age and weight-matched trial pair was carefully placed on opposite sides of an arena separated by a dark Plexiglas barrier standing ~10cm high. Pairs acclimated in the arena for 15 min before the barrier was lifted. Once the barrier was lifted, each trial pair was allowed to interact and establish social rank. Establishment of social rank occurred when one cricket chased the other cricket, produced a rival song, and made aggressive body jerking movements (winner/dominant), while the other cricket retreated (loser/subordinate) (Stevenson et al., 2000). After establishment of social rank, the pair was allowed to interact for an additional 5 min to confirm social rank. Behavioral trials were recorded with a Sony HDR-CX290 camera mounted above the arena and were projected onto a monitor. In between each trial, the arena and barrier were cleaned and dried, and a fresh piece of white paper was placed onto the arena floor to remove residual pheromones. Trials were evaluated by a researcher during the experiment and were re-examined at a later date by watching the recording.

The number of contacts to initiate a fight were recorded. Males had to physically separate by one body length and then come back together to be scored as another contact. We also recorded the

identity of the individual that initiated the agonistic behavior. The initiator was the cricket that approached the other male cricket and touched it with his antennae. The fight level and the identity of the winner and loser of the interaction was also recorded for each trial. Trials were scored according to Stevenson and colleagues (Stevenson et al., 2000), and consisted of seven different levels of interaction. Mutual avoidance (level 0) applied to interactions where both crickets avoided one another and showed no sign of aggressive behavior. This level of interaction was not observed during any of our behavioral trials. Instead, most agonistic trials reached one of the following levels of interaction: pre-established dominance (level 1) occurred when one cricket retreated from an approaching aggressor; antennal fencing (level 2) occurred when the antennae of both crickets made contact; unilateral mandible spreading (level 3) occurred when one cricket spread its mandibles while facing the other cricket; bilateral mandible spreading (level 4) occurred when both crickets spread their mandibles while facing one another; mandible engagement (level 5) occurred when the spread mandibles of both crickets were engaged; tactical combat (level 6) occurred when crickets climbed on top of each other or flipped the other cricket.

2.8 Statistical Analyses

Student's *t*-tests were performed to determine if there were significant differences ($p < 0.05$) in relative expression between treatment groups for qPCR assays. For agonistic behavior, Student's *t*-tests were conducted to determine if there were significant differences in the amount of time to first contact. A χ^2 analysis with Pearson's post-hoc test was used to determine if there were effects of treatment on initiation of agonistic interactions, social rank, and pre-established dominance. Comparisons of mean maximum fight level scores between groups at the same time point were analyzed with a one-way ANOVA followed by Dunnett's post-hoc test with $\alpha = 0.05$. We performed a three-way factorial ANOVAs to assess the effects of treatment, dose, and time of dsRNA injection on the number of total eggs. The data set was tested for normal distribution using a Kolmogorov-Smirnov normality test, and if necessary, data were log-transformed prior to analysis of variance. Since the interaction term Treatment x Dose x Time was not significant, we removed it from the model. We also found that the interaction term Dose x Time was not significant, so we removed it from the model. To assess the effects of treatment, dose, and day of

injection on the proportion of melanized eggs we performed two different statistical analyses. Beta Regression was used to model the mean proportion of melanized eggs based on the treatment and dose level. Since the interaction term Treatment x Dose was not significant, we removed it from the model. Then, we did a multiple comparison with Tukey's adjustment to check the difference effect for the proportion of melanized eggs on treatment by dose. We used the same strategy to model the proportion of melanized eggs on treatment and day by applying Beta Regression. We examined the significance of the interaction term Treatment x Day and found that it was significant. A Linear Regression with log transformation was applied to model the count of melanized eggs on treatment and day. Since the variance of residual was not constant, we used log transformation, and found Treatment x Day was also significant. Multiple comparison with Tukey's adjustment was applied to check the difference effect exited for melanized eggs on treatment by day at dose level 3.5μg for both proportion and count. All statistical analyses were performed using JMP Pro 14 (SAS Institute Inc., Cary, N.C.) software.

3. Results

3.1 Increasing the dose of dsRNA does not lead to a more effective knockdown in the brains of adult females

Adult RNAi was used to characterize the knockdown (KD) of *Fmr1* in the brain tissue of female crickets injected with 2.5μg, 3.5μg, or 7μg of dsRNA at 2dpi (D3), 8dpi (D9) and 20dpi (D21). Crickets are particularly suitable for RNAi studies because a single injection of dsRNA initiates systemic silencing of a target gene, which can last for more than two months in certain species (Moriyama et al., 2008; Tomioka et al., 2009). There was a statistically significant decrease in the relative expression of *Fmr1* in the brain of KD females injected on D1 with 2.5μg and dissected either 2dpi ($p = 0.018$), 8dpi ($p = 0.006$), or 29dpi ($p = 0.010$) (Fig. 1A). We also examined *Fmr1* expression in females injected with 3.5μg of dsRNA on D1 (Fig. 1B). Although expression levels were reduced by 35% (2dpi), 17% (8dpi), and 23% (29dpi), there was not a statistically significant decrease in *Fmr1* at this dose due to variability in the qPCR data for the three biological replicates. The relative expression of *Fmr1* in females injected with 7μg of dsRNA on D1 was also not significantly different from controls 2dpi, 8dpi, or 29dpi (Fig. 1C).

Overall, the effects of adult RNAi were quite variable in female brains and increasing the dose of dsRNA did not impact the strength or duration of the knockdown.

3.2 Repeated dsRNA injection decreases the number of parental RNAi offspring

Previous studies have shown that you can prolong the efficacy of RNAi by repeatedly injecting experimental animals with dsRNA (Coleman et al., 2015; Fishilevich et al., 2016). However, injections can have detrimental effects on an organism. In both planarians (Rouhana et al., 2013) and house flies (Sanscrainte et al., 2018), injections with dsRNA can cause tissue damage and injury-related mortalities. Since our ultimate goal was to raise pRNAi offspring, we examined the impact of multiple injections on the number of pRNAi nymphs that hatched. We counted the total number of nymphs that hatched from eggs laid by females that were injected once, and found that a single injection with either control DsRed2 or Fmr1 dsRNA produced a similar number of offspring (Fig. 2). We also counted the number of nymphs that hatched from females that had received a second injection of dsRNA. There was a large decrease in the number of offspring when compared to treatment-matched offspring from females that received a single injection on D1 (Fig. 2). Furthermore, females that received a second injection of Fmr1 dsRNA produced 50% fewer viable offspring than double-injected control females. These findings support that multiple dsRNA injections greatly reduce the number of offspring produced, and that this decrease was enhanced in females injected with Fmr1 dsRNA.

3.3 A single injection of Fmr1 double-stranded RNA has detrimental effects on eggs

In order to optimize pRNAi in *Acheta domesticus* we assessed the effects of treatment, dose, and day of injection on the proportion of clear versus melanized eggs in the ovaries of D18 adult females (Fig. 3A). As we dissected the ovaries apart, we were able to identify five different egg phenotypes. In addition to clear eggs, we identified four primary phenotypes of melanized eggs ranging from light tan to black (Fig. 3B). The chorion of insect eggs contains phenoloxidase and can become melanized (Li, 1994; Kim et al., 2005). These four primary phenotypes of melanized eggs were all classified as melanized and were assumed to be non-viable based upon previous studies showing that cricket eggs turn black just before they die, in response to damage (Masaki,

1960). We counted the total number of eggs within both ovaries for each female and determined if treatment, dose, or time of injection affected the number of eggs (Fig. 3C). A three-way factorial ANOVA was conducted on the influence of three independent variables: treatment, dose, and time on the total number of eggs. We did not find any significant interactions, indicating that the total number of eggs was unaffected (Table 2). Finally, we examined the proportion of clear versus melanized eggs and found that treatment alone caused a significant difference in the proportion of melanized eggs, regardless of the concentration (Fig 3D). *Fmr1* KD females had significantly more melanized eggs than control females injected with 2.5μg ($p < 0.0001$), 3.5μg ($p < 0.0001$), or 7μg ($p < 0.0001$) of dsRNA (Table 3). When we compared the proportion of melanized eggs between females injected on D1 and D5, we found that *Fmr1* KD females injected on D1 had significantly more melanized eggs than controls ($p = 0.0014$). There was no difference in the proportion of melanized eggs between females injected on D5 and controls (Table 4). These results support that a single injection of Fmr1 dsRNA can impact the viability of eggs in the ovaries of injected females.

3.4 Increased concentrations of dsRNA decrease the number of hatched offspring

After examining the effects of different doses on egg melanization, we counted the total number of nymphs that hatched from eggs laid by females that were injected with 2.5μg, 3.5μg, or 7μg on D1, or 3.5μg on D5. We found that females that received a single injection of 2.5μg or 3.5μg on D1 produced a similar number of Fmr1 offspring (Fig. 4). However, when the dose was increased to 7μg Fmr1 dsRNA there was a drastic reduction in the number of Fmr1 nymphs that hatched. For all concentrations, females injected with control dsRNA on D1 produced more offspring than females injected with the same dose of Fmr1 dsRNA, suggesting that Fmr1 KD is sufficient to reduce the number of offspring that hatch. We also counted the number of nymphs that hatched from females that were injected on D1 or D5 with the same dose of dsRNA to determine if the day of dsRNA injection would affect the total number of offspring. Females injected with Fmr1 dsRNA on D5 did not produce fewer nymphs than females injected on D1, however, females injected with control dsRNA on D5 did produce fewer hatchlings than the control females injected on D1.

3.5 The relative expression of Fmr1 is reduced in offspring following parental RNAi

We determined the efficacy of RNAi in the F1 generation of experimental and control females by examining the relative expression of *Fmr1* during every stage of nymphal development (1st – 9th instars). Females were injected with 2.5μg, 3.5μg, or 7 μg of control or Fmr1 dsRNA on D1 of adulthood, or 3.5μg of control or Fmr1 dsRNA on D5 of adulthood, so that we could assess the effects of dose and timing of dsRNA application. When females were injected with 2.5μg of dsRNA on D1 there was a significant decrease in *Fmr1* expression during the 2nd instar (p = 0.028) and 5th instar (p = 0.002) when compared to controls (Fig. 5A). There was no change in expression during any other stage of development for these offspring. The progeny of females injected with 3.5μg of dsRNA had a significant decrease in *Fmr1* expression during the 2nd (p = 0.023) and 3rd (p = 0.040) instars (Fig. 5B). During the 7th instar, there was a significant increase in *Fmr1* expression (p = 0.035). When females were injected with 7μg of dsRNA, *Fmr*1 expression was decreased during the 6th instar (p = 0.027) in the pRNAi offspring (Fig. 5C). The progeny of females injected on D5 with 3.5μg did not experience a significant decrease in *Fmr1* expression during any stage of development, however, there was a significant increase in expression during the 3rd (p = 0.018) and 4th (p = 0.023) nymphal instars (Fig. 5D). Collectively, these results support that the RNAi effect was transmitted to the next generation, and the expression of *Fmr1* was variable throughout the different stages of development.

3.6 Agonistic behavior of 3.5μg parental RNAi offspring

Agonistic behavior follows a highly stereotyped sequence of increasingly aggressive motor acts that is triggered by chemosensory antennal contact (Adamo and Hoy, 1995; Stevenson et al., 2000; Hofmann and Schildberger, 2001). The only time that antennal contact does not occur is during mutual avoidance (level 0), when both crickets avoid one another and display no signs of aggressive behavior. We did not observe mutual avoidance during behavioral trials conducted on 3.5μg pRNAi offspring, so all 116 pairs engaged in agonistic behavior, and we determined the average time to first physical contact for these pairs. The average time for control pairs (DsRed2 *vs.* DsRed2) to make contact was 102 ± 11 s, while the average time for experimental pairs was 42 ± 10 s (Fig. 6A). These results showed that experimental pairs took significantly less time to

make physical contact during a behavioral trial ($p = 0.009$). After antennal contact, the fights followed a stereotyped sequence of aggressive acts that were scored. We determined the number of pairs exhibiting pre-establishment of social rank (level 1), where one male shows behaviors of social dominance, and the other male shows behaviors associated with subordinance, upon initial physical contact. We found that the number of control pairs that exhibited pre-establishment (10/56 fights) was not significantly different from the number of experimental pairs exhibiting pre-establishment (11/60 fights; $\chi^2 = 0.103$, $p = 0.748$). There was also no significant difference in the mean maximum fight level reached by control and experimental pairs (Fig. 6B; $F_{1,114} = 0.032$, $p = 0.858$). Taken together, these results support that pRNAi may have an effect on the *Fmr1* males level of activity or motivation to engage in an agonistic interaction since the time to first contact was significantly shorter in experimental pairs.

Next, we looked at agonistic interactions between experimental pairs to determine if the motivation to initiate a fight or ability to win a fight was affected in pRNAi offspring. The initiator made the first aggressive action by approaching the other cricket, followed by antennal touching if the fight escalated. We found that *Fmr1* pRNAi males initiated fights just as often as controls ($\chi^2 = 0.834$, $p = 0.361$) (Fig. 6C), indicating that their motivation to fight was not impacted. Finally, we determined the percentage of fights won by each treatment in an experimental pairing (Fig. 6D). *Fmr1* pRNAi males won agonistic interactions just as often as controls ($\chi^2 = 0.533$, $p = 0.465$), indicating that a pRNAi KD does not impact the outcome of a fight.

3.7 Agonistic behavior of 7μg parental RNAi offspring

We examined agonistic behavior in the pRNAi offspring of females injected with a larger 7μg dose of dsRNA. As with the 3.5μg pRNAi fights, we did not observe mutual avoidance during any trials conducted on 7μg pRNAi offspring. All 104 pairs engaged in agonistic behavior, and the average time for the males of control pairs (DsRed2 *vs.* DsRed2) to make contact was 92 ± 12 s, which was not significantly different from that of experimental pairs (85 ± 11 s; $p = 0.717$; Fig. 7A). After antennal contact, male interactions could escalate, and all trials were scored as to the maximum fight level reached by each pair in order to determine if experimental pairings

showed different levels of aggression than control pairings. First, the number of pairs exhibiting pre-establishment of social rank (level 1) was not significantly different for the two groups ($\mathcal{X}^2$ = 0.308, p = 0.579). Only 12/51 (24%) of control pairs and 15/53 (28%) of experimental pairs exhibited pre-establishment. In addition, the average maximum fight level reached by control and experimental pairs (Fig. 7B) was not significantly different ($F_{1,102}$ = 0.423, p = 0.517). Taken together, these findings support that pRNAi did not affect the aggression of 7µg *Fmr1* offspring.

Next, we looked at agonistic interactions between experimental pairs to determine if there was an impact on fight initiation or social status in *Fmr1* pRNAi crickets (Fig. 7C, D). We found that *Fmr1* males initiated fights significantly less often than did the control males of these pairings ($\mathcal{X}^2$ = 10.906, p = 0.001; Fig. 7C)). In addition, despite initiating fights significantly less often than controls, *Fmr1* males were just as likely as control males to become dominant during these agonistic trials ($\mathcal{X}^2$ = 1.247, p = 0.536; Fig. 7D), indicating that decreased *Fmr1* does not impact the outcome of a fight. These results support that, while decreased *Fmr1* may affect a male's motivation to engage in a fight, these males were just as aggressive and likely to become dominant as control males.

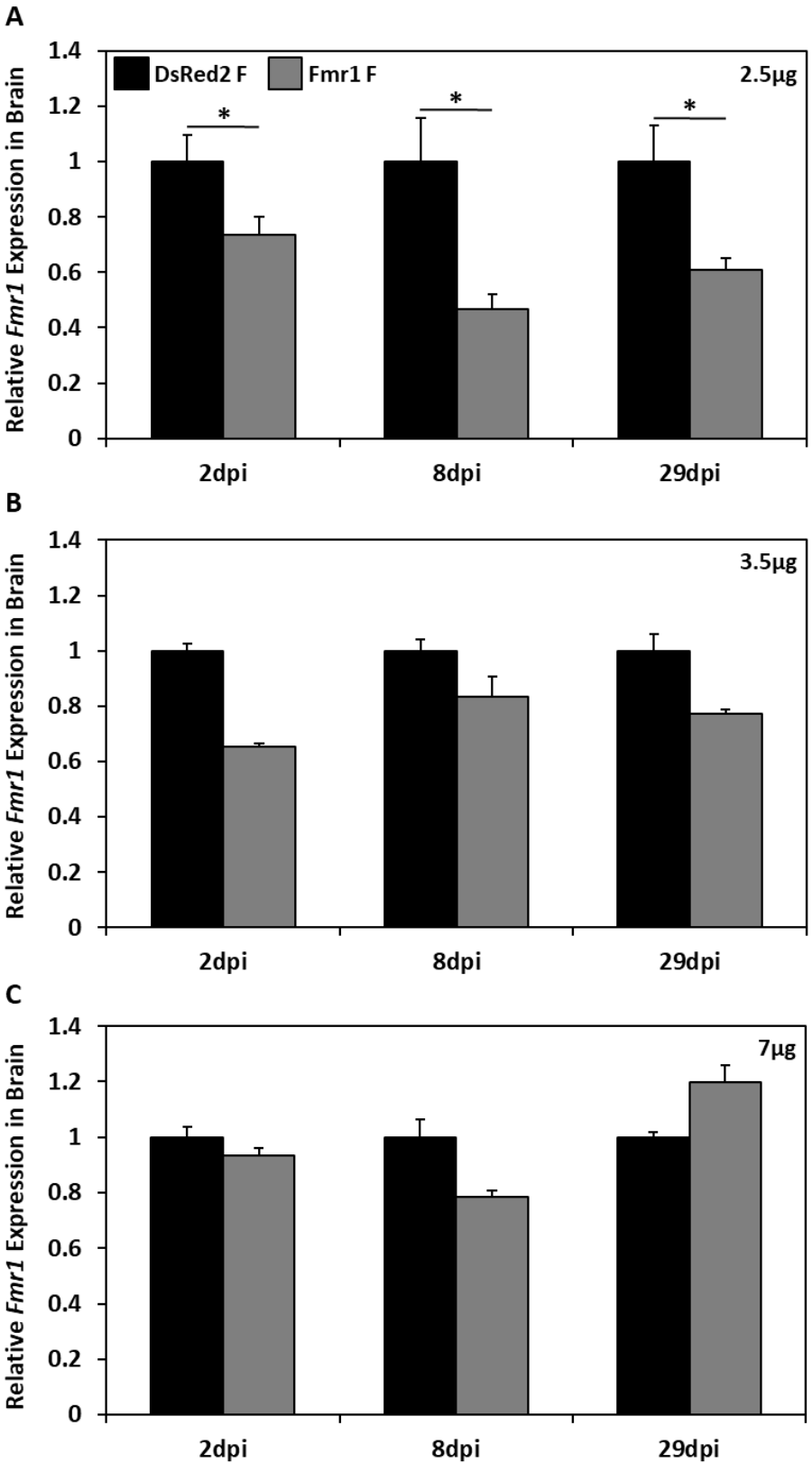
A
DsRed2 F
Fmr1 F
2.5µg
Relative Fmr1 Expression in Brain
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
2dpi
8dpi
29dpi
B
3.5µg
Relative Fmr1 Expression in Brain
2dpi
8dpi
29dpi
C
7µg
Relative Fmr1 Expression in Brain
2dpi
8dpi
29dpi

Figure 1

Figure 1. *Fmr1* expression is decreased in the adult female brain. Relative *Fmr1* mRNA expression in the brain of adult females (A-C) as determined by quantitative Real-Time PCR (qPCR). Crickets were injected with 2.5μg (A), 3.5μg (B), or 7μg (C) of DsRed2 dsRNA (negative control) or Fmr1 dsRNA (experimental) on Day 1 of adulthood and the relative expression was determined 2 days post-injection (2dpi), 8dpi, or 29dpi. (A) Females injected with 2.5μg of Fmr1 dsRNA had a significant reduction in *Fmr1* expression in the brain 2dpi ($p = 0.018$), 8dpi ($p = 0.006$), and 29dpi ($p = 0.010$). There was not a significant change in expression at any timepoint examined following either a 3.5μg (B) or 7μg injection (C) of dsRNA. Relative mRNA expression is the average of three independent runs (biological triplicates) ± SEM. Significance is denoted by an asterisk, Student's T-test, $p < 0.05$.

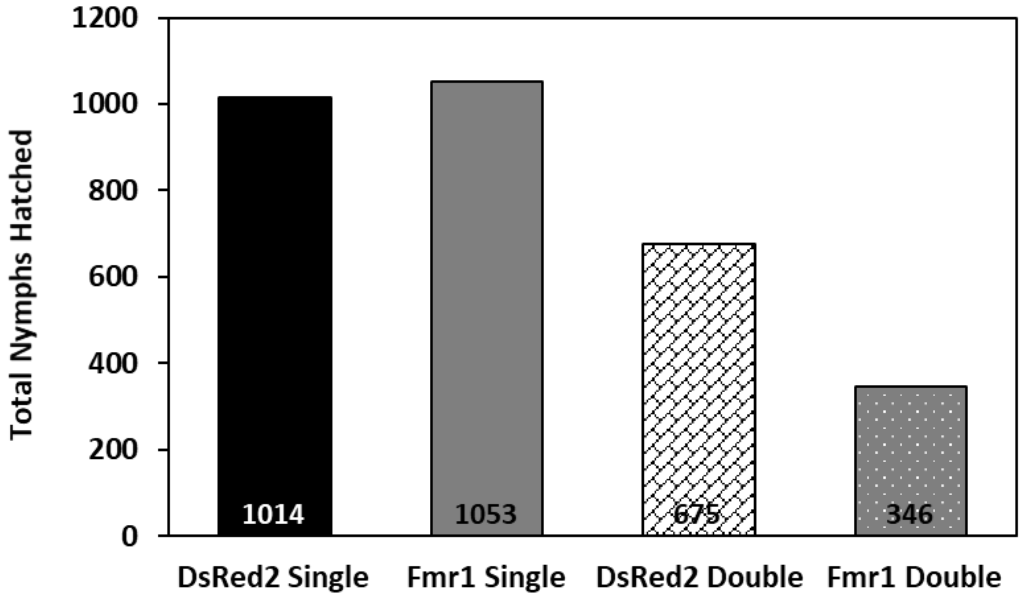
1200
1000
800
600
400
200
0
Total Nymphs Hatched
1014
1053
675
346
DsRed2 Single
Fmr1 Single
DsRed2 Double
Fmr1 Double

Figure 2

Figure 2. Repeated dsRNA injection decreases the number of pRNAi hatchlings. Females received a single (D1) or double (D1 and D9) injection of 3.5μg of DsRed2 (control) or Fmr1 (experimental) dsRNA and then the total number of nymphs that hatched were counted. A second injection with dsRNA greatly reduced the number of offspring. Bars represent the total number of nymphs hatched for each treatment group.

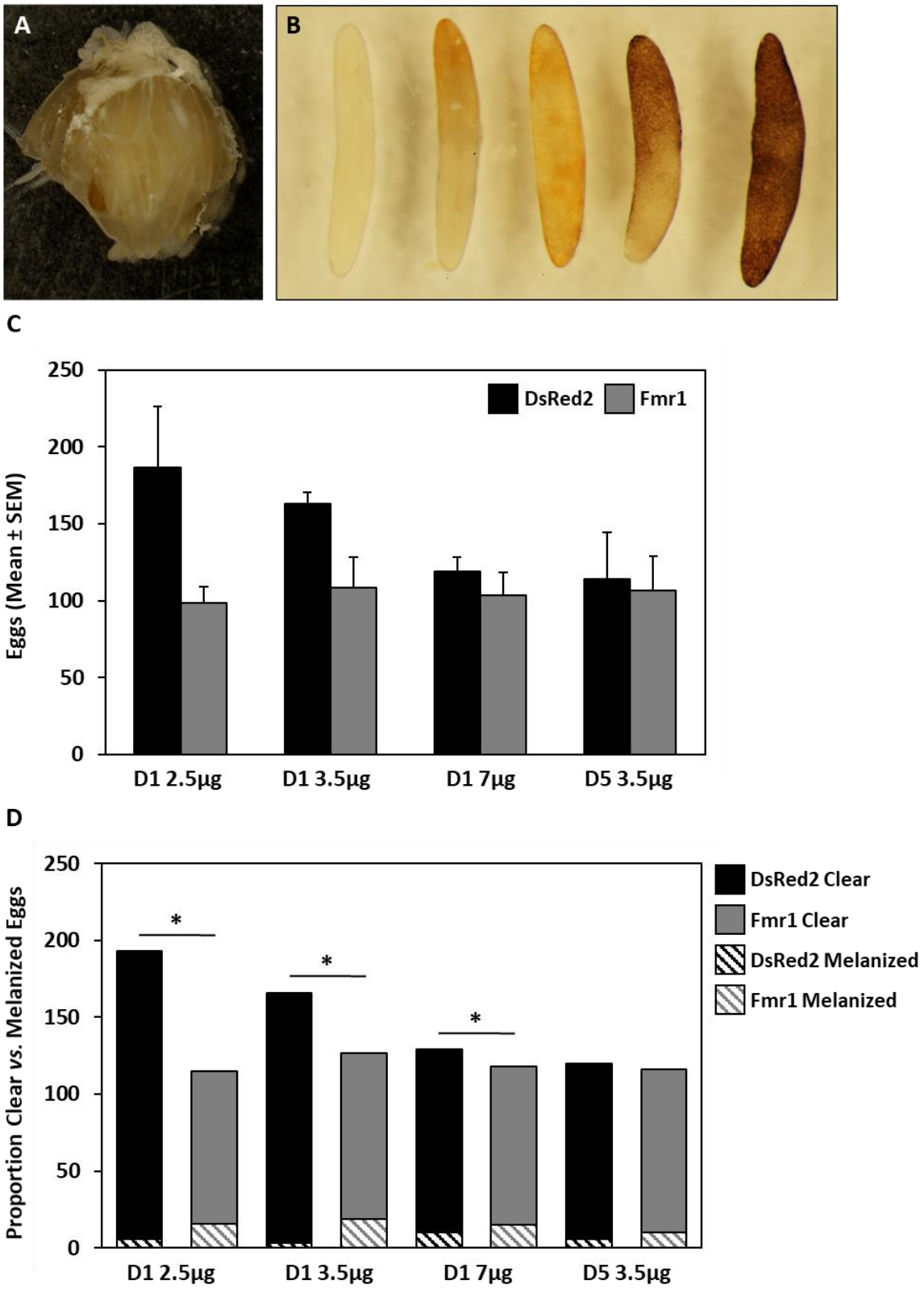
A
B
C
250
200
150
100
50
0
Eggs (Mean ± SEM)
DsRed2
Fmr1
D1 2.5µg
D1 3.5µg
D1 7µg
D5 3.5µg
D
250
200
150
100
50
0
Proportion Clear vs. Melanized Eggs
*
*
*
DsRed2 Clear
Fmr1 Clear
DsRed2 Melanized
Fmr1 Melanized
D1 2.5µg
D1 3.5µg
D1 7µg
D5 3.5µg

Figure 3

Figure 3. A single injection of Fmr1 double-stranded RNA has detrimental effects on egg viability. (A) Representative image of an ovary dissected from a female on D18 of adulthood. Both ovaries were dissected from each female and the total number of eggs counted. A single melanized egg is visible in this ovary. (B) Ovaries contained five different egg phenotypes including clear eggs (far left) and four phenotypic variations of melanized eggs. (C) A three-way multifactorial analysis of variance was conducted to assess the effects of treatment, dose, and time of injection on the number of eggs in the ovaries. There was not a significant effect of any of these variables on the number of eggs. *P*-values for the significance of main and interactive effects are shown in Table 2. Bars represent the mean number of eggs ± SEM in the ovaries of six D18 adult females. (D) An injection with Fmr1 dsRNA significantly increased the proportion of melanized eggs in the ovaries of D18 females when compared with controls, regardless of concentration. Bars represent the proportion of total eggs that were clear and melanized for each treatment group and asterisk indicates significant differences in the proportion of melanized eggs ($p < 0.05$).

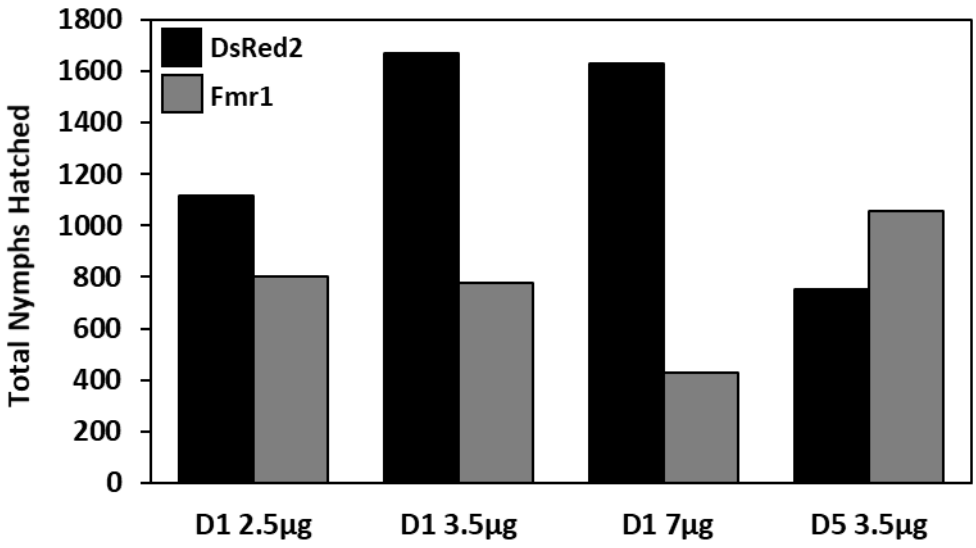
1800
1600
1400
1200
1000
800
600
400
200
0
Total Nymphs Hatched
DsRed2
Fmr1
D1 2.5µg
D1 3.5µg
D1 7µg
D5 3.5µg

Figure 4

Figure 4. Increased concentrations of dsRNA decrease the number of offspring. Females received a single injection with 2.5μg, 3.5μg, or 7μg of dsRNA on D1 or D5 of adulthood and the total number of nymphs that hatched from matings with control males were counted. A single injection of 2.5μg or 3.5μg produced a similar number of Fmr1 offspring, and when the dose was increased to 7μg there was a drastic reduction in the number of Fmr1 nymphs. We also counted the number of nymphs that hatched from females injected on D1 and D5 with the same dose of dsRNA to determine if the day of dsRNA injection would affect the total number of offspring. An injection on D5 did not affect the number of Fmr1 nymphs that hatched when compared to an injection on D1 with the same dose, but the number of control nymphs that hatched were drastically reduced with an injection on D5.

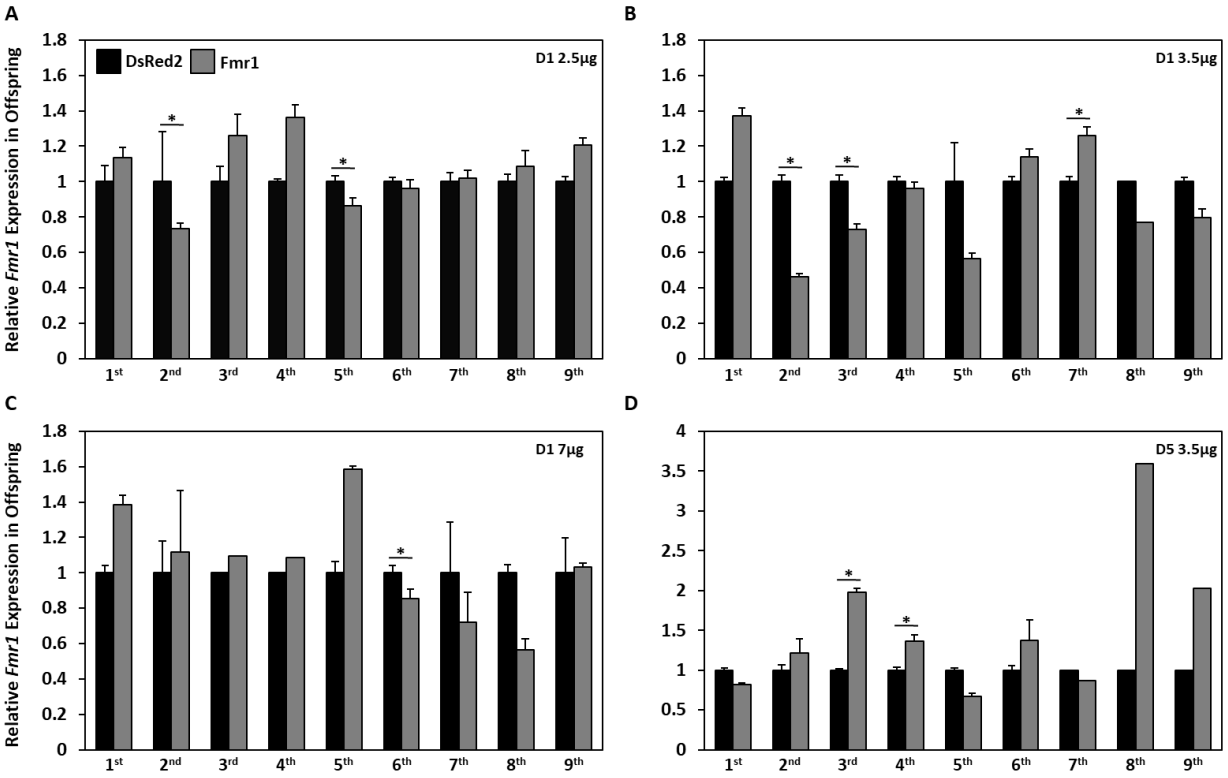
A
B
C
D
DsRed2
Fmr1
D1 2.5µg
D1 3.5µg
D1 7µg
D5 3.5µg
Relative Fmr1 Expression in Offspring
1st
2nd
3rd
4th
5th
6th
7th
8th
9th

Figure 5

Figure 5. Relative *Fmr1* mRNA expression in pRNAi offspring. Females were injected with 2.5μg (A), 3.5μg (B), or 7 μg (C) of DsRed2 dsRNA or Fmr1 dsRNA on D1 of adulthood, or 3.5μg of dsRNA on D5 of adulthood (D) and the relative expression of *Fmr1* was determined in offspring during every stage of nymphal development (1^{st} – 9^{th} instars). (A) There was a significant decrease in *Fmr1* expression during the 2^{nd} instar ($p = 0.028$) and 5^{th} instar ($p = 0.002$) when compared to age-matched controls. There was not a significant change in expression during any other stage of development in the offspring of females injected with 2.5μg of dsRNA. (B) When adult females were injected with 3.5μg of dsRNA, offspring had a significant decrease in expression during the 2^{nd} ($p = 0.023$) and 3^{rd} ($p = 0.040$) instars. During the 7^{th} instar there was a significant increase in *Fmr1* expression ($p = 0.035$). (C) *Fmr*1 expression was decreased during the 6^{th} instar ($p = 0.027$) in the offspring of females injected with the largest dose of dsRNA. (D) There was a significant increase in the relative expression of *Fmr1* during the 3^{rd} ($p = 0.018$) and 4^{th} ($p = 0.023$) instars when females were injected on D5 of adulthood. Relative mRNA expression is the average of two independent runs (biological duplicates) ± SEM. Only one run is represented for: D1 3.5μg 8^{th} instar, D1 7μg 3^{rd} and 4^{th} instars, and D5 3.5μg 8^{th}-9^{th} instars. Significance is denoted by an asterisk, Student's T-test, $p < 0.05$.

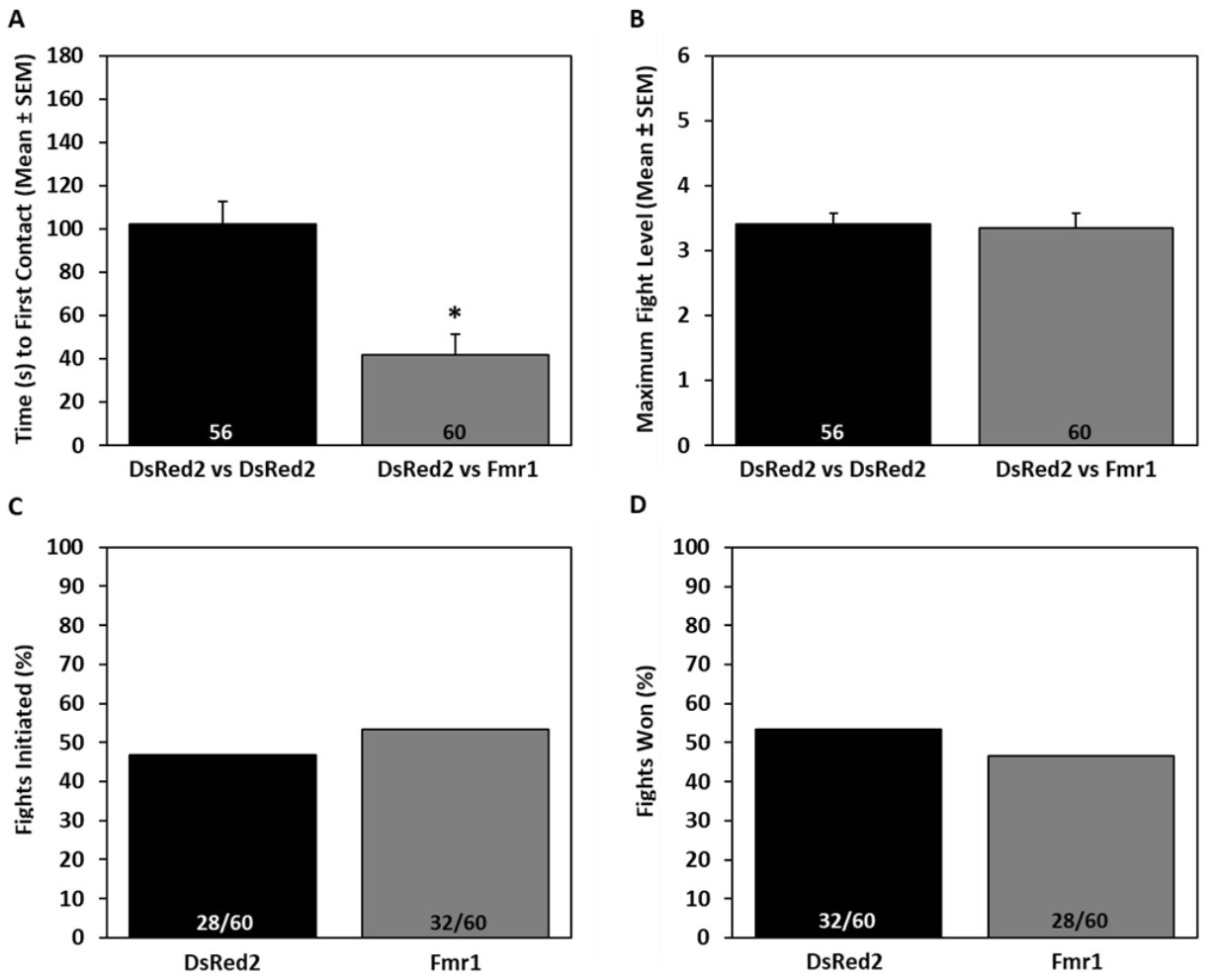
A
Time (s) to First Contact (Mean ± SEM)
180
160
140
120
100
80
60
40
20
0
*
56
60
DsRed2 vs DsRed2
DsRed2 vs Fmr1
B
Maximum Fight Level (Mean ± SEM)
6
5
4
3
2
1
0
56
60
DsRed2 vs DsRed2
DsRed2 vs Fmr1
C
Fights Initiated (%)
100
90
80
70
60
50
40
30
20
10
0
28/60
32/60
DsRed2
Fmr1
D
Fights Won (%)
100
90
80
70
60
50
40
30
20
10
0
32/60
28/60
DsRed2
Fmr1

Figure 6

Figure 6. Agonistic behavior of 3.5μg parental RNAi offspring. The male offspring of females injected with 3.5μg of dsRNA were raised to adulthood and underwent behavioral trials on D9 of adulthood. (A) The average time to first contact during an agonistic trial was significantly lower for experimental pairs (DsRed2 *vs.* Fmr1) than control (DsRed2 *vs.* DsRed2) pairs ($p = 0.009$). (B) The maximum fight level reached during agonistic trials was not significantly different between control and experimental pairs ($F_{1,114} = 0.032$, $p = 0.858$). The average fight level included pairs reaching fight levels ranging from pre-established dominance (level 1) to tactical combat (level 6). For A and B, bars represent the mean ± SEM and the numbers within bars indicate sample sizes for each treatment group. (C) The percentage of fights initiated by DsRed2 or *Fmr1* males from experimental pairs. The percentage of fights initiated did not differ between control and *Fmr1* pRNAi males ($\chi^2 = 0.834$, $p = 0.361$). (D) The percentage of fights won did not differ between control and *Fmr1* pRNAi males ($\chi^2 = 0.533$, $p = 0.465$). Experimental pairs examined in C and D are the same experimental pairs from A and B. For C and D, bars represent the percentage of initiations or wins for the indicated group. The numbers within bars indicate the number of initiations/wins for the indicated group followed by the total number of agonistic trials.
Significant differences ($p < 0.05$) between groups denoted by an asterisk *.

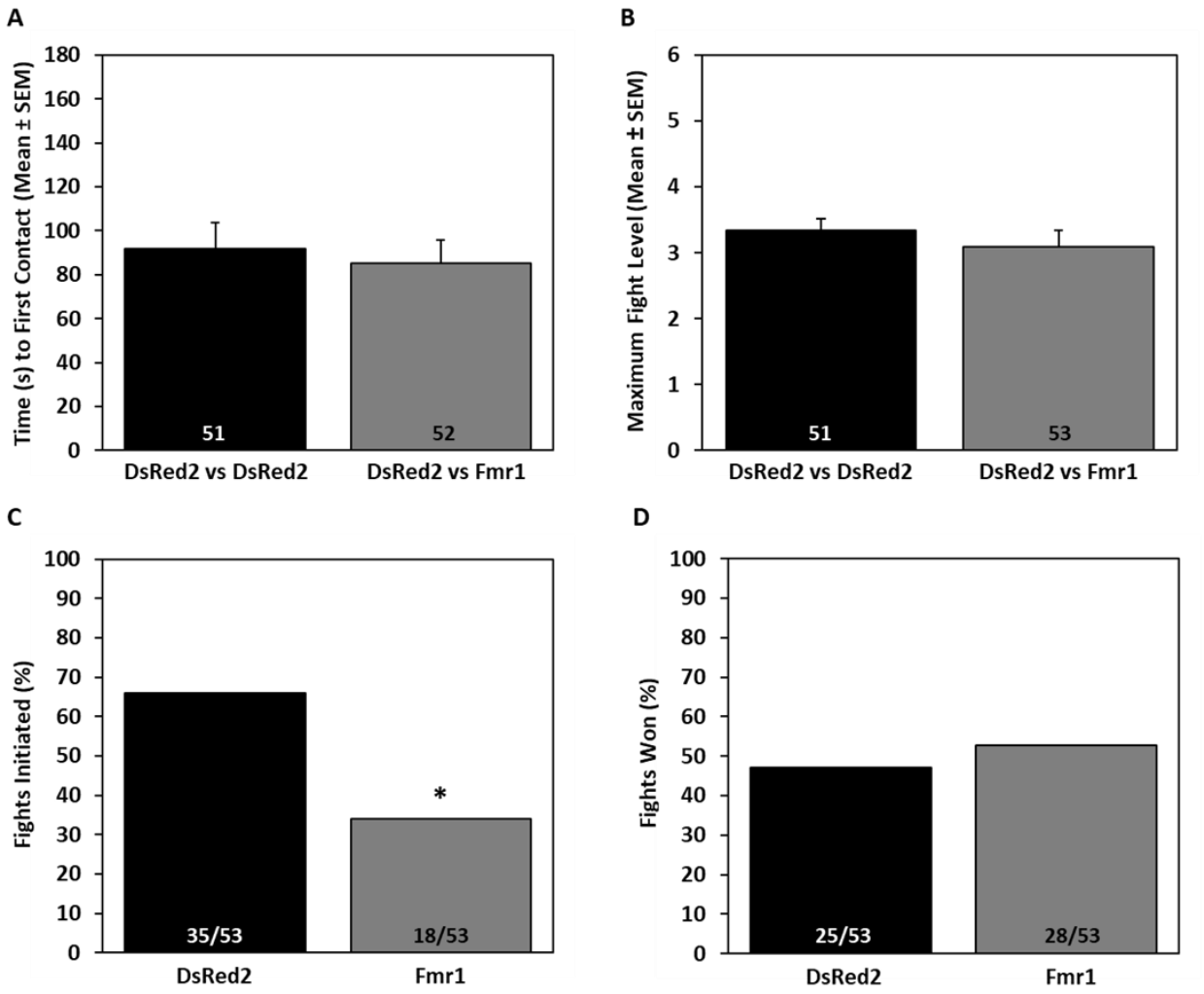
A
Time (s) to First Contact (Mean ± SEM)
180
160
140
120
100
80
60
40
20
0
51
52
DsRed2 vs DsRed2
DsRed2 vs Fmr1
B
Maximum Fight Level (Mean ± SEM)
6
5
4
3
2
1
0
51
53
DsRed2 vs DsRed2
DsRed2 vs Fmr1
C
Fights Initiated (%)
100
90
80
70
60
50
40
30
20
10
0
*
35/53
18/53
DsRed2
Fmr1
D
Fights Won (%)
100
90
80
70
60
50
40
30
20
10
0
25/53
28/53
DsRed2
Fmr1

Figure 7

Figure 7. Agonistic behavior of 7μg parental RNAi offspring. The male offspring of females injected with 7μg of dsRNA were raised to adulthood and underwent behavioral trials on D9 of adulthood. (A) The average time to first contact during an agonistic trial did not differ between experimental pairs (DsRed2 *vs*. Fmr1) and control (DsRed2 *vs.* DsRed2) pairs ($p = 0.717$). (B) The maximum fight level reached during agonistic interactions was not significantly different between control and experimental pairs ($F_{1,102} = 0.423$, $p = 0.517$). The average fight level included pairs reaching fight levels ranging from pre-established dominance (level 1) to tactical combat (level 6). For A and B, bars represent the mean ± SEM and numbers within bars indicate sample sizes for each treatment group. (C) *Fmr1* pRNAi males initiated fights significantly less often than control males ($\chi^2 = 10.906$, $p = 0.001$). (D) The percentage of fights won did not differ between control and *Fmr1* pRNAi males ($\chi^2 = 1.247$, $p = 0.536$). For C and D, bars represent the percentage of initiations or wins for the indicated group. The numbers within bars indicate the number of initiations/wins for the indicated group followed by the total number of agonistic trials. Significant differences ($p < 0.05$) between groups denoted by an asterisk *.

Table 1. Primers used for qPCR analysis.

Primer	Sequence	Amplicon	Efficiency
16s rRNA F	CTTCTCGTCCCCTATTTTCATTTG	150 bp	95.1%
16s rRNA R	GGTATATTGACCGTGCAAAGG		
18s rRNA F	TGTTTTCCTTGCTCGAGAGG	112 bp	96.1%
18s rRNA R	AATGATTCCCGCTTACTAGGC		
Fmr1 F	CTCATGGGTTTGGCAATTGG	96 bp	100.9%
Fmr1 R	GCACGAGTTTTCTTCCAGTTC		

Table 2. Three-way analysis of variance assessing the effects of treatment, dose, and time on total number of eggs.

Source of Variation	**d.f.**	**F Ratio**	***P* Value**
Treatment	1, 40	1.7757	0.1902
Dose	2, 40	1.1615	0.3233
Time	1, 40	1.3636	0.2498
Treatment x Dose	2, 40	1.3721	0.2652
Treatment x Time	1, 40	1.1587	0.2882

Significant effects ($P < 0.05$) are shown in bold. d.f., degrees of freedom

Table 3. The effects of treatment and dose on the proportion of clear versus melanized eggs.

Dose	Treatment	Estimated Proportion (Standard Error)	Contrast	Estimated Proportion Difference (Standard Error)	*P*-value
2.5μg	DsRed2	0.045 (0.101)	DsRed2 – Fmr1	-0.128 (0.023)	**<0.0001**
	Fmr1	0.173 (0.026)			
3.5μg	DsRed2	0.040 (0.010)	DsRed2 – Fmr1	-0.115 (0.023)	**<0.0001**
	Fmr1	0.156 (0.027)			
7μg	DsRed2	0.045 (0.011)	DsRed2 – Fmr1	-0.128 (0.023)	**<0.0001**
	Fmr1	0.173 (0.026)			

Significant effects ($P < 0.05$) are shown in bold.

Table 4. The effects of treatment and day of injection on the proportion of clear versus melanized eggs.

Day	Gene	Estimated Proportion (Standard Error)	Contrast	Estimated Proportion Difference (Standard Error)	P-value
1	DsRed2	0.038 (0.014)	DsRed2 – Fmr1	-0.133 (0.037)	**0.0004**
	Fmr1	0.170 (0.035)			
5	DsRed2	0.070 (0.020)	DsRed2 – Fmr1	-0.027 (0.031)	0.3874
	Fmr1	0.097 (0.024)			

Significant effects ($P < 0.05$) are shown in bold.

4. Discussion

In the present study, we evaluated the parameters required to generate a successful pRNAi response in the cricket *Acheta domesticus*. Double-stranded RNA was injected into females on the first day of adulthood, and the efficacy of RNAi was evaluated at several time points post-injection. These females were permitted to mate with control males and the RNAi efficacy was monitored through each stage of nymphal development in the F_1 offspring to assess parental transmission. Once the pRNAi offspring reached adulthood, we examined their agonistic behavior to determine the functional effect of decreased *Fmr1* during development. This represents the first report of parental RNAi transmission to the next generation that also resulted in a functional effect in *Acheta domesticus* male crickets.

We used RNAi to decrease *Fmr1* expression in adult females and determined the temporal endurance of the RNAi effect at three time points post-injection (2dpi, 8dpi, and 29dpi). Several studies have demonstrated that crickets are highly susceptible to RNAi, because a single injection of dsRNA initiated systemic down-regulation of a target gene, and the effects lasted for more than two months (Moriyama et al., 2008; Tomioka et al., 2009). Here we demonstrated that RNAi against *AdFmr1* significantly reduced mRNA expression in the adult female, and the effects lasted for at least 30 days. In a previous study, we found that an injection with 7μg of Fmr1 dsRNA on D1 of adulthood caused a significant decrease in *Fmr1* expression in male brains 2dpi (see Chapter 3). By 8dpi, there was no longer a decrease in *Fmr1* expression in the brain, suggesting that RNAi does not always have long-lasting effects. In a similar study, Coleman et al. (2015) examined the persistence of RNAi efficacy in the green peach aphid *Myzus persicae*. Initially, gene expression was significantly reduced in the aphid, but within six days of dsRNA exposure, gene expression had returned to wildtype levels (Coleman et al., 2015). We did not determine the endurance of the RNAi effect past 30 days, but it would be interesting to see how long the effects of a single injection can last.

We also investigated if larger concentrations of dsRNA injected into adult females could impact the strength of the gene knockdown that resulted in those females. Surprisingly, injecting a larger dose did not increase the strength of the knockdown, overall the larger concentrations produced

weaker effects. Unlike our findings, a previous study in the red flour beetle *Tribolium castaneum* found that the efficiency of RNAi correlated with the dose of dsRNA injected into larvae (Miller et al., 2012). Similar to our findings, an RNAi study in the blood feeder *Rhodnius prolixus* showed that doubling the concentration of dsRNA injected into 3rd instar nymphs did not have an additive effect (Paim et al., 2013). Taken together, these findings support that RNAi is highly variable across insect species, reconfirming the need to identify the parameters that are important for successful RNAi.

In order for RNAi effects to be passed on to the next generation, there needs to be enough dsRNA available to be spread to the germline cells. The duration of RNAi efficacy varies across insect species, and may last longer in some species because the dsRNA is not depleted as quickly (reviewed in Belles, 2010; Cooper et al., 2018). In the red flour beetle *Tribolium castaneum*, pRNAi is highly efficient with nearly 100% of the F_1 progeny displaying an RNAi phenotype after the first egglay (Bucher et al., 2002). The number of offspring displaying an RNAi phenotype gradually decreases, as does the strength of the phenotype in preceding egglays, supporting that dsRNA is being depleted (Bucher et al., 2002). In some insects, such as the western corn rootworm and the brown stink bug, the effects of pRNAi can be extended by repeated exposure of the female to dsRNA through feeding or injection (Coleman et al., 2015; Fishilevich et al., 2016). We wanted to determine if repeated injections of dsRNA could be administered without negatively impacting other experimental parameters, such as the number of F_1 progeny. We found that a second injection of dsRNA drastically decreased the number of nymphs that hatched and, furthermore, a second injection with Fmr1 dsRNA produced 50% fewer progeny than a second injection with control dsRNA. These findings support that multiple injections with dsRNA greatly reduce the number of offspring produced. Since our ultimate goal was to raise F_1 progeny for use in future experiments, we decided not to move forward with repeated exposure to dsRNA through injection. Studies have shown that pRNAi can be achieved in insects through repeated ingestion of dsRNA (Fishilevich et al., 2016), so an alternative method of dsRNA application may prove to be useful in the cricket, and future studies will explore the possibility of administering dsRNA through feeding.

We also examined the dose effect on the number of progeny, to see if higher doses of dsRNA would impact the number of nymphs produced in the F_1 generation. When we injected 2.5μg or 3.5μg of dsRNA into adult females, more control nymphs hatched than did *Fmr1* nymphs, but the number of *Fmr1* nymphs did not differ between the two doses of dsRNA injected. However, an injection with 7μg of dsRNA reduced the number of *Fmr1* nymphs by nearly half, while the number of controls remained similar. These results suggest that the Fmr1 dsRNA being administered is responsible for the decreased number of nymphs, and not the injection itself. This decrease in nymphs could be the result of fewer eggs being laid, or the eggs that were laid may not have been fertilized or could have died prior to hatching. Ultimately, this may be attributed to the role of FMRP. While FMRP is most highly expressed in neurons, it is also expressed in nearly every cell type, including the oocytes of the ovaries (Antar and Bassell, 2003; reviewed in Drozd et al., 2018). Patients with reduced levels of FMRP, but not a complete loss of FMRP, have disease phenotypes that are distinct from FXS (Li and Zhao, 2014), which includes Fragile X-associated Primary Ovarian Insufficiency (FXPOI) (reviewed in Man et al., 2017). FXPOI causes reduced ovarian function due to insufficient egg development which leads to reduced fertility (reviewed in Man et al., 2017). A recent study using a FXPOI mouse model showed that knockout mice had decreased fertility and reduced litter sizes when compared to wildtype mice (Shelley et al., 2018). Taken together, these findings support that the decrease in offspring may be attributed to decreased *Fmr1* expression.

The chorion of insect eggs can become melanized because they express the enzyme phenoloxidase (Li, 1994; Kim et al., 2005). Once activated, phenoloxidase converts phenols to quinones in a complex cascade of events that results in the production of melanin (reviewed in González-Santoyo and Córdoba-Aguilar, 2012; Satyavathi et al., 2014; Dubovskiy et al., 2016). We observed different degrees of melanization ranging from light tan to black, and we considered all of these eggs to be non-viable. Previous research has shown that cricket eggs will turn black in response to extreme temperatures (Masaki, 1960). After turning black, these cricket eggs failed to hatch and ultimately died (Masaki, 1960). Our previous findings suggest that cricket eggs turn black after exposure to a viral infection, indicating that they are no longer viable (Kathleen Killian, personal communication). In order to optimize pRNAi in *Acheta domesticus,* we also assessed the effects of treatment, dose, and day of dsRNA injection on the

proportion of clear versus melanized eggs in female ovaries. We found an injection with Fmr1 dsRNA significantly increased the proportion of melanized eggs in the ovaries, regardless of the concentration. Additionally, injecting on D5 instead of D1 did not affect the proportion of unhealthy eggs. Clearly, decreased *Fmr1* impacts the viability of the eggs, but the female is still able to oviposit, and nymphs are able to hatch and develop so it is feasible to use pRNAi in future studies, however, reduced numbers of progeny will need to be accounted for in the experimental design.

We found that RNAi was transmitted to the F_1 offspring after a single injection of dsRNA into the mother on D1 of adulthood. While dsRNA was transmissible, the efficacy in the F_1 generation was highly variable across doses, and even within the same dose. For example, we saw a significant decrease in *Fmr1* transcripts in the 2nd and 5th nymphal instars of 2.5μg pRNAi offspring, while we only saw a significant decrease in *Fmr1* expression during the 6th nymphal instar in 7μg pRNAi offspring. Similar to our findings, a pRNAi study in the blood feeder *Rhodnius prolixus* found that RNAi effects were not observed in the 1st instar of F_1 generation nymphs (Paim et al., 2013). However, by the 2nd instar they saw an 89% reduction in the mRNA levels of the target gene in the salivary glands (Paim et al., 2013). Unfortunately, this study did not monitor the efficacy of RNAi past the 2nd instar in the F_1 generation. It has been proposed that the delay in pRNAi response may be attributed to the rate at which the RNAi effect spreads in the insect, as well as protein turnover rate (Fishilevich et al., 2016). In another study, Miller et al. (2012) found that beetles injected with the same concentration of dsRNA showed drastic differences in the amount of time that the RNAi effect took to wear off. Larvae were injected with dsRNA targeting EGFP, so that no fluorescence indicated a knockdown while the return of fluorescence indicted that the effects of RNAi had worn off. The first individuals began to express EGFP 28 dpi, while EGFP was not expressed in all individuals until 77dpi (Miller et al., 2012). Several factors contribute to the variable efficiency of RNAi seen in insects including: dsRNA stability, cellular uptake of dsRNA, expression of core RNAi machinery components, and the amplification and spreading of RNAi (reviewed in Belles, 2010; Cooper et al., 2018). In the migratory locust, it was found that dsRNA uptake was reduced in oocytes and resulted in a less efficient ovarian RNAi response (Ren et al., 2014). Additionally, in the desert locust *Schistocerca gregaria*, the ovaries and testes were less susceptible to RNAi because of reduced

expression of core RNAi components (Wynant et al., 2012). A number of factors could have contributed to the inconsistent RNAi response seen in the F_1 generation. It is possible that dsRNA was not taken up equally well among the oocytes, and led to differential effects of RNAi in the developing nymphs. It is also feasible that nymphs did not experience the same degree of knockdown because of differences in the individual's ability to amplify and spread the RNAi signal. Since we pooled four individuals at every developmental stage to assess the knockdown, it is possible that the individuals experienced differential effects of RNAi that would contribute to the variability seen throughout the stages of development.

In the current study, we also varied the timing of the dsRNA exposure by injecting on D1 or D5 of adulthood, because this parameter has been shown to affect the efficacy of the RNAi response in insects (see Paim et al., 2013 for example). We found that injecting females with 3.5μg of dsRNA on D1 resulted in a more efficient knockdown in the pRNAi offspring than did an injection of the same dose on D5. A study in the roundworm *Caenorhabditis elegans* also demonstrated the importance of the timing of dsRNA application (Marre et al., 2016). When dsRNA was ingested from hatching until the fourth larval stage, gene silencing occurred in 10% of the pRNAi offspring. However, when dsRNA was ingested after the fourth larval instar gene silencing occurred in 100% of progeny (Marre et al., 2016). In another study, pRNAi was only successful in the blood feeder *Rhodnius prolixus* when females were injected with dsRNA during the 5^{th} instar, the last nymphal stage prior to adulthood, (Paim et al., 2013). When females were injected with the same dose of dsRNA during the 3^{rd} nymphal instar, there was no knockdown of the target gene in any of the F_1 progeny. They tried doubling the dose of dsRNA and still saw no pRNAi effects in the offspring. To determine if this was a tissue-specific effect, they targeted the midgut instead of the salivary glands and found that the effects of RNAi were only seen in the F_1 generation when females were injected during the 5^{th} nymphal instar (Paim et al., 2013). This difference in parental transmission may be attributed to the developmental stage of the oocytes at the time of dsRNA injection. For example, in *R. prolixus* the oocytes do not develop until the 5^{th} nymphal instar (Paim et al., 2013). When the dsRNA is injected into the 3^{rd} instar it could be taken up by any cell that has an uptake mechanism, but RNAi amplification would only occur in cells where endogenous target mRNA was expressed. By the time the insect reaches the 5^{th} instar and the oocytes are developed, all of the dsRNA that was injected during the 3^{rd} instar is

depleted. This would not leave any dsRNA to be taken up by the oocytes and passed on to the next generation, whereas an injection during the 5th instar would allow dsRNA to be taken up by all cells, including the developed oocytes. Taken together, these findings suggest that the oocytes may need to be developed in order to take up dsRNA. It would be interesting to test this theory in *Acheta domesticus* by injecting females prior to oocyte development, during oocyte development, and just after oocyte development.

We also examined the agonistic behavior of these pRNAi males once they reached adulthood. We tested offspring from adult females that received a 3.5μg injection of dsRNA because *Fmr1* expression was significantly decreased during two consecutive instars during early stages of development (2nd and 3rd instar). We also tested offspring from adult females that received an injection with 7μg of dsRNA because it significantly reduced *Fmr1* expression during a later stage of development (6th instar), allowing us to compare the effects of an early knockdown with a late knockdown. During behavioral trials of 3.5μg offspring, we did not see an impact on the maximum fight level, or the ability to initiate or win a fight. However, we found that experimental pairs took significantly less time to make physical contact than control pairs. This decrease in the amount of time to make contact may be attributed to hyperactivity that is often exhibited in FXS patients (Baranek et al., 2005; Reiss and Hall, 2007; Bailey et al., 2008) and in animal models of FXS (Wu et al., 2017). A study by Wu et al. (2017) found that *Fmr1* KO zebrafish showed increased hyperactivity and anxiety during novel tank tests and light/dark tests when compared to controls. In agreement with these findings, *Fmr1* KO mice were significantly more active in an open field test when compared to control mice (Gurney et al., 2017). We did not determine if *Fmr1* KD crickets displayed increased movement or hyperactivity during behavioral trials in this study. Future studies could assess the activity level of crickets by determining the amount of time that each cricket spends moving around the arena prior to initial contact, in order to address this hypothesis directly.

Next, we examined the agonistic behavior of pRNAi males whose mothers received an injection with 7μg of dsRNA. We did not see an impact on the time to first physical contact, however, we did find that *Fmr1* KD males were less likely to initiate a fight than control males. In an agonistic interaction, the initiator is the individual that makes the first aggressive act, and begins

with one cricket approaching the other cricket, followed by antennal touching if the interaction escalates. Similar to our findings, *Fmr1* mutant flies were less likely to initiate a social interaction with a wildtype fly when the pair was placed into a chamber together (Bolduc et al., 2010). In the same study, Bolduc et al. (2010) also determined that *Drosophila* mutant flies interacted with other flies less often by maintaining larger inter-fly distances.

It has been proposed that the individual who initiates the first encounter during a fight is more likely to emerge as the winner of the fight (Chen et al., 2002). We examined the fight outcome between experimental pairs and found that *Fmr1* KD males won agonistic interactions just as often as controls, even though they initiated fights significantly less. Additionally, we found that the average maximum fight level between control pairs and experimental pairs was similar, suggesting that fights were just as aggressive. In a previous study we found that decreased *Fmr1* expression during adulthood had a similar effect on agonistic behavior (see Chapter 2); *Fmr1* males initiated fights significantly less often than controls, but won just as often as controls. We propose that this behavioral effect may be due to an inability to process sensory input (see Chapter 2) since numerous studies have shown that the ability to integrate multisensory input is impaired in *Fmr1* KD animals (Bolduc et al., 2010; Truszkowski et al., 2016; Wu et al., 2017). Our findings suggest that even short periods of decreased *Fmr1* expression can have long-term effects on an individual. Future studies will focus on optimizing the parameters for successful pRNAi so that we can extend the efficacy of RNAi in the F_1 generation and begin to test this hypothesis.

In summary, our study has identified some key parameters for successful pRNAi in the model *Acheta domesticus*, and demonstrated that the effects of RNAi could be passed on to the F_1 generation. The strength and duration of the *Fmr1* knockdown varied during different stages of development. Despite this variation, the decreased *Fmr1* expression during short windows of time in development was sufficient to impact the social behavior of pRNAi offspring once they reached adulthood. Moving forward, pRNAi can be a useful tool for the continued study of FMRP and its role in neurophysiology and behavior in the insect model *Acheta domesticus.*

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Chapter 5: General Discussion

This body of work sought to provide important insight into Fragile X Mental Retardation Protein (FMRP) and its effects on immune function and behavior using a novel insect model of FXS. As FXS is a neurodevelopment disorder, most research in animal models has focused on the effects of decreased FMRP during development, and very little work has focused on the effects of decreased FMRP during adulthood. One of our primary goals was to contribute to this field of knowledge by decreasing *Fmr1* specifically during adulthood and assessing the impacts.

In Chapter 2 we examined the effects of decreased *Fmr1* on the innate immune system of adult males and females, using a novel insect model of FXS. Most FXS research has relied heavily on vertebrate models such as the mouse, rat, and zebrafish. However, over the past twenty years, the fruit fly *Drosophila melanogaster* has proved to be an invaluable model, and has provided important functional insights on FXS. The fruit fly has been used to study the effect of FXS on everything from social behavior (Bolduc et al., 2010) and adult locomotion (Zhang et al., 2001) to phagocytosis (O'Connor et al., 2017). In this study, we proposed the cricket *Acheta domesticus* as another valuable insect model to provide even more insight into FXS. We identified the *Fmr1* ortholog in *A. domesticus* and found that it was highly homologous to the fruit fly, as well as to all vertebrate models of FXS including the mouse, rat, zebrafish, and frog. Most importantly, we found that the *AdFmr1* ortholog was highly homologous to human *FMR1*, supporting that *A. domesticus* is a promising new model for the study of FXS.

Despite FXS and ASD patients displaying immune defects (Careaga et al., 2014; reviewed in Estes and McAllister, 2015), very little work has been done to investigate the role of FMRP on immune function. In order to address this, we used RNAi to decrease *Fmr1* expression in adult males and females and determined the effects on several immune parameters. We found that there was a significant impact on the cellular and humoral defense mechanisms of the innate immune system. While males and females exhibited similar changes in immune parameters as a result of decreased *Fmr1*, there were sex-specific differences in their ability to respond to an immune challenge. These novel findings support that FMRP has a significant impact on the immune system, and that the effects are sex specific. This reinforces the need for continued studies using both sexes, as most FXS studies have most often focused on male subjects or organisms.

As a result of the neural deficits associated with decreased FMRP, patients with FXS exhibit impaired social behaviors which are typically manifest as social anxiety, withdrawal, and aloofness (reviewed in Bhakar et al., 2012). Several studies carried out in animal models have also shown impaired social behaviors in response to decreased FMRP (see Hamilton et al., 2014; Wu et al., 2017 for examples), but a majority of this research has focused on the effects of FMRP when it is depleted during development. In Chapter 3, we examined the agonistic behavior of male crickets that underwent RNAi-mediated knockdown of *Fmr1* during adulthood. Our goal was to assess whether decreased *Fmr1* expression outside of development was sufficient to impair this social behavior. We found that two different aspects of agonistic behavior were affected. First, we found that experimental pairs (DsRed2 *vs* Fmr1) took significantly more time to make physical contact during a behavioral trial than control pairs. This increase in the amount of time to make contact could be attributed to changes in exploratory behavior or locomotor activity in *Fmr1* KD crickets. Several studies have shown that decreased *Fmr1* impairs locomotor activity and exploratory behavior in animal models of FXS. For example, Bolduc et al. (2010) found that *Drosophila Fmr1* mutants stopped for longer lengths of time and covered less overall area when placed into a novel test chamber. In agreement with these findings, *Fmr1* KD tadpoles had lower overall swimming speeds when compared to controls (Truszkowski et al., 2016). Additionally, Xu et al. (2004) demonstrated that *Drosophila Fmr1* larvae spent less time crawling than control larvae when placed into vertical tests chambers, indicating that locomotion was impaired in these mutants. We plan to determine if the locomotor activity of *Fmr1* KD males is impaired, and may have contributed to this increase in the amount of time to make initial contact. Since all of our behavior is videotaped, we will re-watch our recordings and examine the amount of time that each cricket in the pairing spent moving prior to the first contact. This will allow us to easily determine if the *Fmr1* KD males were moving less than control males. Additionally, we would like to determine if other social behaviors are impaired in *Fmr1* KD crickets. Previous studies in the fruit fly *Drosophila melanogaster* have demonstrated that the courtship activity of *Fmr1* mutant males is impaired, and appears to be attributed to their inability to maintain interest in courtship (Dockendorff et al., 2002). We would like to examine the impact of decreased FMRP on the mating behavior of males and females. It would be interesting to inject both males and females, and determine if both sexes are impacted by decreased FMRP, or if the effects are sex specific.

We also found that *Fmr1* KD males initiated fights less often than controls, but were just as likely to become dominant, and also fought just as aggressively. Previous studies have shown that *Fmr1* mutants are less likely to initiate a social interaction (Bolduc et al., 2010). Bolduc et al. (2010), used *Drosophila* to examine social behavior in wildtype and *Fmr1* mutant flies by placing two isogenic flies in adjacent chambers separated by mesh. They determined the spatial probability distributions and measured inter-fly distances to determine the likelihood of a social interaction. They found that *Fmr1* mutant flies stayed farther away from the divider and maintained larger inter-fly distances than controls, indicating that *Fmr1* mutant were less likely to initiate a social interaction, or to interact with other flies (Bolduc et al., 2010). But the fact remains that the male crickets used in the current study still became dominant as often as controls. So why would decreased *Fmr1* expression affect the likelihood of a male to initiate a fight but not its aggressiveness or ability to attain dominance status? Evidence supports that the ability to integrate multisensory input is impaired in *Fmr1* KD mutants (Bolduc et al., 2010; Truszkowski et al., 2016; Wu et al., 2017), and we would like to assess the cricket's ability to respond to sensory stimuli to determine if this may have contributed to our findings. The ability of a cricket to respond to sensory stimuli can be assessed by repeatedly touching different body areas with a small paintbrush and scoring those responses (see Killian et al., 2006). We will determine sensory response scores for both control and *Fmr1* KD males to assess differences. Taken together our findings from Chapter 3 suggest that modest decreases in *Fmr1* expression are sufficient to change the behavioral phenotype of adult crickets.

In Chapter 4 we evaluated the parameters required to generate a successful pRNAi response in the cricket *Acheta domesticus,* so that this model could be used for both adult and developmental studies of FXS. We found that the effects of RNAi were passed on to the next generation, however, the strength and duration of the knockdown varied considerably during development. We also found that large doses of Fmr1 had detrimental effects on the unfertilized eggs within the ovaries and severely decreased the hatch size. While these results are promising, I believe that the parameters for pRNAi could continue to be optimized in order to obtain an even stronger pRNAi knockdown. Since we found that large doses were detrimental and that repeated injections reduced the hatch size, it might be interesting to try different methods of dsRNA application. Previous studies have shown that pRNAi can be achieved in insects through repeated ingestion of dsRNA (Fishilevich et al., 2016), so an alternative method of dsRNA application

may prove to be useful in the cricket. If we fed the dsRNA to our crickets, instead of injecting it, we might be able to obtain a stronger knockdown. We could begin by administering a single dose of 2.5μg to D1 adult females and then assessing the knockdown in the brain at several times post-injection, just as we did with this study. Depending upon the results, we could try increasing the dose of dsRNA or we could administer a second dose and see if continuous feeding extends the efficacy of the RNAi effect. If this is unsuccessful, we could try feeding dsRNA to nymphs during the stage of development that we wish to knockdown *Fmr1* expression.

In closing, these findings demonstrate the importance of continuing to examine the effects of decreased FMRP outside of the developmental window of time, because it can provide important insight into the functions of Fragile X syndrome and other Fragile X-related disorders.

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