EPIGENETIC EFFECTS OF PATERNAL PERCEPTION OF PREDATION RISK ON OFFSPRING DEVELOPMENT, STRESS REACTIVITY, AND NEURAL GENE EXPRESSION

By

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Abstract: In stable environments, parents able to transmit information such as predation risk should have offspring that are pre-adapted to the environment they will encounter as adults. While intergenerational epigenetic transmission of paternal experience has been demonstrated in mammals, whether paternal perception of predation risk can alter offspring phenotypes has not been investigated. We exposed male mice to a predator odor (2-4-5-trimethylthiazoline, TMT) and measured offspring behavioral phenotypes throughout development as well as adult neural gene expression and stress reactivity. We predicted that offspring of males exposed to TMT would exhibit decreased activity and increased anxiety-like behaviors relative to controls because these behaviors are analogous to anti-predator behaviors in the wild. Unexpectedly, we found that offspring of TMT-exposed males tend to be more active and exhibit fewer anxiety-like behaviors relative to controls. In the prefrontal cortex, we found evidence of decreased relative expression of *Bdnf* and increased relative expression of *Nr3c2* in experimental offspring. Additionally, offspring of TMT-exposed males exhibited decreased baseline plasma CORT relative to controls. Previous work suggests that prey increase risk-taking behavior in areas with high predator density, suggesting potential tradeoffs between anti-predator behavior and activities such as foraging and reproduction. When considering such trade-offs, these results suggest that fathers exposed to predation threat produce offspring that are resilient to stress and, potentially, better adapted to a high predation environment. Importantly, this study provides evidence that ecologically relevant paternal experience can be transmitted through the germline and can exert consistent effects on offspring phenotypes throughout development.

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CHAPTER I

INTRODUCTION

Although death is the most severe fitness consequence exerted by predators no prey, predators can also have nonlethal (nonconsumptive) effects that significantly impact prey behaviors and, in turn, fitness. For example, songbirds exposed to auditory predator cues reduced both the total number of offspring produced per year (Zanette et al. 2011) and the amount of late-state parental care provided to offspring (Dudek et al. 2018). Likewise, chronic predation risk resulted in increased oxidative stress and slowed escape speed in damselfly larvae (Janssen & Stoks 2014). In order to survive, prey alter foraging behaviors (Peacor & Werner 2000) and habitat use, often at the expense of reproduction (Marshall & Sinclair 2010, Zanette et al. 2011, Dudek et al. 2018). Prey that can readily detect and optimally respond to predator cues are more likely to successfully avoid predators, forage efficiently, and reproduce while minimizing the trade-offs between chronic stress and reproduction.

One of the strongest non-consumptive effects predator exert upon prey is stress. At the organismal level, chronic stress has adverse effects on reproduction (Marshall & Sinclair 2010), immune response (Adamo et al. 2017, Sapolsky et al. 2000), and physical performance (Jannssen

& Stoks 2014). At the molecular level, interactions between corticosteroids and their receptors (*Nr3c1*, *Nr3c2*) are central to negative regulation of the stress response and the restoration of homeostasis following stressful events like predator encounters (Schwabe et al. 2013). While dopamine is more commonly associated with reward circuitry within the brain (Kandel et al. 2013, Pignatelli & Bonci 2015, Thanos et al. 2016), it also encodes aversion to stressful stimuli, especially when the fear response is initiated (Pignatelli & Bonci 2015). Neurotrophins, such as *Bdnf*, promote growth and differentiation of neurons in the central nervous system (Osorio et al. 2017). In humans, altered *Bdnf* expression is associated with anxiety-like disorders (Osorio et al. 2017) and acutely stressful events can increase *Bdnf* expression for over a week (Denhardt 2017).

Stressful events during early development can alter brain development (Green et al. 2017), with behavioral effects that manifest later in development or even in adulthood. For example, gravid crickets exposed to wolf spiders produced offspring with greater anti-predator behavior and increased survivability in the presence of predators relative to controls (Storm & Lima 2010). Numerous studies in mammals demonstrate that maternal stress can alter both behavior and physiology of offspring (Champagne et al. 2006, St-Cyr & McGowan 2014, St-Cyr et al. 2017). In sheep, increased exposure to synthetic glucocorticoids during gestation altered negative feedback systems in mothers and offspring, resulting in fewer glucocorticoid and mineralocorticoid receptors in offspring hippocampus (Sloboda et al. 2008). Exposing pregnant female mice to predator odor increased anxiety-like and anti-predator behavior in all offspring, with elevated corticosterone (CORT) in response to predator odor in female offspring (St-Cyr & McGowan 2014). These sex-specific effects persisted throughout development and into adulthood (St-Cyr et al. 2017). Such studies indicate that non-consumptive effects of predators on prey exert potentially important effects both within and across generations. .

If environmental conditions are relatively stable, parents able to transmit key information such as predation risk should have offspring that are pre-adapted to the

environment they will encounter as adults (Crews et al. 2012). According to the match-mismatch hypothesis, animals that have received specific adaptive information in early life that is well-suited to their adult environment (e.g. sensitivity to predator odors in a high predation risk environment) are more likely to survive in that environment. Conversely, individuals who do not receive this information, or received information pertinent to a different environment, are less likely to survive (Schmidt 2011). In either case, the question remains: how is such information passed from parent to offspring?

Inheritance is typically viewed as the transmission of genetic information encoded in DNA from parent to offspring. However, individual experience can induced lasting changes in when and how genes are expressed. Known as epigenetic changes, these alterations involve chemical modifications (e.g. methylation) to DNA or histones that do not alter the nucleotide sequence (Williams 2013). Furthermore, epigenetic changes accumulated during an individual's lifetime can be transmitted to offspring via the germline (Allegrucci et al. 2005, Bohacek et al. 2016, Curley et al. 2011, Crews et al. 2012, Dias & Ressler 2015, Rankin 2015). The lasting effects of chronic stress are perpetuated by epigenetic changes that alter expression of stress response-associated genes, both within and outside the hypothalamic-pituitary-adrenal (HPA) axis (Denhardt 2017).

While there is ample evidence that chronic parental stress can result in altered stress reactivity in offspring (Champagne et al. 2006, Dias & Ressler 2014, Sloboda et al. 2008, St-Cyr & McGowan 2014, St-Cyr et al. 2017), obligate interactions between mammalian mothers and their young (i.e. gestation and lactation) make it hard to discriminate between direct effects of maternal environment and true epigenetic inheritance. In contrast, most mammalian fathers contribute only sperm to their offspring. Therefore, tests for paternal transmission of experience

represent an important alternative approach in the study of mammalian epigenetic inheritance that permits separation of germline-mediated and behaviorally- or physiologically-mediated epigenetic effects.

Controlled experimental work with alternative reproductive techniques in mice demonstrates that epigenetic information can be transmitted through the germline alone. For example, the offspring of male mice exposed to foot shocks had reduced body weight relative to controls, regardless of whether they were conceived via artificial insemination or standard mating practice (Bohacek et al. 2016). Thus, the physiological effects of paternal stress on offspring were not explained by pre-mating interactions between mother and father (Bohacek et al. 2016; but see Masoodh et al. 2018). Similarly, offspring of male mice presented with a neutral odor paired with an electrical shock demonstrated strong paternal epigenetic inheritance in a laboratory setting: subsequent generations displayed a fear response to the same odor and also had altered DNA methylation on the olfactory receptor that binds the odor's main ligand (Dias & Ressler 2014). Because fathers only contributed sperm, offspring response to the odor stimulus could be attributed solely to paternal epigenetic inheritance (Dias & Ressler 2014).

While shocks evoke a strong response, they are not an ecologically relevant stimulus. Paternal exposure to ecologically relevant cues such as changes in food availability (Carone et al. 2010, Kaati et al. 2002, Masoodh et al. 2018), diet composition (Weyrich et al. 2018), and heat exposure (Weyrich et al. 2016) all cause epigenetic changes in offspring. Collectively, these studies indicate that the paternal germline can transmit epigenetic information between generations, that this information can be influenced by environmental conditions, and that these inherited epigenetic changes could impact offspring fitness in nature. However, no mammalian study to date has tested for effects of paternal exposure to chronic predation stress.

Here, I investigate the effects of paternal perception of predation risk on offspring anxiety-like phenotypes throughout development, and on neural gene expression in adulthood, by exposing adult male mice to 2,4,5-trimethylthiazoline (TMT), a component of fox feces that is aversive to rodents (Buron et al. 2007, Green et al. 2017, Hacquemand et al. 2013, Janisky et al. 2014, St-Cyr & McGowan 2014, St-Cyr et al. 2017). I hypothesize that offspring of males exposed to predator cues prior to mating will exhibit 1) behaviors that would promote survival in a high predation environment relative to offspring of control males and 2) hormonal and gene regulatory phenotypes that reflect a more efficient stress response relative to offspring of control males. Specifically, I predict that offspring of males exposed to predator cues will be more anxious and stress reactive relative to controls, and will exhibit greater avoidance behaviors when presented with those same cues. I predict that, in association with increased stress-reactivity, offspring of males exposed to predator cues will have higher expression of dopamine, glucocorticoid, and mineralocorticoid receptors in regions key to mediating the stress and fear responses (hypothalamus, hippocampus, amygdala, and prefrontal cortex) relative to offspring of control males. This would result in more efficient termination of the stress response, which would be critical for conserving energy in a predator-rich environment. Finally, I predict that offspring of males exposed to predator odor will have decreased *Bdnf* expression, consistent with previous work demonstrating downregulation following chronic stress paradigms (Shi et al. 2010).

CHAPTER II

METHODS

Mouse Husbandry

Mus musculus (C57BL/6J) mice were purchased from Jackson Laboratory. Male mice (paternal generation) were singly housed in polycarbonate cages with Sanichip® bedding and *ad libitum* access to food (LabDiet® 5001 Rodent Diet) and water. During the 5-day pairing period, one male and one female were housed per cage. Offspring were housed in same sex groups of up to 4 individuals/cage after weaning. The colony was maintained on a 12-hour light: dark cycle with lights on at 0930. The Oklahoma State University Institutional Animal Care and Use Committee (IACUC) approved all of the following procedures (AS-14-1).

Paternal Treatment

Sexually naïve, adult male mice (62-150 days old, mean 103.2 ± SD 2.9) were exposed to either 10% TMT (BioSRQ, SKU 1G-TMT-90) in propylene glycol (experimental; n=15) or 1% banana extract in propylene glycol (control; n=13) 5 minutes daily for 8 consecutive days. TMT is a component of red fox feces commonly used as a predation stress stimulus for rodents (Buron et al. 2007, Green et al. 2017, Hacquemand et al. 2013, Janisky et al. 2014, St-Cyr & McGowan 2014, St-Cyr et al. 2017) because rodents do not habituate to TMT (Green et al. 2017) and TMT produces lasting anxiety-like behaviors (Janisky et al. 2014). During the exposure period, mice

were housed in a separate room (to avoid exposing the main mouse colony to either odorant) with 24 hours acclimation prior to the first exposure. I administered treatment on filter paper (50 μL/day) placed in the home cage for 5 minutes within the first three hours of the light cycle (0930-1230 hours).

I conducted open field trials (OFT) prior to the first exposure and 24 hours after the last exposure to determine if there was an immediate effect of paternal treatment on anxiety-like behaviors (scored as amount of time spent in the center of the apparatus) and activity levels (scored as the total number of grid lines crossed). The open field arena consisted of a 16 square grid enclosed by a clear Plexiglas box with no lid (60.96 cm x 60.96 cm x 60.96 cm). Mice were placed in a PVC tube in the center of the arena, and each five-minute trial began after the mouse left the center of the grid following the removal of the PVC tube. Each trial was video recorded and scored by an observer blind to treatment. Trials were conducted in the main mouse colony between 1000-1400 hours.

TMT-exposed males were paired twice with sexually naïve females (60-234 days old, mean 115.58 ± SD 5.78), at 17 and 25 days after the first exposure to the predator cue (Figure 1A). Mature spermatozoa at 17 and 25 days were at the postmeiotic round spermatid and premeiotic spermatogonial stages, respectively, at first exposure (Fallahi et al. 2010, Oakberg 1956). This design allowed me to test for an effect on offspring phenotypes of spermatogenic stage during paternal TMT exposure. Control males were paired once only at 17 days after first exposure (Figure 1A). All pairs were split after 5 days to minimize potential effects of male behavior on mothers. After splitting pairs, females were placed in a fresh cage with a cotton nestlet and paper hut and left undisturbed, except for routine handling associated with cage changes. Females were observed every other day to determine pregnancy, and once it was determined a dam was pregnant (usually 10-12 days following the split), she was checked daily until parturition. I tested for the effect of spermatogenic stage on offspring behaviors in the

experimental group with linear mixed models including timing as a fixed effect and litter ID as a random effect (Table 2).

Two days after pairs were split, males were sacrificed by cervical dislocation. Sperm was collected from caudal epididymides and brains were preserved in RNAlater for future studies.

Offspring Behavioral Assays

Offspring were used in a series of behavioral assays, from post-natal day (PND) 3 through adulthood, before sacrifice as adults for tissue or blood collection (Figure 1B). On PND 3, I weighed offspring and recorded ultrasonic vocalizations (USVs) for 3 minutes. USVs are alarm cries that promote maternal pup retrieval when neonates are displaced from the nest (Mogi et al. 2017), and are used as a measure of anxiety in infant rodents (Winslow 2009). Pups were removed individually from the nest and placed in a cage with clean bedding inside the recording chamber, a 52 x 36 x 30 cm anechoic foam-lined PVC box with a microphone (UltraSoundGate CM16/CMPA, Avisoft Bioacoustics) positioned ~15 cm above the floor of the box. Recording began immediately after placement and vocalizations were sampled at 192 kHz, 16 bits using Avisoft (version 4.2.24) software and hardware (UltraSoundGate 116hb). The number of vocalizations (distinct notes) per minute over a three minute period was scored manually in Raven Pro.

On PND 21, offspring were weaned, weighed, and five minute OFTs were conducted as described above to determine if there was an effect of paternal treatment on offspring activity and time spent in the center of the apparatus. At approximately PND 84 (mean 84.6 ± SD 0.85), all offspring were exposed to 50 μL TMT on filter paper at one end of a standard polycarbonate mouse cage (11.5”x 7.5” x 5”) for five minutes to measure fear response to the same predator cue experienced by fathers. I randomized which side the filter paper was on in each trial to eliminate side bias. Trials were video recorded and scored by hand. Response was measured by 1)

calculating the ratio of time spent on the side of the cage with TMT relative to the side without odor and 2) scoring the following behaviors: sniffing the TMT dish, touching the TMT dish, rearing, and digging. OFTs were conducted the day before and immediately after TMT exposure to measure adult baseline and post-acute stress behavior, respectively.

I analyzed open field, the total number of ultrasonic vocalizations, and time spent with TMT with generalized linear mixed models, with paternal treatment and sex as fixed effects and litter as a random effect. I ran a linearized mixed model on the ratio of baseline: stressed behaviors to determine if there was an effect of paternal treatment on the change in open field behavior. I analyzed weight, TMT behavioral data, and remaining USV data (first minute, second minute, latency to first call) with linear mixed models, including paternal treatment as a fixed effect and litter ID as a random effect (Table 3)**.** Models were selected using Akaike information criterion adjusted for small sample sizes (AICc). All analyses were conducted in R Version 3.5.2 "Eggshell Igloo".

Offspring Tissue Collection

After all behavioral assays were complete, I randomly assigned adult offspring to use for either tissue collection to measure gene expression in the brain (glucocorticoid receptor *Nr3c1,* mineralocorticoid receptor *Nr3c2,* dopamine receptors *Drd1* and *Drd2*, and *Bdnf*), or blood collection to quantify plasma corticosterone. At approximately PND 90, one individual per sex per litter was sacrificed by cervical dislocation and weighed. Adrenal glands were collected and weighed. Adrenal gland mass was corrected for body mass (mean adrenal mass/body mass) prior to analysis. All mass analyses were conducted with linearized mixed models (LMM). For neonatal mass, I included paternal treatment as a fixed effect and litter ID as a random effect. For juvenile and adult body mass, and adrenal mass analyses, I included paternal treatment and sex as fixed effects and litter ID as a random effect.

Brains were extracted into RNAlater, stabilized at 4°C for 24-48 hours, and stored at -20°C until dissection. Target brain regions (amygdala, hypothalamus, hippocampus, prefrontal cortex) were dissected into RNAlater with a scalpel under a dissecting microscope (Chiu et al. 2007, Zapala et al. 2005), using the mouse brain atlas (Paxinos and Franklin 2013) as a guide. DNA and RNA were extracted using the AllPrep DNA/RNA Mini Kit (Qiagen) according to the manufacturer's instructions.

qPCR

RNA was converted to cDNA with Bio-Rad iScript Reverse Transcriptase Supermix for RT-qPCR (cat. no: 1708841) with target RNA input of 0.6 µg (prefrontal cortex), 0.5 µg (amygdala, and hippocampus), or 0.3 µg (hypothalamus). However, due to variation in total RNA yield, target RNA input was not met for all samples (n=10 samples/region: amygdala, mean 0.46 µg ± SD 0.02; hippocampus, mean 0.47 µg ± SD 0.04; hypothalamus, mean 0.29 µg ± SD 0.03; prefrontal cortex, mean 0.52 µg ± SD 0.05).

Primers for qPCR (Table 1) were designed in Primer-BLAST (Ye et al. 2012) using sequences and intron-exon structure from Ensembl Mouse (GRCm38.p6). When possible, primers spanned an intron-exon boundary. Experiments were performed on a CFX Connect Real-Time System (Bio-Rad)**.** Each 10 µl reaction contained 4 µl cDNA (diluted 1:10), 5 µl SsoAdvanced™ Universal® SYBR Green Supermix (Bio-Rad; cat. no: 1725270), and 0.3 µM of each primer. Plates were balanced for sex and paternal treatment and included three technical replicates per sample for experimental genes, and the internal control (beta-actin). Relative mRNA expression was calculated using the comparative C_T method (Schmittgen & Livak 2008), with threshold crossing (C_T) values normalized relative to beta-actin. I tested for an effect of paternal treatment and sex on gene expression with analysis of variance (ANOVA). To account

for tests on each gene across four brain regions, alpha was set to 0.0125 using a Bonferroni correction. Litter ID was not included in this analysis because I used one individual per litter.

Blood Collection and Corticosterone Assay

At approximately PND 100 (mean 103.2 ± SD 1.6) blood for baseline CORT measurements was collected by submandibular bleeding from a minimum of one individual per sex per litter. Two to five days later, the same individuals were stressed by 30 minutes restraint inside a small plastic tube (Kaytee Critter Trails Fun-nels, 6.35 cm diameter, 8.85 cm long), placed in their home cage. After removal from the tube, a second blood sample (stressed sample) was collected by submandibular bleeding. One hour later, mice were sacrificed by cervical dislocation and a third sample was collected by cardiac puncture (recovery sample). Blood samples were spun down (5 min at 8500 rpm in an Eppendorf 5424 benchtop centrifuge) and plasma was stored at -80°C.

CORT levels were assayed with an ELISA (Enzo Life Sciences; cat. no: ADI-900-097) optimized for mouse plasma (1:40 plasma dilution, 1% SDB). Plates were read at 405 nm on a Biotek EL808 plate reader. Standards were run in triplicate, and samples were run in duplicate. Sample sizes for experimental litters were n=15 baseline, n=13 stress, and n=14 recovery. Sample sizes for control litters were n=9 baseline, n=10 stressed, and n=11 recovery. Plasma was pooled from same sex littermates when available to increase volume of plasma for the assay. Inter-plate variation was 5.00%, and average intra-plate variation was 7.74%. To determine whether offspring of predator cue-exposed and control males differed in baseline glucocorticoid activity, I tested for an effect of paternal treatment on baseline CORT with ANOVA. The effect of paternal treatment on CORT levels at all three time points was tested with repeated measures ANOVA (Bonferroni $\alpha = 0.017$).

CHAPTER III

RESULTS

Effect of chronic stress paradigm on fathers' behavior

Control and TMT-exposed fathers did not differ in activity level (number of lines crossed) or time in the center in the open field, either before or 24 hrs. after the 8-day exposure paradigm (Figure 2). Both activity and time spent in the center of the apparatus were best explained by the null model.

Effect of pair timing on experimental offspring behaviors

There was not an effect of spermatogenic stage on USVs, open field behavior, or behavior in the TMT-assay in experimental offspring (LMM, $p>0.15$).

Effect of paternal predation experience on body mass and adrenal gland mass

Offspring of males exposed to predator cues did not differ in mass from offspring of males without predator experience as neonates (control=10 litters$^{60\ individuals}$, experimental=19 litters$^{117\ individuals}$) (LMM, 0.06 ± 0.15, $z=27.0$, $p=0.70$). Males (M) were heavier than females (F) as juveniles (control F=9 litters$^{34\ individuals}$, control M= 7 litters$^{15\ individuals}$, experimental F =15 litters$^{54\ individuals}$, control M=15 litters$^{34\ individuals}$) (LMM, estimate ± SE: 0.46 ± 0.16, $z=2.93$, $p=0.0041$) and adults (control F=11 litters$^{13\ individuals}$, control M=7 litters$^{7\ individuals}$, experimental F=16 litters^{20}

individuals, experimental M=15 litters[19 individuals]) (LMM, estimate ± SE: 3.88 ± 0.38, z=10.34, p<0.001), regardless of paternal predator experience. Similarly, adult body mass-corrected adrenal glands were significantly heavier in males (LMM, estimate ± SE:-0.032 ± 0.12, z=-2.72, p=0.009) but there was not an effect of paternal treatment.

Ultrasonic Vocalizations

Offspring of TMT exposed males (19 litters[120 individuals]) and offspring of males without predator experience (11 litters[60 individuals]) did not differ in vocalizations produced during a 3-minute trial (Figure 3). With generalized linear mixed models, the number of vocalizations produced was best explained by the null. Vocalizations produced in the first minute, the second minute, and the latency to the first call, were best described by linear mixed models with paternal treatment as an effect. However, the difference between experimental and control offspring was not significant for these measurements (LMM, all p>0.4).

TMT Assay

In the TMT assay, I did not find a difference in behaviors of experimental (18 litters[89 individuals]) and control offspring (11 litters[59 individuals]) (Figure 4). There was a significant effect of sex on the amount of time spent on the side of the cage with TMT: male offspring spent less time on the TMT side than females, regardless of paternal treatment (GLMM, estimate ± SE: -0.246 ± 0.027, p<0.001). Similarly, female offspring tended to touch the dish containing TMT more often than males (LMM, -1.703 ± 0.954, t=-1.785, p=0.07). There were no effects of paternal treatment or sex on the number of times individuals sniffed TMT, reared, or dug during the five minute trial (LMM, all p>0.3).

Open Field Trials

In the juvenile open field trial, there was a significant effect of sex (GLMM, estimate ± SE: -0.11 ± 0.018, z=-5.76, p<0.001) and paternal treatment (GLMM, estimate ± SE: -0.46 ± 0.12, z=-3.82, p=0.001) on overall activity (Figure 5A). Offspring of males with predator odor experience (15 litters$^{77\ individuals}$) were more active than offspring of males without predator odor experience (10 litters$^{52\ individuals}$). Within groups, females (control: 10 litters$^{35\ individuals}$, experimental=15 litters$^{48\ individuals}$) were more active than males. The amount of time spent in the center of the open field was best explained by a model incorporating the interaction between paternal treatment and sex (Figure 5B). Offspring of TMT-exposed males spent more time in the center relative to control offspring (GLMM, estimate ± SE: -0.49 ± 0.15, z=-3.37, p<0.001). This effect was driven by experimental male offspring, who spent the most time in the center relative to all other groups (GLMM, estimate ± SE: 0.22 ± 0.0.068, z= 2.25 p=0.001) (Figure 5B).

In the adult offspring baseline open field trial, I found significant effects of sex (GLMM, estimate ± SE: -0.091± 0.015, z=-6.02, p<0.001) and paternal treatment (GLMM, estimate ± SE: 0.13 ± 0.062, z=2.03, p=0.042) on activity (Figure 6A). Within groups, females were more active than males and offspring of TMT exposed males (18 litters$^{89\ individuals}$) were more active than control offspring (11 litters$^{60\ individuals}$) (Figure 6A). The amount of time offspring spent the center of the apparatus at baseline was best explained by the model that included paternal treatment only. Offspring of males with predator experience tended to spend less time in the center of the open field relative to controls (GLMM, estimate ± SE: 0.16 ± 0.089, z =1.82, p=0.069) (Figure 6B).

Open field activity following predator cue exposure was best explained by a model incorporating paternal treatment, sex, and treatment by sex. Offspring of TMT-exposed males crossed more lines relative to control offspring (GLMM, estimate ± SE: 0.17 ± 0.076, z=2.24, p=0.025). Overall, sons crossed more lines than daughters (GLMM, estimate ± SE: 0.055 ± 0.027, z=2.024, p=0.043). The direction of sex-specific effects was treatment-dependent: whereas

sons of TMT-exposed fathers were less active than daughters, sons of control fathers were more active than daughters (GLMM, estimate ± SE: -0.12 ± 0.034, z= -3.54, p<0.001). I also found effects of paternal treatment, sex, and treatment by sex on the amount of time offspring spent in the center of the apparatus following predator cue exposure (Figure 6B). Collectively, offspring of TMT-exposed males spent more time in the center relative to offspring of males without predator experience (GLMM, estimate ± SE: 0.32 ± 0.12, z=2.7, p=0.0067). Female offspring spent more time in the center of the apparatus than males (GLMM, estimate ± SE: 0.16 ± 0.067, z=2.32, p=0.02). This sex-specific effect was driven by female offspring of TMT-exposed males: daughters of predator-exposed males spent more time in the center relative to all other groups, while daughters of males without predator exposure spent the least amount of time in the center (GLMM, estimate ± SE: -0.18 ± 0.081, z=-2.25, p=0.024) (Figure 6B). Notably, daughters of TMT-exposed males spent more time in the center of the apparatus relative to sons of TMT-exposed males), indicating reduced anxiety-like behavior following stress relative to males in the same treatment group (Figure 6B).

There was no effect of paternal treatment or sex on the change in behavior following exposure to the predator cue, measured as the ratio of line crosses or time spent in center before and after predator cue exposure (LMM, p<0.2) (Figure 6C and D). In both cases, the null model best explained the data.

qPCR

Offspring of males that experienced predator stress prior to mating had increased relative expression of mineralocorticoid receptor, *Nr3c2* (ANOVA, $F_{1,19}$=10.53, p=0.0045), and tended to have decreased relative expression of *Bdnf* in the prefrontal cortex (ANOVA, $F_{1,19}$=7.31, p=0.015) (Figure 7A and C). *Bdnf* and *Nr3c2* expression did not differ in hippocampus, hypothalamus, or amygdala (ANOVA, all p≥0.3) (Figure 7A and C) and there was no effect of

sex in any brain region (ANOVA, all $p \geq 0.025$). There was no effect of paternal treatment or sex on the relative expression of *Drd1, Drd2,* or *Nr3c1* in any brain region (ANOVA, all $p \geq 0.1$) (Figure 7B, D and E).

Corticosterone Assay

Baseline plasma CORT was significantly lower in offspring of TMT-exposed males relative to offspring of control males (ANOVA, $F_{1, 23}=5.32$, $p=0.026$) (Figure 8A). However, there was no effect of paternal treatment on stress and recovery CORT (repeated measures ANOVA, $F_{1,23}=51.14$, $p=0.33$) (Figure 8B).

CHAPTER IV

DISCUSSION

In this study, I tested for effects of chronic paternal predation stress on offspring behavioral, neural, and hormonal phenotypes. I hypothesized that offspring of males exposed to predator cues prior to mating would exhibit phenotypes associated with increased survival in a predator-rich environment. Specifically, I expected increased anxiety-like behaviors and reduced activity in offspring of males with predator experience relative to offspring of control males. Additionally, I expected higher expression of glucocorticoid, mineralocorticoid, and dopamine receptors in areas of the brain key to modulating the stress response (amygdala, hypothalamus, prefrontal cortex, and hippocampus). However, I expected offspring of males exposed to predator cues would have decreased *Bdnf* expression, consistent with chronic stress (Shi et al. 2010). I also expected that offspring of males exposed to predator cues prior to mating would exhibit a more efficient stress response: specifically, offspring of TMT-exposed males would exhibit increased plasma CORT relative to control offspring following restraint, but would also recover faster (recovery values closely resembling baseline CORT values). Based on neonatal, juvenile, and adult behavioral phenotypes, my hypothesis was not supported. Strikingly, I found evidence of overall decreased anxiety-like behavior and increased activity in juvenile and adult offspring of TMT-exposed males with evidence of altered mineralocorticoid receptor and *Bdnf* expression in the prefrontal cortex of adults. While there was no overall effect of paternal treatment on plasma CORT during and following stress, baseline CORT was lower in the offspring of TMT-exposed

males. I also found evidence of sex-specific effects on anxiety-like behavior across development: relative to daughters of TMT-exposed fathers, sons exhibited less anxiety-like behavior as juveniles but more anxiety-like behavior after exposure to predator odor as adults.

Taken together, behavioral and hormonal results suggest that offspring of predator cue exposed males have increased stress resilience relative to controls. Below I consider two non-mutually exclusive mechanisms that could explain this effect on offspring phenotypes. First, rather than enhancing offspring stress reactivity, paternal stress may buffer offspring against mild to moderate stressors (e.g. the open field test and predator cue exposure). Second, females mated to males exposed to chronic predation threat may compensate for paternal stress, resulting in offspring that are less stress reactive than those of females mated to non-stressed males (Braun & Champagne 2014).

Paternal buffering and offspring stress reactivity

While paternal care is rare in mammals, mammalian fathers do make other types of investment; when mated with preferred females, male house mice produced more offspring with increased survivorship relative to those mated to non-preferred females (Gowaty 2003). If fathers can alter investment based on female quality, they may also be able to do so based on environmental cues. Because epigenetic machinery can be energetically costly to maintain (reviewed in Macartney et al. 2017), epigenetic transmission of experience represents an important aspect of indirect paternal investment in offspring fitness.

Male mice that have enough energy to manage and alter the epigenome in response to environmental cues can provide key information to their offspring about the environment those offspring are likely to experience. While it is advantageous for prey animals to avoid predators, launching a stress response every time a predator cue is detected is costly, especially in predator-

rich areas. Thus, predator-rich environments may favor more stress resilient animals**.** In this study, I found behavioral patterns that indicate that offspring of TMT-exposed males are more resilient than control offspring (Figures 5 and 6). Furthermore, adult daughters of TMT-exposed males may be more resilient than sons (Figure 6). This result is particularly interesting when considered from the perspective of sex differences in dispersal and reproductive investment. Since mammalian females are typically the philopatric sex (Mabry et al. 2013) and always invest more in reproduction than males, the probable match between parent and offspring environments may be higher for fathers and daughters than for fathers and sons. Likewise, stress resiliency in a high predation environment may particularly benefit females, allowing them to conserve metabolic energy for reproduction.

Given the conservation of the mechanism underlying the stress response, it is striking how different paternal stress paradigms result in varied offspring phenotypes. Whereas in this study offspring of males exposed to predation threat exhibited reduced anxiety-like behavior and increased exploratory behavior, offspring of male mice exposed to a chronic defeat stress paradigm exhibited increased depressive and anxiety-like behaviors (Dietz et al. 2011). In lines of mice exposed to paternal postnatal trauma (i.e. early maternal separation), offspring and grand-offspring of traumatized fathers showed reduced aversion to open space relative to controls and increased risk-taking in the elevated plus-maze (van Steenwyk et al. 2018). Strikingly, male and female descendants of traumatized fathers also exhibited increased risk-taking through the fourth generation (van Steenwyk et al. 2018). Thus, the type of paternal stress paradigm seems to influence offspring behavioral phenotypes.

My original hypothesis, that offspring of predator-exposed males would show increased sensitivity to predator odor, reduced activity and increased anxiety-like phenotypes, was based on the assumption that a stress-reactive and risk-averse phenotype should benefit a prey species in a high predation environment. However, this hypothesis did not take into account potential trade-

offs between predator avoidance and other activities such as foraging and reproduction. Indeed, behavioral studies in wild rodent populations suggest that both familiarity with the predator (Abom & Schwarzkopf 2016) and increased predator density (Orrock & Fletcher 2014) promote higher activity and more risk-taking behavior (e.g. foraging in open areas). Given that fathers in the present study were chronically exposed to ecologically-relevant doses of TMT (Buron et al. 2007), it is possible that increased exploratory behavior and stress resilience in offspring would be adaptive responses in a predator-dense environment. While less anxious, less stress-reactive individuals should have more time and metabolic energy to invest in foraging and reproduction, the hypothesis that these phenotypes are adaptive under high predation threat is contingent on whether paternally-buffered offspring are able to effectively avoid predation, a question not addressed in the present study.

Restraint elicits an acute stress response in rodents, regardless of parental or developmental experience. Whether an effect of parental stress is detected against the background of such an acute stressor differs among studies. For example, the adult offspring of stressed female lab mice had a more extreme response to restraint stress relative to controls (St-Cyr & McGowan 2014). However, in wild-caught tuco tucos, juvenile offspring of predator-exposed dams were less active in the open field and more anxious in the elevated-plus maze, but had equivalent cortisol before and after immobilization stress relative to controls (Brachetta et al. 2018). Given that I did not find an effect of paternal predation threat on plasma CORT during restraint stress or recovery (Figure 8B), it would be interesting to investigate CORT levels in conjunction with ecologically-relevant stressors such as predation threat or food stress that elicit less extreme responses.

Maternal buffering against paternal stress

Animals that experience high predation risk alter investment in young by changing aspects of parental care or how many young are produced (Zanette et al. 2011, Dudek et al. 2018). As a general rule, females invest more in reproduction than males, and mammalian mothers must carry, give birth to, and provide maternal care for offspring. Thus, it is in a mammalian mother's best interests to find the highest-quality mate possible while still maximizing her fitness by reproducing. When high quality males are not available, females can maximize offspring fitness by altering maternal investment in those offspring (Braun & Champagne 2014, Gowaty et al. 2007, Masoodh et al. 2018). The compensation hypothesis proposes that, when paired with a low-quality male, females increase their investment in offspring to alleviate paternally-derived disadvantages (Gowaty et al. 2007). For example, dams that were artificially inseminated with sperm from food-deprived males produced offspring with growth deficits and depression-like behaviors, whereas offspring conceived from natural mating between a normal female and a food-deprived male did not exhibit these deficits (Masoodh et al. 2018). In the presentstudy, males were paired with females for five days, so mothers could have detected and altered investment to compensate for potential deficits in predator-exposed males. However, chronic predator experience does not visibly alter phenotypes in a comparable manner to food deprivation. As evidence, behavioral differences in fathers were not detected 24 hours following the stress paradigm (Figure 2), thus paternal behavior likely was not strongly altered at the start of mating, which occurred 9 days following the last day of predator odor exposure (Figure 1).

Parental effects on offspring neural gene expression

In the rodent brain, the prefrontal cortex is involved in managing the stress and fear responses (Arnstern 2009), in spatial memory (Laubach et al. 2018), and in working memory and executive

function (McEwen et al. 2016). Chronic stress can alter both structure and function of the prefrontal cortex (Nelson 2011, Arnstern 2009). These changes are often mediated by other brain regions (Arnstern 2009). Relative to controls, offspring of predator-exposed males had increased mineralocorticoid receptor (*Nr3c2*) expression and tended to have decreased *Bdnf* expression in the prefrontal cortex (Figure 7A and C).

In non-stressed (basal) conditions, mineralocorticoid receptors are activated by circulating glucocorticoids. In stressed conditions, both mineralocorticoid and glucocorticoid receptors are activated by increased circulating CORT, and glucocorticoid receptors are critical to inducing downstream effects. However, both corticosteroid receptors are key to mediating the acute stress response (de Kloet et al. 1998). I found offspring of TMT-exposed males may be more sensitive to circulating glucocorticoids prior to stress due to increased *Nr3c2* expression in the prefrontal cortex; this may contribute to effective negative regulation of the stress response and decreased anxiety-like behaviors (ter Heegde et al. 2015). These results are of particular interest when examined in the context of baseline glucocorticoid maintenance because glucocorticoids have 10x greater affinity for mineralocorticoid receptors than glucocorticoid receptors (reviewed in de Kloet et al. 1998, Gomez-Sanchez & Gomez-Sanchez 2014, Joels et al. 2012). Because of this, most *Nr3c2* receptors are occupied by circulating glucocorticoids in basal conditions, and genomic mineralocorticoid receptors set the HPA-axis activation threshold (reviewed ter Heegde et al. 2015). Furthermore, increased forebrain mineralocorticoid receptor expression is associated with reduced anxiety (Lai 2007, Harris et al. 2013). While mRNA expression and functional protein levels are considered positively correlated, these correlations can be weak or even negative in some cases due to post-transciptional regulatory processes (Nagaraj 2011, Vogel & Marcotte 2012), and including proteomic analyses in would add power to future studies. Some work suggests the ratio of mineralocorticoid to glucocorticoid receptor function mediates successful adaptation to novel environments and proper HPA-axis

maintenance (Gomez-Sanchez & Gomez Sanchez 2014), although altering mineralocorticoid receptor expression does not alter glucocorticoid receptor expression and vice versa (Harris et al. 2013). In contrast to current findings, transgenic overexpression of mineralocorticoid receptors does not alter baseline plasma corticosterone relative to control individuals (Harris et al. 2013, Lai et al. 2007). Increased *Nr3c2* expression in conjunction with the pattern of decreased baseline plasma CORT in offspring of predator-exposed males relative to controls suggests that offspring of TMT-exposed males may be more sensitive to circulating glucocorticoids and have a higher threshold for HPA axis activation.

Bdnf is key to maintaining synaptic plasticity and neural growth (Nelson 2011; Kandel et al. 2013). Work in rodents has established that *Bdnf* is epigenetically altered by stress (e.g. reduced maternal care, restraint, or predator presence/social defeat), but that different stressors have different effects on *Bdnf* expression (reviewed in Bennett & Lagopoulos 2014). In rats, individuals whose fathers experienced predator stress have altered brain morphology in the prefrontal cortex and adults exhibited increased spine density in the orbital frontal cortex (Harker 2018), which could be consistent with increased *Bdnf* activity (Bennett & Lagopoulos 2014). Interestingly, glucocorticoid and mineralocorticoid receptors have opposing effects on *Bdnf* action in the hippocampus and amygdala, although their effects on *Bdnf* in the prefrontal cortex remain unclear (Bennett & Lagopoulos 2014). Collectively, these data indicate that paternal stress can affect offspring brain morphology and that these effects may be mediated by *Bdnf*.

In this study, offspring of predator-exposed males tended to have decreased *Bdnf* expression relative to offspring of males without predator exposure in conjunction with increased mineralocorticoid receptor (*Nr3c2*) expression in the prefrontal cortex (Figure 7A and C). Repeated restraint stress in rats resulted in HPA-axis dysregulation in conjunction with decreased hippocampal *Bdnf* and increased plasma CORT (Makhathini et al. 2017). Here, there was no evidence of altered expression in the hippocampus or differences in plasma CORT during the

stressed or recovery time points between treatment groups. The hippocampus, prefrontal cortex, and amygdala are all key to integrating context and cues to launch the fear response (especially in contextual fear condition paradigms; reviewed in Rozeske et al. 2015), and dysregulation in many of these circuits can lead to altered stress reactivity or anxiety-like behaviors. In studies investigating anxiety-like phenotypes following paternal stress for four generations (summarized above), van Steenwyk and colleagues (2018) found that depressive behavioral phenotypes were conferred as well as increased risk-taking behavior in some (but not all) generations. While the present study did not measure depressive behavioral phenotypes, future work on the relationship between depressive and stress resilient phenotypes and *Bdnf* expression could provide insight into complex interactions between fear circuitry, anti-predator behavior, and stress resilience.

CHAPTER V

CONCLUSION

In this study, I investigated the effects of paternal stress on offspring neural, hormonal, and behavioral phenotypes. I found evidence of altered *Nr3c2* and *Bdnf* expression in the prefrontal cortex, reduced baseline plasma corticosterone, and decreased anxiety-like behavioral phenotypes in offspring of male mice exposed to TMT. These results suggest that fathers exposed to predation threat produce offspring that are resilient to stress and, potentially, better adapted to a high predation environment. Importantly, this study provides evidence that ecologically relevant paternal experience can be transmitted through the germline and can exert consistent effects on offspring phenotypes throughout development. Future studies should examine the mechanism (e.g. altered DNA methylation, histone acetylation, miRNA activity) responsible for the transmission of paternal experience.

Work in both natural and laboratory systems demonstrates that paternal effects do not act in isolation (Guillaume et al. 2016; Masoodh et al. 2018). For example, plastic offspring phenotypes in marine tubeworms are the product of interactions between environmental, maternal and paternal effects, and the strength and directionality of parental effects depend on the match between parent and offspring environments, and the sex of the parent (Guillaume et al. 2016). In mammals, the developmental environment is maternally-defined and even subtle differences in maternal behavior can have lasting effects on offspring phenotypes. Therefore, if mothers in my study were sensitive to paternal stress, a maternal contribution to offspring phenotypes is probable. Further work on this system should employ artificial insemination and cross-fostering

to parse paternal from maternal contributions to offspring phenotypes. However, this study demonstrates that paternal contributions exert stable epigenetic changes on offspring phenotypes, thus providing insights into alternate methods of paternal investment.

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APPENDICES

APPENDIX A

TABLES

Table 1. Primers used for qPCR analyses.

Gene	Ensembl Gene ID	Forward Primer	Reverse Primer
Nr3c1	ENSMUSG00000024431	CAAGGGTCTGGAGAGGACAA	CTGGACGGAGGAGAACTCAC
Nr3c2	ENSMUSG00000031618	TTGGTGTGTGGAGATGAGGC	TGCAGGCAGGACAGTTCTTT
Drd1	ENSMUSG00000021478	GCAAATCCGGCGCATCTCA	AGCCAGCAGCACACGAATA
Drd2	ENSMUSG00000032259	GCCATCAGCATCGACAGGTA	ATGACAGTAACTCGGCGCTT
Bdnf	ENSMUSG00000048482	TGCATCTGTTGGGGAGACAAG	TGGTGGAACATTGTGGCTTTG

Table 2. *Linear mixed models used to determine effect of spermatogenic stage on offspring behaviors. This analysis was conducted on offspring of experimental individuals to determine if spermatogenic stage had an effect on offspring behaviors. To determine pair timing, offspring were assigned "1" if they were born after the first pairing and "2" if a result of the second pairing.*

Type of Model	Behaviors Measured	Code
Linear mixed model	USVs, OFT, TMT assay.	lmer(Behavior~Pair Timing +(1|Litter ID)

Table 3. *Mixed models used to determine effect of paternal treatment and sex on behaviors.*

Type of Model	**Behaviors Measured**	**Code**
Generalized linear mixed model	Total number of USVs	glmer (Behavior~Paternal Treatment + (1| Litter ID)) glmer (Behavior~ 1 + (1| Litter ID))
Linear mixed model	USVs (first minute, second minute, latency), TMT exposure assay, weight	lmer (Behavior~Paternal Treatment + (1| Litter ID)) lmer (Behavior~ 1 + (1| Litter ID))
Generalized linear mixed model	OFT (time in center/activity), number of USVs, time spent with TMT in predator odor	glmer(Behavior~Paternal Treatment*Sex + (1|Litter ID)) glmer(Behavior~Paternal Treatment + Sex + (1|Litter ID)) glmer(Behavior~Paternal Treatment + (1| Litter ID)) glmer(Behavior~Sex + (1|Litter ID)) glmer(Behavior~ 1 + (1| Litter ID))
Linear mixed model	OFT (ratio baseline:stressed)	lmer (Behavior~Paternal Treatment*Sex +(1|Litter ID)) lmer (Behavior~Paternal Treatment + Sex + (1| Litter ID)) lmer (Behavior~Paternal Treatment + (1|Litter ID)) lmer (Behavior~Sex + (1|Litter ID) lmer (Behavior~1 + (1|Litter ID))

APPENDIX B

FIGURES

A

Paternal treatment timeline

Day 0	Days 1-8	Days 17-23	Day 25	Day 30
Acclimate	TMT or banana extract exposure	Pair males	Experimental males paired 2nd time Control males sacrificed	Second experimental male pairs split

B

Offspring treatment timeline

Day 0	Day 3	Day 21	Day 83	Day 84	Day 100
Birth	Weigh Record USVs	Weigh Wean Juvenile OFT	Adult baseline OFT	TMT exposure Stress OFT	Weigh Collect blood Harvest tissue

Figure 1. *Experimental timelines for A) paternal and B) offspring treatments. Offspring adult treatments occurred over a range of ~10 days. OFT, open field test.*

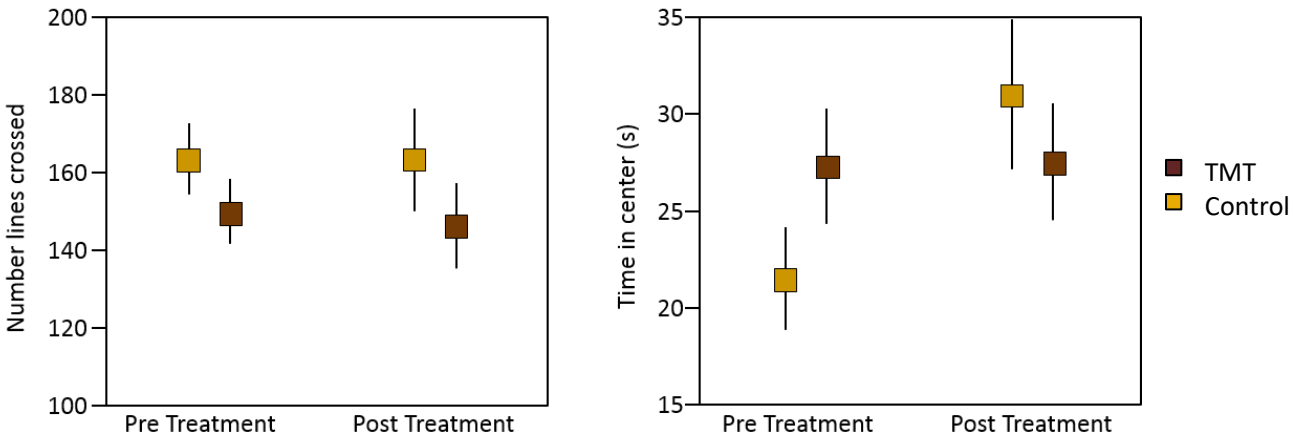

Figure 2*. Fathers' A) overall activity and B) time in the center of the open field before and after paternal stress paradigm. There was no effect of treatment on A) fathers' activity before or after treatment or B) the amount of time fathers spent in the center of the apparatus before or after treatment. n=15/treatment. Error bars ± 1 SE of mean.*

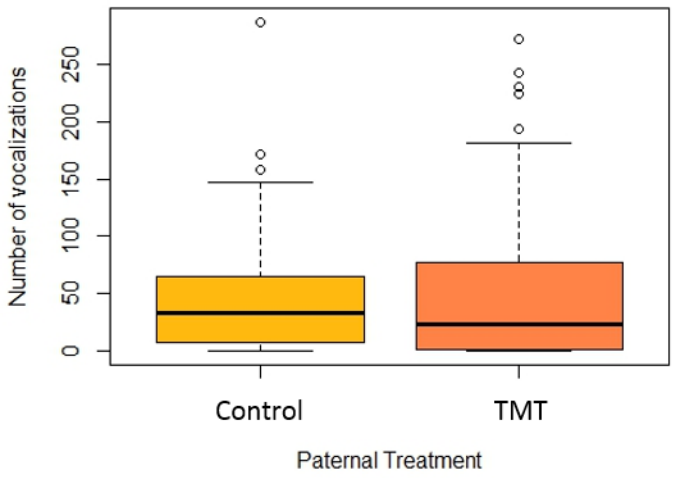

Figure 3. *Number of ultrasonic vocalizations produced by 3 day old pups. There was no effect of paternal treatment on the total number of vocalizations produced during a 3 minute trial. Box plot includes average and interquartile range. Sample size: # litters* $^{\#individuals}$*: experimental offspring =* 19^{120}*, control offspring =* 11^{60}.

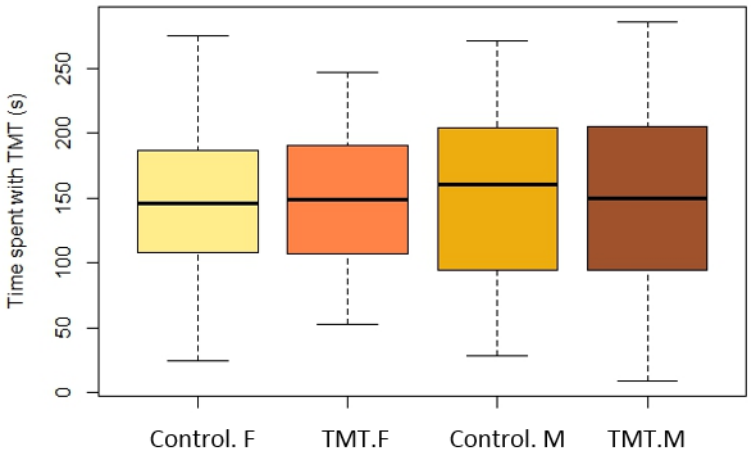

Figure 4. *Amount of time spent on the side of a cage with 10% TMT on filter paper in adult offspring. There was no effect of paternal treatment on time spent with TMT ($p=0.6$). Male offspring spent less time on the side of the cage containing TMT than females, regardless of paternal treatment ($p<0.001$). Boxplot includes average and interquartile range. Sample size: # litters$^{\#\ individuals}$: control females: 11^{33}, TMT females=18^{54}, control males=10^{26}, TMT males=18^{35}.*

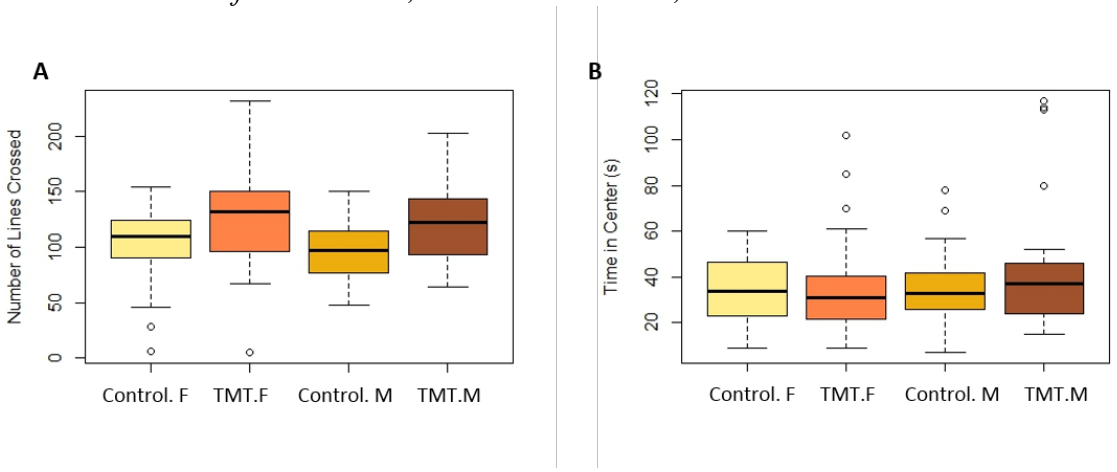

Figure 5. *Juvenile behavior in the open field. A) Juveniles from litters sired by fathers exposed to predator odor were more active relative to offspring of males without predator experience ($p<0.001$). Within groups, males were less active than females ($p<0.001$). B) Offspring of TMT-exposed males exhibited reduced time in the center of the open field relative to control offspring ($p< 0.001$). This effect was driven by male offspring of TMT-exposed males, who spent the least amount of time in the center of the open field relative to other groups ($p=0.001$). Boxplots include average and interquartile range. Sample size: # litters$^{\#\ individuals}$: control females=10^{35}, TMT females=15^{48}, control males=9^{17}, TMT males=14^{29}.*

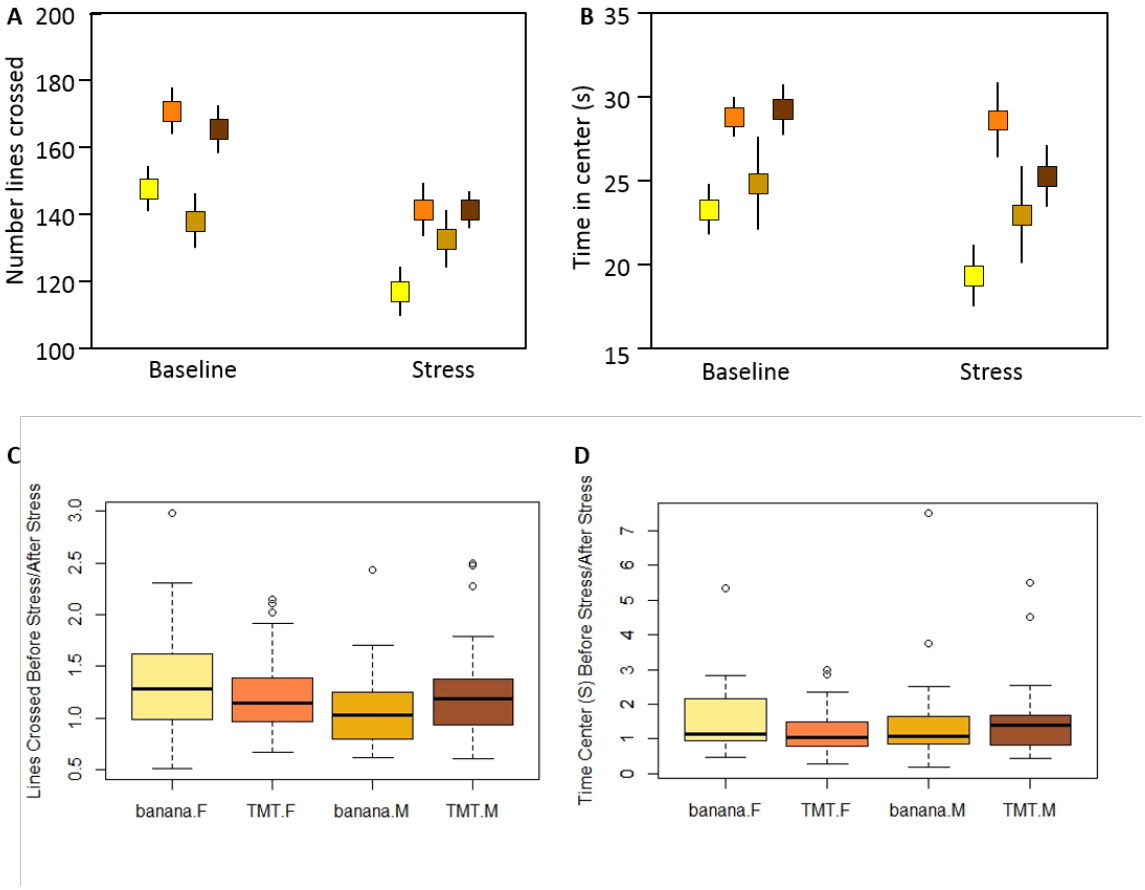

Figure 6. *Open field results for adult offspring. A) Overall activity and B) time spent in the center of the open field before and after stress. A) Before stress, offspring of TMT exposed males crossed more lines than control offspring ($p=0.042$). Within groups, females cross more lines than males ($p<0.001$). Following stress, offspring of TMT-exposed males crossed more lines relative to controls ($p=0.025$). Sons were overall more active than daughters ($p=0.043$). This sex specific effect was treatment dependent: control male offspring crossed more lines than their female counterparts, but sons of TMT-exposed males crossed fewer lines than daughters ($p<0.001$). B) Before stress, offspring of TMT exposed males tended to spend more time in the center of the apparatus relative to offspring of control males ($p=0.069$). Following stress, offspring of TMT-exposed males spent more time in the center ($p=0.0067$). Female offspring spent more time in the center than males ($p=0.02$), but this effect was driven by female offspring of TMT-exposed males, who spent the most time in the center relative to other groups ($p=0.024$). There was no effect of paternal treatment on C) the ratio of grid lines crossed or D) the ratio of time spent in the center of the apparatus before : after TMT exposure. Boxplots include average and interquartile range. Error bars ± 1 SE of mean. Sample size: # litters$^{\#\ individuals}$: control females: 11^{37}, TMT females=18^{52}, control males=9^{23}, TMT males=16^{37}.*

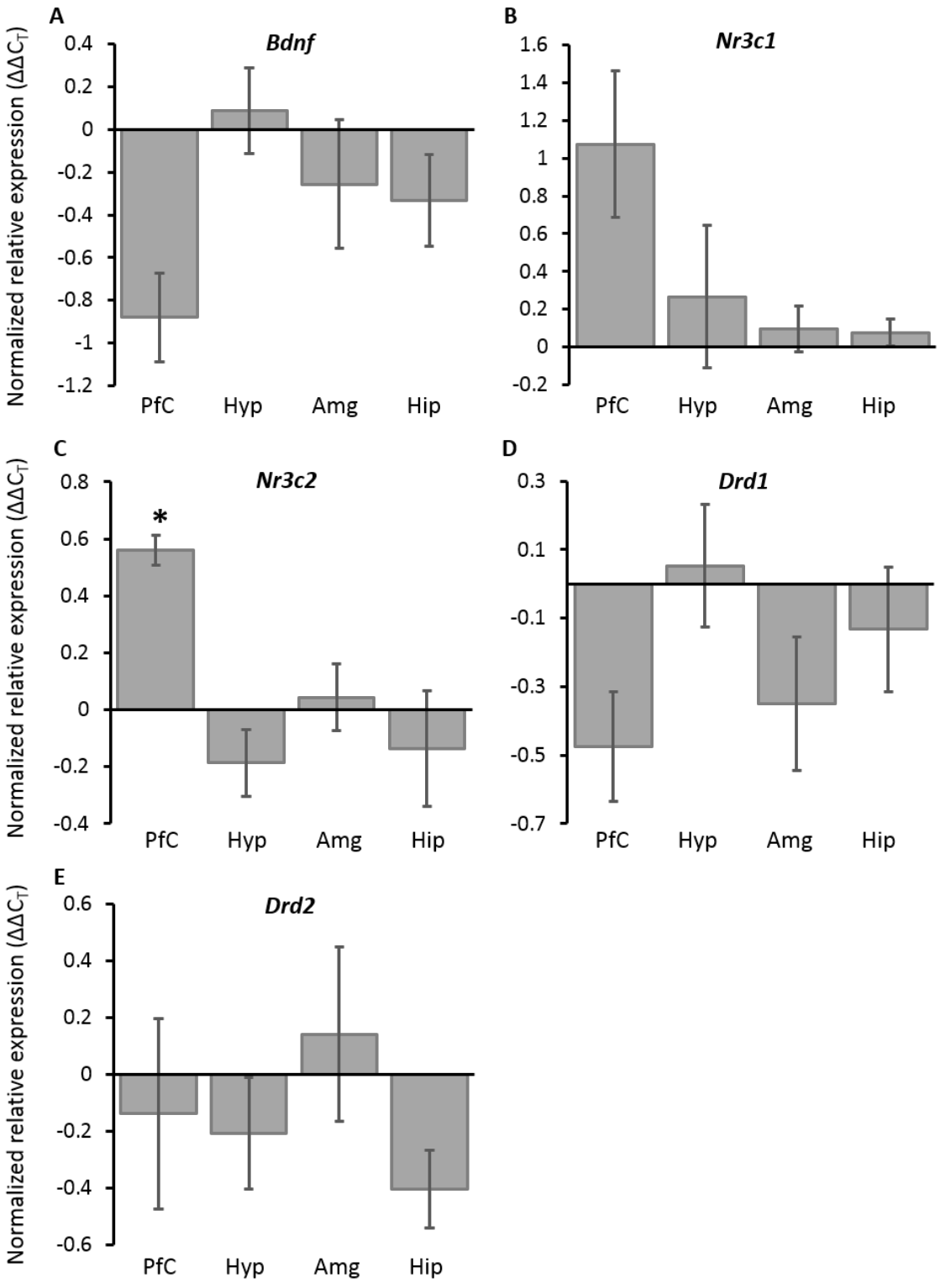

Figure 7. *Normalized difference in expression (ΔΔC$_T$) between experimental and control offspring for A)* Bdnf*, B)* Nr3c1*, C)* Nr3c2*, D)* Drd1*, and E)* Drd2*. Positive values*

indicate increased expression in experimental individuals relative to control individuals. Negative values indicate decreased expression in experimental individuals relative to control. Offspring of predator odor exposed males A) tended to have decreased Bdnf *expression (p=0.014) and C) had increased* Nr3c2 *expression (p=0.0045) in the prefrontal cortex relative to control individuals. There was no effect of paternal treatment on* Bdnf *or* Nr3c2 *expression in the hypothalamus, amygdala, or hippocampus (p=0.292-0.858). There was no effect of paternal treatment on offspring gene expression for* Drd1, Drd2, *and* Nr3c1 *(p=0.15-0.887) in the prefrontal cortex, hypothalamus, amygdala, or hippocampus. n=5/sex/treatment. Error bars ± 1 SE of mean $\Delta\Delta C_T$. Sample sizes: 5 per sex per treatment.*

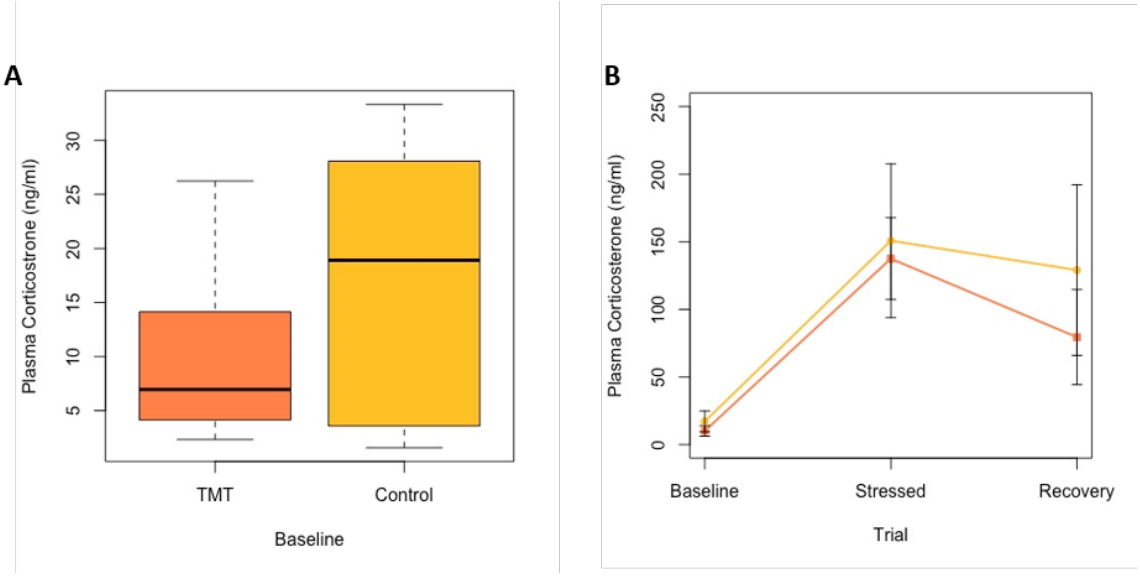

Figure 8. *Effects of paternal stress on offspring plasma corticosterone A) at baseline and B) at baseline, during and after stress. A) Offspring of fathers exposed to predator odor had reduced baseline plasma corticosterone relative to controls (p=0.026). B) There was no overall effect of paternal treatment on offspring plasma CORT across the three time points. Boxplot includes mean and interquartile range. Error bars ± 1 SE of mean. Sample sizes (litters): baseline: n=15 experimental, 9 control; stress: n=13 experimental, 10 control; recovery: n=14 experimental, 11 control.*

VITA

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Master of Science

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