Effects of semi-natural environmental conditions on phenotypic plasticity in *Rattus norvegicus*

by

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ABSTRACT

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Controlled laboratory experiments find there is normal variation in maternal care that regulates the development of the endocrine, cognitive and behavioral responses to stress in rats. As housing conditions of laboratory rats can have pronounced effects on experimental outcomes, we examined how semi-naturalistic environmental conditions affect maternal care and how /if variation in maternal care affects neural and behavioral development in adult female offspring. Specifically, we assessed maternal behaviors in semi-natural enclosures and what effects, if any, alternative maternal strategies might have on the development of stress physiology and cognition in adult offspring. We found semi-natural housing conditions decreased variation in maternal care, litter size, and offspring survival regardless of the amount of maternal care provided to pups, but increased pregnancy success in dams phenotyped as lower maternal care providers in laboratory housing, suggesting there could be direct fitness benefits to providing lower levels of maternal care. I also found significant environmental effects on offspring stress and cognitive profiles that are not mediated by our standard laboratory measures of maternal care. Collectively these analyses underscore the critical role of environmental context on maternal behavior and its behavioral and physiological outcomes.

DEDICATION

For Nico and Penelope

"*What goes on inside is just too fast and huge and all interconnected for words to do more than barley sketch the outlines of at most one tiny little part of it in any given second"* -David Foster Wallace

TABLE OF CONTENTS

INTRODUCTION

A wealth of data from the past 50 years has documented the effects of early life on offspring development. The majority of this research has focused on the development of neuroendocrine systems that play a role in stress-related behavior in the hopes of identifying factors impacting resilience and risk-sensitivity (Denenberg *et al.,* 1964; Levine, 1957; Meaney, 2001). However, most of what we know about stress has been produced in laboratory mice and rats housed in highly artificial environments unlikely to be experienced in the natural world, making any interpretation of behavior from an adaptive perspective difficult if not impossible. While controlled studies have generated many valuable biological insights, it has also generated a large amount of phenomena that has not been validated in field settings or is not present in the field (for reviews see Wolff, 2003; Calisi and Bentley, 2010). Clearly, leaving these questions solely to the domain of biomedical research leaves out the possibility of understanding stress from an ecological and evolutionary perspective (Armein, 2008; LaDage *et al.,* 2009; Barnea and Nottebohm, 1994; Barnea, 2010; Boonstra, 2013).

Stereotypic behaviors (e.g. repetitive behaviors such as excessive grooming) are a known phenomenon observed in a wide range of animal taxa housed in impoverished captive environments, including rats housed in standard laboratory conditions (Kalueff et al., 2007; Garner and Mason, 2002; Harrison et al., 2001).
Rodents in animal facilities are typically housed alone (if pregnant or involved in invasive experimental testing) or paired-housed with another individual of the same sex in 'shoe box' sized cages (20 x 20 x 32 cm), under standardized temperature (22 C) and humidity (40% to 50%), entrained to a constant 12hrs of light, with unlimited food. As a rule, external stimuli are purposely limited to regular animal husbandry to control for environmental 'noise'-unwanted environmental effects on experimental outcomes. Often these conditions result in obesity, glucose intolerance, less aggression, and more or less responsiveness to stressors compared to wild counterparts (Miller et al., 2002; Wolff, 2003; Martin et al., 2010)

It is widely known that enriched housing conditions can dramatically affect physiology and behavior (Altman and Das, 1969; Diamond, 1964; Kempermann, 1997), but what is not as well understood is how life outside of laboratory paradigms influences the development of stress phenotypes. My objective for this dissertation was to document maternal care and the development of the stress axis in 'reality' (e.g. in an ecologically relevant setting). To accomplish this, I examined stress and stress related phenomena in female Long-Evans rats that were born and

housed in semi-natural enclosures and compared these findings to laboratory data from age-matched females housed in animal facility conditions.

In chapter one, I examined the effects of semi-natural conditions on maternal care and measures of reproductive fitness by comparing data from females housed in semi-natural enclosures at the Field Station for Behavioral and Ecological Research (FSBER) with females housed in standard animal facility conditions. Laboratory evidence suggests there is naturally occurring variation in maternal care that has pronounced affects on offspring gene expression, stress and reproductive physiology and behavior (Caldji et al., 1998; Francis *et al.,* 2003; Liu *et al.,* 1997). My data indicate that semi-natural conditions decrease reproduction and fitness, possibly as a result of HPA axis changes. I also found that maternal care was not a stable pattern at the field station, suggesting it might not be a valuable mediator of stress development in free-living populations subject to even more complexity.

In chapter two, I examined factors mediating long-term developmental plasticity of the hypothalamic pituitary adrenal (HPA) axis and anxiety- and depression-like behavior. Here, I tested the hypothesis that the early maternal environment is a significant mediator of HPA axis development and behavior. My data indicate that unlike laboratory findings, individual stress phenotypes are not mediated by maternal care received but are instead mainly influenced by the housing environment as well as partially influenced by maternal corticosterone phenotypes.

Because maternal care did not play a significant role in individual stress phenotypes, in chapter three, I focused on the effects of semi-natural housing versus laboratory conditions on measures of cognition and neural plasticity in aged adult females housed long-term in semi-natural conditions. Recent work indicates living in complex natural habitats is associated with dramatic structural changes in the brain, including increased neurogenesis in the DG, across a broad range of taxa (Barnea and Nottebohn, 1996; Boonstra *et al.,* 2001; Barnea, 2010; LaDage *et al.,* 2010; Dunlap *et al.,* 2010). These resulting data indicate the physical environment is an important determinant of neural plasticity and cognition. Collectively these analyses yield important insights into the physiological and behavioral consequences of semi-natural conditions.

Acknowledgments

I would like to express my deepest gratitude for everyone who has beena source of encouragement through the challenges of graduate school. I am particularly grateful to my advisor Darlene Francis. She allowed me complete academic freedom and encouraged me to pursue my interests-both in research and in pursuits outside of academia. I was fortunate to have an advisor that truly values life outside of the lab. I would also like to thank my dissertation committee, in particular Daniela Kaufer, for their support and guidance and for being role models of decency, kindness, and truth. Most importantly I thank my family. The loves of my life, Nico and Penelope, who were born into the madness of late night rat emergencies and the general stress of graduate school; my husband, Gerard Dunn, who sacrificed so much for me to finish; and last but not least my mom, dad, and Ellen who encouraged me over the years and fostered my love of the outdoors.

Chapter 1

Environmental modulation of maternal care and reproductive success

INTRODUCTION

Parental behavior has been demonstrated to influence offspring development across a range of taxa and outcomes. In humans, there is a large literature exploring how features such as harsh parenting or maternal warmth are capable of influencing the physiological and behavioral development of children. For example, young girls raised in homes with greater maternal harshness at age 5 are more likely to become sexual risk-takers as adolescents (Belsky, Steinberg, Houts *et al.,* 2010). One working hypothesis suggests that harsh maternal parenting contributes to early menarche that ultimately contributes to increased sexual risk-taking earlier in life.

From an ecological perspective, one common approach to the study of parental care has focused on the Trivers and Willard (1973) finding which states that, by the process of natural selection, variability in parental investment is capable of varying the sex ratio of offspring. That is, as maternal conditions decline or become more challenging, females tend to produce lower ratios of males to females. Support of the Trivers-Willard hypothesis has been equivocal. However, a great deal of research has focused on addressing variation in mammalian sex ratios often resulting in contradictory results.

In the laboratory, the rat has become the model of choice to investigate how early-life experiences, particularly maternal care, can influence the developing offspring. The study of maternal care in the laboratory rat has documented that naturally occurring intra-specific variation in postnatal maternal care has pronounced effects on gene expression, stress-sensitive measures and reproductive physiology and behavior (Caldji *et al.,* 1998; Francis *et al.,* 2003; Liu *et al.,* 1997). Individual differences in maternal behavior profiles are reported to be stable across multiple cohorts (Champagne *et al.,* 2003) resulting in the transmission to offspring. In particular, female rats born and reared by a highly 'maternal' mother (assessed by licking and grooming scores), later in adulthood, will then become a highly 'maternal' mother herself (Francis, 1999; Champagne *et al.,* 2003).

Maternal care and measures of individual fitness are not static traits in an individual, but can shift throughout an individual's lifetime as a consequence of environmental variation (phenotypic plasticity), age, and reproductive history (Williams, 1966). Phenotypic plasticity in parenting strategies is documented in fish (Bagenal, 1971; Wright and Gibb, 2005), birds (Fontaine and Martin, 2006) insects (Tollrian and Von Elert, 1994; Grindstaff, 2006) as well as mammals (Gonzalez *et al.,* 2001; Lovic, 2001; Fleming *et al.,* 2002; Shapiro *et al.,* 1995). Maternal variation in response to environmental variation is found in primates as well. For example, free-living populations of Bonnet-macaque mothers change their responses to their infants depending on food scarcity. When food availability is unpredictable, there is pronounced conflict between the macaque mother and infant (Coplan *et al.,* 1996; Rosenblum and Andrews, 1994).

Although phenotypic plasticity and its role in developmental programming is a well-documented phenomenon, much less is known about the stability of between-individual differences across diverse environments, particularly in the female laboratory rat (Beery and Francis, 2011). Indeed, the bulk of research investigating maternal care in the research laboratory has been conducted under conditions that bear little resemblance to a natural environment. Recent research on natural variation in maternal care in the Long Evans rat found that, in impoverished or enriched environments laboratory environments, between-individual variation shifts in predictable patterns. A recent study examining the effects of adversity on maternal L/G found that after seven days of prenatally stressing high L/G mothers, higher care mothers decreased L/G to levels indistinguishable from low L/G dams (Champagne and Meaney, 2000). Another study by the same group found that maternal licking differences between high and low dams were abolished when rats were weaned into impoverished, or enriched cages, high L/G dams remained constant but low L/G dams shifted to high phenotypes in environmental enrichment and low L/G remained constant but high L/G shifted to lower phenotypes in impoverished conditions (Champagne and Meaney, 2007).

The study of rodent maternal behavior in free-living populations or in populations housed in semi-natural conditions is virtually non-existent. Although some studies have been able to document 'proxies' of maternal care in free-living populations (e.g. offspring weight, time on nest) the majority of research on maternal care in free-living populations has focused on the study of wild-trapped rats brought into captivity (Barnett 1963; Boice, 1968; Price 1970). Many phenomena often observed in rodents under laboratory conditions have either not been validated or even observed in free-living populations (Wolff, 2003). It is important to know if naturally occurring variations in maternal care (assessed by licking and grooming scores) are stable phenotypes under 'non-laboratory' conditions. While observing maternal care in the wild is ultimately the only sure way of knowing how rats behave in free-living populations, quantifying licking and grooming in a nocturnal animal that burrows (a behavior that is not easily quantified in laboratory conditions) would be very difficult. Semi-natural conditions include potentially salient features of the female burrow system (e.g. dark nest boxes, ambient environmental conditions) that allow for the emergence of some natural behaviors, while still allowing for detailed observations of behavior.

The current study assessed how robust maternal L/G phenotypes are between two environments: i) a controlled standard animal facility condition (AFH) and ii) a less-controlled and more naturalistic semi-natural enclosure condition at the UC Berkeley Field Station for Behavioral and Ecological Research (FSBER). If there is between-individual variation in licking and grooming and fecundity, then we expect to see consistency in individual behaviors across environmental conditions. Alternatively, if individuals are as variable in L/G behavior and fecundity as the entire population (not consistent) then we can conclude the variation we observe at the field station is variation within-individuals and not between individuals. Because reproduction is affected by environmental variation, we quantified

reproductive success by measuring fecundity, litter size, and offspring morbidity and mortality to assess how these measures differed within individuals and between higher or lower L/G dams. Finally we measured female corticosterone (CORT) levels during and after a mild stressor to gauge the hormonal stress response.

METHODS

Animals and experimental conditions

Standard Laboratory Housing

Two cohorts of fifty-day old female Long-Evans rats (cohort 1, n = 25; cohort 2, n = 50) arrived in our facility from Charles River Laboratories, (Wilmington, MA) and were provided a 4-day acclimation period prior to manipulation. Rats were housed in standard animal facility housing (AFH) conditions. AFH consists of same sex same pair-housed rats in 'shoe box' sized cages (20 x 20 x 32 cm), under standardized temperature (22°C) and humidity (40% to 50%) conditions, and on a 12:00 h light/dark cycle.

After assessing estrous cyclicity for 21 days, cohort 2 rats were subjected to a brief 15 minute restraint stress with tail blood samples collected basally, during and after the stressor to measure CORT levels (described in detail below). Several days after the stressor application females were individually housed and paired with a "stud" male to allow mating to occur. Males were removed after six days, and except for standard care, females were left alone to give birth and rear offspring. Offspring were weaned at PND21. Maternal care was assessed.

Semi-Natural Housing at Berkeley Field Station

A smaller pool of females that successfully gave birth in the AFH (cohort 1, n = 18; cohort 2; n = 29) were transferred to the Field Station for Behavioral and Ecological Research (FSBER) shortly after their first litters were weaned. While housed at the field station all rats were exposed for the first time to natural ambient light, temperature, humidity and predator cues. After a forty-day habituation period estrous cyclicity was assessed followed by another brief restraint stress exposure (similar to what was first assessed when all rats were housed under AFH conditions). These same females were subsequently placed in new cages and bred with "stud" males for six days. On gestational day 19, females previously characterized as providing lower (n = 7) and higher (n = 5) levels of maternal care in AFH (and that gained more than 80 grams, confirming pregnancy) were removed from cages and allowed access to semi-natural enclosures (1 dam/enclosure). Each enclosures (2.4 × 3.1 × 2.7m) had five walls, three solid walls and two constructed of ¼-inch mesh hardware cloth. The larger of the mesh walls was open to an outdoor

walkway allowing for seasonally dependent environmental conditions. The upper half of the smaller wall, which faced onto an inner hallway, was 50% mesh /50% observation window allowing for unobtrusive observations. In an attempt to simulate the hidden burrow system, all rooms contained black nest boxes located on 0.6m × 0.6m, platforms accessible by 1.3m ramps. Ambient conditions were monitored and ranged from -6°C to 31°C, humidity was approximately 70-85%, and lighting ranged from 10-15 hours per day. Beginning on PND1, maternal behavior was recorded as described below. On PND 21, offspring were weaned and dams were sacrificed.

Controls for effects of parity

A smaller pool of nulliparous females housed in cages (n = 5) or housed in rooms (n = 5) ordered from Charles River and transported to the FSBER were used as a control for the effects of parity on litter size and composition. All animals in this study had *ad libitum* access to food and water.

Maternal behavior

Animal facility maternal behavior was scored from Day 1 through Day 6 postpartum. Each day consisted of five hours of observations starting at 6am-7am, at an hour before lights are turned on, 07:00 h-08:00 h, 12:00 h, and 20:00 h -21:00 h an hour before lights are turned off, and 9pm-10pm. Within each observation period, the behavior of each mother was scored every 2 min (60observations/period × 5periods per day = 300 observations/mother/day = 1800 observations per mother over the 6 days). The following behaviors were scored-mother off pups, mother licking and grooming any pup (both body and anogenital licking were included), and self-grooming.

Field station maternal behaviors were scored from dams housed in enclosures (n = 8). To best match laboratory light cycles and to allow for comparison, we modified observations so that they started one hour before sunrise and one hour after sunrise, noon, and one hour before sunset and 24:00 h. The same behaviors as described above were recorded over the first six postnatal days.

Restraint Stress Protocol

All rats were stressed and blood samples taken between 08:00 h and 12:00 h in AFH and at sunrise to 12:00 h at the FSBER. Basal blood samples were collected from all rats between 08:00 h-09:00 h in AFH conditions and at sunrise to an hour after sunrise at the FSBER. Blood was collected from the tail after a positive vaginal smear indicating estrous (basal) was confirmed. All basal blood samples were collected within 45sec after removal from the cage. Animals were then placed in plastic

decapi-cones and not allowed to escape for 20 minutes. Blood samples were also collected at 15, 30, 60, 90 minutes from the onset of restraint. (Meaney *et al.,* 1989). All blood samples were collected into microcentrifuge tubes and stored on ice until the end of the experiment. Samples were centrifuged at 4°C and plasma extracted and stored at 20°C until assayed. To decrease intra and inter-assay error, all stress procedures in AFH and at the FSBER were performed by the same individual.

ELISA for corticosterone

CORT was quantified using a commercially available ELISA kit (Assay Designs, Inc). All procedures were completed according to the manufacturer's instructions without modification. Samples were diluted 1:20 in buffer solution that allowed for them to fall within the linear portion of the standard curve. Intra-assay variation was less than 8% and inter-asssay variation was less than 9%. All samples were assayed in duplicate. Statistical analysis was conducted using the average of duplicates. All CORT values are reported as μg/dl.

Quantifying fitness and survival

Reproductive success was measured as the rate of successful pregnancies that resulted in parturition. Direct fitness between higher and lower L/G dams and between FSBER and AFH conditions was assessed by recording the number of pups that were successfully weaned at PND 21. Offspring survival was determined as the number of adult offspring at three months surviving to 15 months. Offspring deaths included fatalities (animals found dead in their cage) and morbidities (any animals that had an illness, such as a visible tumor, paralysis, or large infection, which required euthanasia as ordered by OLA. Multiple rats at the field station had minor wounds and infections that did not require euthanasia and were scored as surviving.

Data analysis

A two-way ANOVA with repeated measures was used to analyze CORT levels (environmental condition × time). When results were significant, multiple comparisons were performed using Sidak's test. Spearman's r correlation (unequal certainty) was used to analyze the relationship between maternal licking and grooming scores between AFH rats and FSBER rats and between maternal LG scores and CORT. Nominal logistic regression was used to analyze the relationship between maternal LG scores and pregnancy success. Maternal off nest bouts were analyzed using a two-way ANOVA (housing × postnatal day) followed by a Sidak's test for multiple comparisons. Litter sizes were compared using Wilcoxan matched-pairs signed rank test. A non-parametric t-test was used to analyze gender composition ratios. An unpaired t-test was used to assess offspring survival.

RESULTS

Maternal behaviors

When L/G profiles were compared across rearing conditions/sites, using Spearman's correlation, there was no relationship between L/G in AFH and FSBER enclosures (Figure 1.1a). Animal facility females spent significantly more time off the nest during observations in PND 3, 4 and 5 ($p < 0.005$; Figure 1.1b).

Corticosterone

CORT was measured basally, during and following a 20 min restraint stressor in the AFH (n = 25) and the FSBER (n = 25) rat dams. Basal CORT levels did not different significantly between AFH and FSBER conditions. There were significant differences during the recovery stage after a stressor. CORT levels were significantly higher in FSC compared to AFH conditions. Statistical analysis revealed a significant main effect of environment on CORT ($F = 8.857$; $p < 0.0001$) as well as an interaction between housing and CORT ($p < 0.0001$). Multiple comparisons test revealed that plasma CORT levels were significantly higher in FS conditions during the 30min ($p < 0.0005$), 60min ($p < 0.05$) and 90min ($p < 0.05$) post stress. There were no significant differences in peak CORT levels during the stressor. (Figure 1.2 for all)

CORT stress-reactivity profiles were correlated across environmental conditions to assess the stability of individual profiles. There was a significant positive correlation between AFH CORT levels and FSBER CORT levels during recovery ($p = 0.0306$ Figure 1.3a).

As maternal L/G has been demonstrated to influence developmental programming of the HPA axis, we investigated the relationship between maternal L/G and CORT in AFH and FSBER conditions. CORT levels were significantly higher 30 min after an acute stressor in lower maternal care dams at the FSBER but not in the AF (Spearman r: $p = 0.0067$; Figure 1.3c). There was a trend towards higher basal CORT and higher peak stress in lower maternal care phenotypes at the FSBER (Spearman r: $p = 0.0568$, $p = 0.0673$; Figure 1.3b,d). There was no relationship between FSBER L/G in enclosures and CORT at the FSBER or in the AF.

Fitness and Survival

Fecundity

All dams that had previously given birth in AFH were rebred at the FSBER. Fecundity, measured as the number of successful pregnancies, was significantly lower at the field station (Fishers exact test: $p = 0.0009$; Figure 1.4a). However, dams previously phenotyped and characterized as low L/G in AF conditions were significantly more likely to give birth in field station conditions (Logistic regression Chi-square: $p = 0.0082$; Figure 1.4b)

Litter size

Linear regression did not show a significant difference between maternal licking and grooming scores on litter size therefore maternal phenotypes were pooled to determine if housing in FSC or AFH influenced the number of pups born. Litter sizes were not significantly different in dams housed in cages or enclosures at the FSBR, so data from these groups was pooled and compared to AFH litters. Environmental housing conditions, but not the maternal LG phenotype, was related to the number of pups born per litter. Litter size was significantly larger in AF compared to FSC (Wilcoxan matched pairs: $p = 0.0010$; Figure 1.5b). Although litter size was related to maternal housing conditions the ratio of female to male pups was not (Figure 1.5a). Gender composition of litters was not significantly correlated with either housing conditions or maternal licking and grooming phenotype.

Offspring survival

The number of female offspring surviving until age 15 months was significantly lower at the field station compared to age matched females housed in animal facility conditions (Fischer's exact test: $p < 0.0050$; Figure 1.5c). Maternal L/G received was not significantly correlated with pup survival.

DISCUSSION

This study assessed the robustness of between-individual variation in maternal L/G in semi-natural environmental conditions, as well as the phenotypic plasticity of maternal and reproductive behaviors between environments. Our results show that FSBER conditions decreased female's ability to recover from stress, confirming our hypothesis that females would interpret the field station as challenging. Not surprisingly, this environment had pronounced affects on fecundity and measures of direct fitness. More surprising, we found maternal care given at the FSBER did not predict FSBER female maternal scores, whereas between-individual CORT differences remained stable.

Although there was phenotypic plasticity in maternal care in dams housed in enclosures, maternal licking was not consistent between environments, nor was there a reliable pattern as observed in laboratory paradigms (Champagne, 2003; Champagne and Meaney, 2008). This suggests that between-individual differences in maternal L/G are potentially an artifact of highly controlled laboratory conditions and high or low maternal L/G is better thought of as falling within the normal range of behavior for any individual in a population. Alternatively, true between-individual variation could exist in free-living populations of rats but in these complex environments distinguishing maternal patterns in L/G would be extremely difficult due to environmental heterogeneity or 'noise'. If the latter is true, it is interesting that subtle stimuli could have such a dramatic effect on maternal profiles and suggests wholesale impoverishment or enrichment paradigms in laboratory settings are not necessary to shift maternal behavior up or down. Future laboratory

experiments could examine this finding by studying L/G transmission under more realistic stimuli. It also suggests that transmission of associated phenotypes would be extremely difficult to predict in free-living populations of rats that are subject to even greater complexity than animals housed in semi-natural enclosures.

Although maternal licking and grooming was inconsistent, dams housed in enclosures spent significantly more time in their nest and on their pups then dams in standard housing, irrespective of their maternal phenotype. Differences in time spent on pups between the two conditions may be proximately affected by differences in pup body temperature. Dams at the FSBER gave birth in March in both cohorts, consequently dams and neonates were subject to a wide range of ambient temperatures. Neonate pups cannot regulate their own body temperature and depend on heat from the mother, especially if litters are smaller, therefore more time spent in contact with pups might be physiologically necessary (Grota and Ader 1969; Leigh and Hofer, 1973; Mendl, 1988). In addition to temperature regulation, dams could spend more time with their pups in order to defend them from real or perceived predators (Leigh and Hofer 1973), whereas vigilance is a trait selected out of standard laboratory animals.

Semi-natural conditions had prominent effects on reproduction; both high and low L/G dams had decreased fecundity (fewer pregnancies carried to term) in field station conditions, with the probability of having a successful pregnancy significantly skewed to lower maternal care givers. Evolutionary theory predicts that female reproductive strategies should be strategies that result in the greatest number of offspring during an individual's reproductive lifetime. Consequently, animals will suppress reproduction if reliable and predictable environmental and physiological cues indicate future conditions are more likely to be favorable than present conditions (Wasser and Barash, 1983). It is probable that females born in benign laboratory conditions experienced ambient conditions at the field station as distressful, as they had a prolonged CORT response post stress, and adjusted reproductive effort. Prolonged CORT levels after a mild stressor, an indication of impaired feedback inhibition of the HPA axis, is a reasonable proxy for the degree of environmental challenge an individual or population is experiencing (Bonier, 2009), and suggests that females were prenatally stressed (Maccari *et al.,* 1995; Barbazanges *et al.,* 1996; Plotsky *et al.,* 1998). The relationship between CORT and reproduction is complicated and not fully understood, but it is known to inhibit reproductive function and behaviors associated with reproduction (Ubuka, 2008; Kreigsfeld *et al.,* 2006; Wu *et al.,* 2009).

Consistent with our findings, females born to high L/G mothers in laboratory conditions also display less sexually receptive behavior, receive fewer ejaculations, release fewer oocytes when ovulating and are less likely to get pregnant (Cameron *et al.,* 2008; Sakhai *et al.,* 2011). These findings, taken together, suggest a general fitness benefit associated with low maternal phenotypes in captive environments. It is likely that high L/G dams have higher fitness under different environmental conditions thus allowing both phenotypes to persist (Mazer and Damuth, 2001). It is

also possible that artificial selection for laboratory-conducive traits, the same traits most associated with higher maternal L/G, allows a phenotype to persist that would be otherwise selected out of free-living populations. Even if this hypothesis is not correct, maternal phenotypes were not consistent with laboratory findings in this study and, without an obvious pattern to this behavior, the question of how reliably these associated reproductive traits are transmitted to offspring remains open.

Housing in semi-natural conditions resulted in smaller litter sizes and decreased offspring lifetime survival, irrespective of maternal L/G. Similar to our findings, a longitudinal study looking at a semi-natural population of rats found the average litter size to be 10 pups per litter (Calhoun, 1969; Boice 1972) and decreased life expectancies are seen in many free-living populations of rodents, compared to laboratory-housed conspecifics (Getz, 1997). It is well documented in ecological literature that adverse environments have immediate effects on fecundity and survival (for a review see Oikos, 1988). Challenging environments require a different set of physiological responses and behaviors and many organisms change parental investment in order to maximize lifetime reproductive success. An animal that must allocate energy to survival will have less energy to allocate to parental care. For example, a smaller litter size can be an adaptive response to challenging environmental conditions because decreasing energy expenditure can increase parent survival, or equal energy expenditure can result in healthier offspring (Beilhartz and Mitpaiboon, 1993). Alternatively, a smaller litter can result in increased parental investment per individual thus increasing direct and indirect reproductive fitness.

Although semi-natural conditions had dramatic effects on maternal and reproductive behavior and HPA axis reactivity, it is important to take into consideration FSBER enclosures, while more realistic than laboratory paradigms, are not replicas of 'real' life. Wild populations of rats, typically live and give birth in burrow systems that are well protected from temperature fluctuations and disturbance (Calhoun, 1962; Price, 1973), and although we attempted to mimic burrow conditions by providing dark nest boxes, dams were noticeably more vigilant of our presence than females in laboratory conditions, possibly affecting maternal behavior. Future studies examining free-living populations of rats, or populations of rats living in even more natural conditions, could shed light on these concerns.

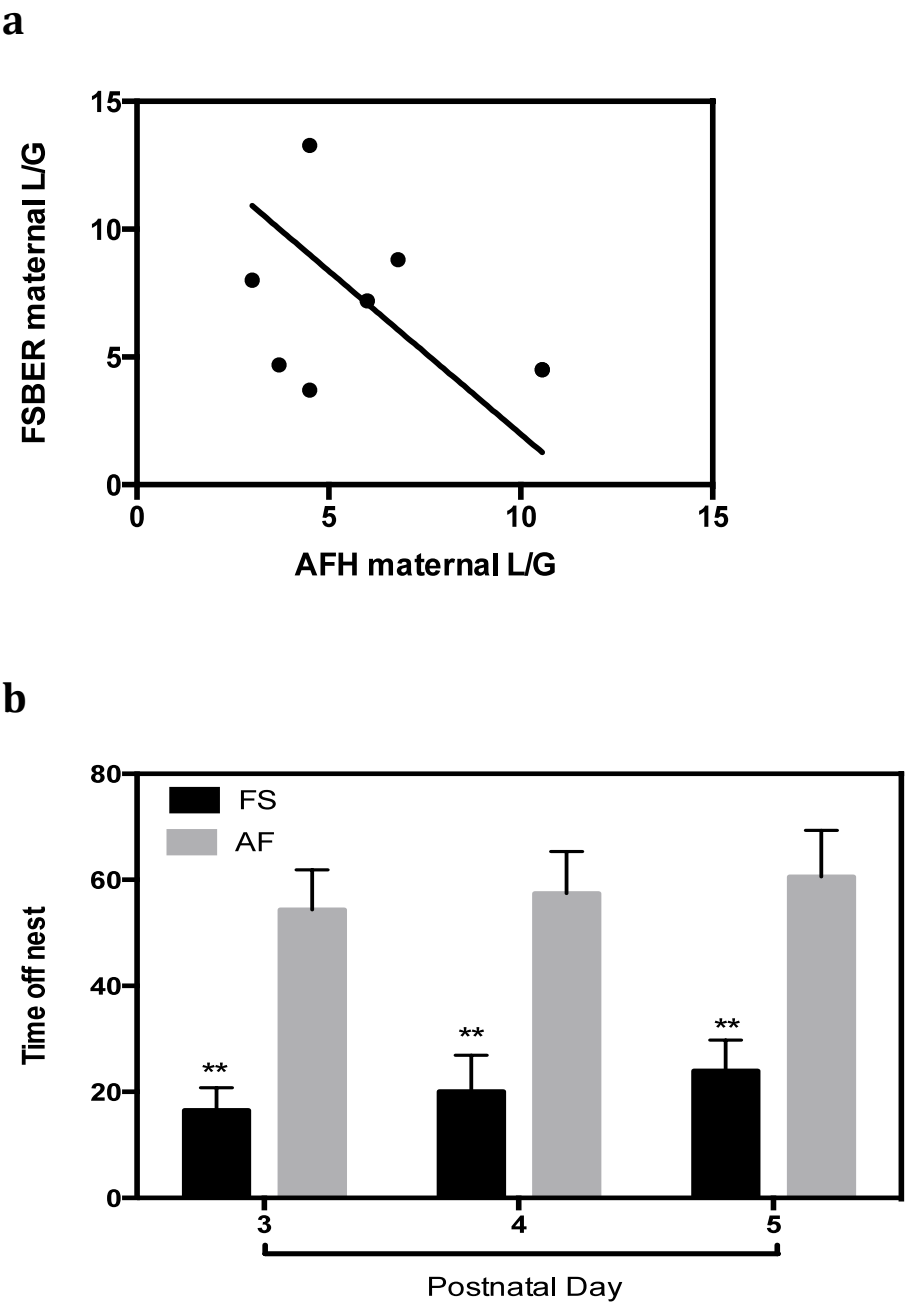


Figure 1.1

Maternal behaviors are not stable in semi-natural conditions. (a) Spearman's correlation between maternal licking and grooming in dams housed in semi-natural enclosures (FSF) and animal facility housing (AFH) environments (Spearman's correlation: $p = 0.3294$). (b) Mean (± SEM) Episodes spent off the nest during PND 3-5 in semi-natural enclosures and animal facility environments (two-way ANOVA: $p < 0.001$)

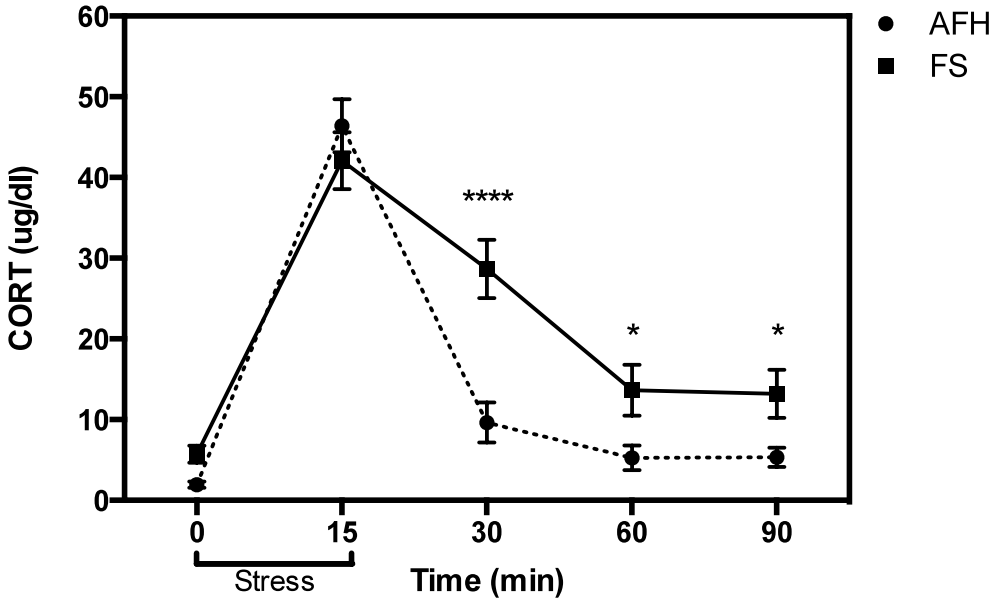


Figure 1.2

FSBER conditions significantly increase post-stress CORT. Mean (±SEM) plasma CORT responses to restraint stress in dams housed in semi-natural (FS) and animal facility (AF) environments (two way ANOVA with repeated measures: * $p < 0.01$, **** $p < 0.0001$)

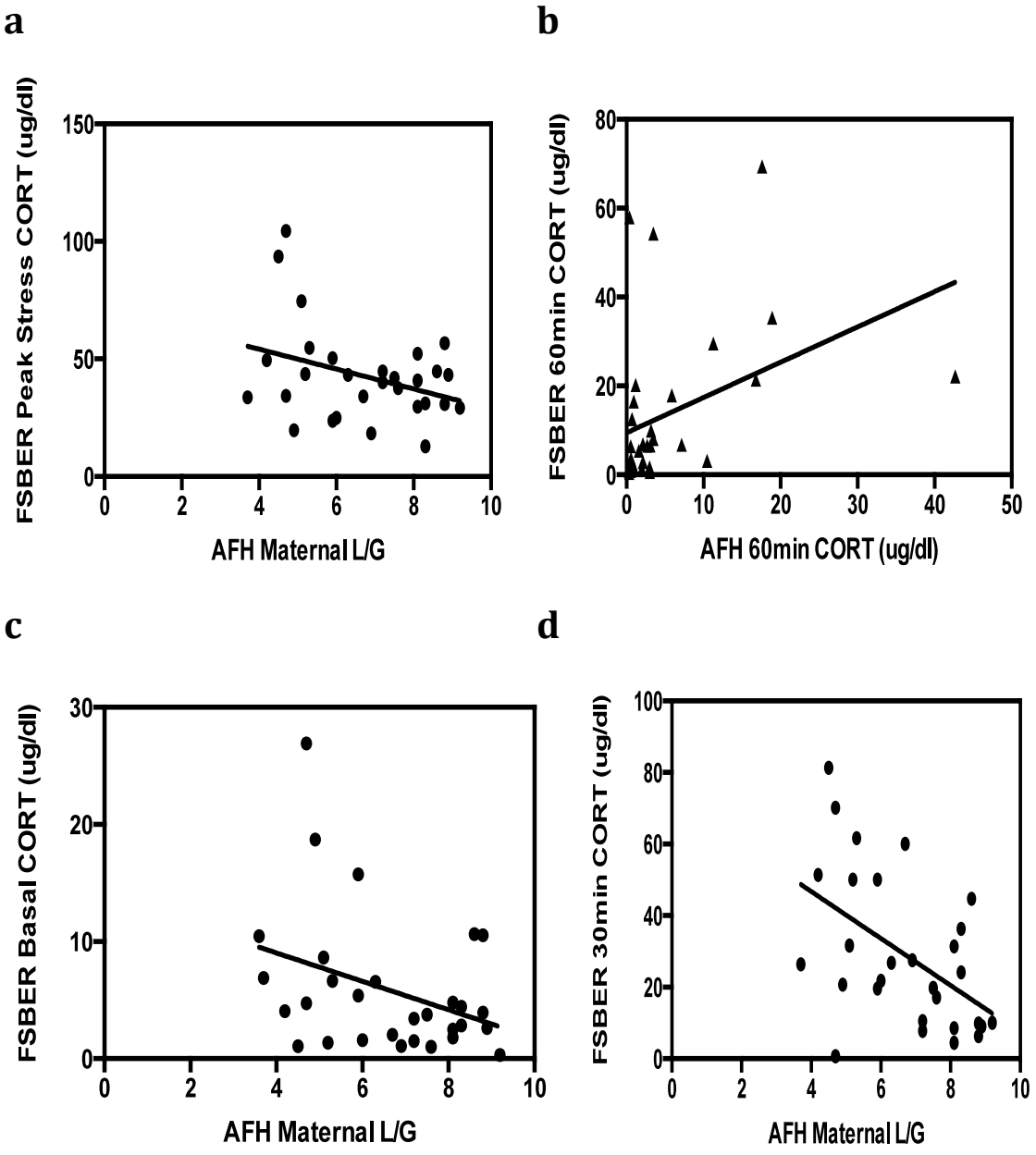
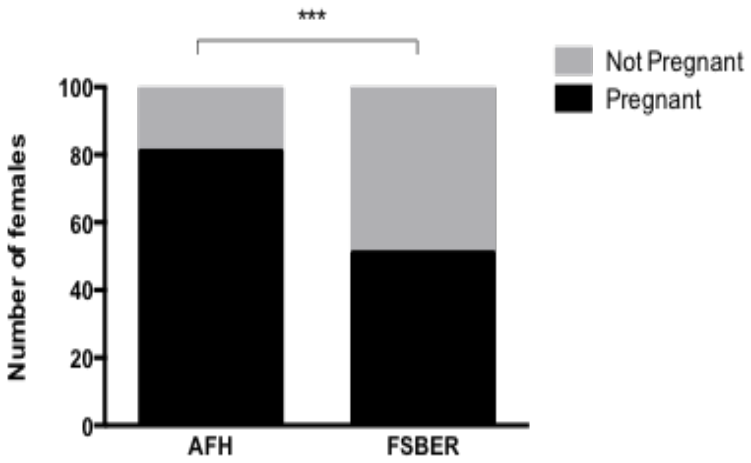


Figure 1.3

CORT values between individuals are stable traits across environments and are correlated to maternal L/G in AFH but not FSBER maternal L/G (a) Negative relationship between AFH CORT and FSBER CORT during recovery (Spearman r: p = 0.0306) Negative relationship between maternal L/G in AFH conditions and FSBER (b) basal (Spearman r: 0.0067) (c) recovery values (Spearman r: p = 0.0568) (d) peak stress (Spearman r: p = 0.0673

a



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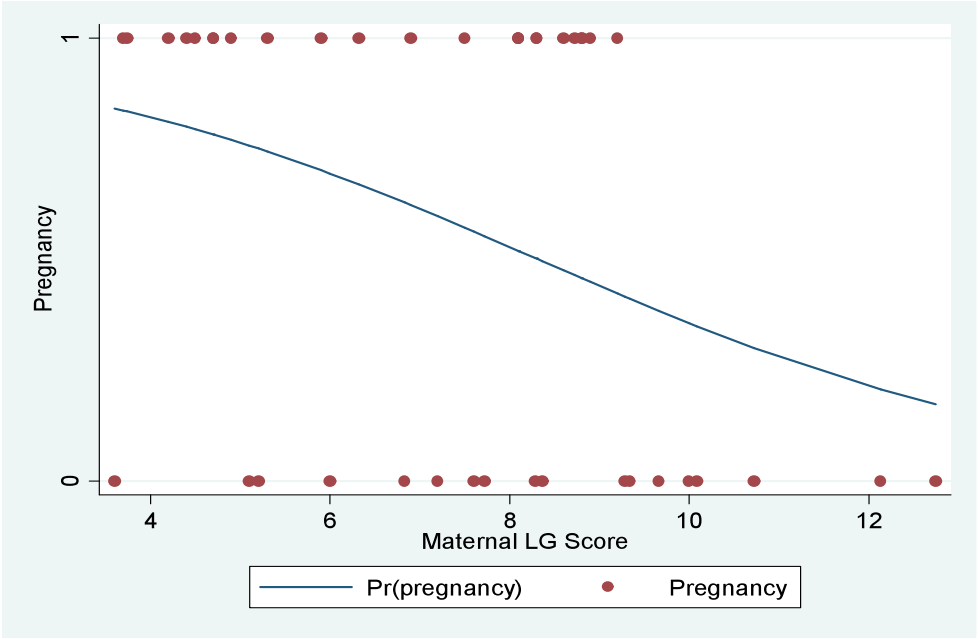


Figure 1.4

FSBER conditions decrease overall fecundity but successful pregnancies are skewed towards low L/G dams. (a) Mean ±(SEM) Percent of females pregnant versus not pregnant (Fishers exact test: p = 0.0009) (b) Logistic relationship between maternal licking and grooming and pregnancy success in dams phenotyped for maternal care in AFH conditions and rebred at the FSBER. (Chi-square, p = 0.0082)

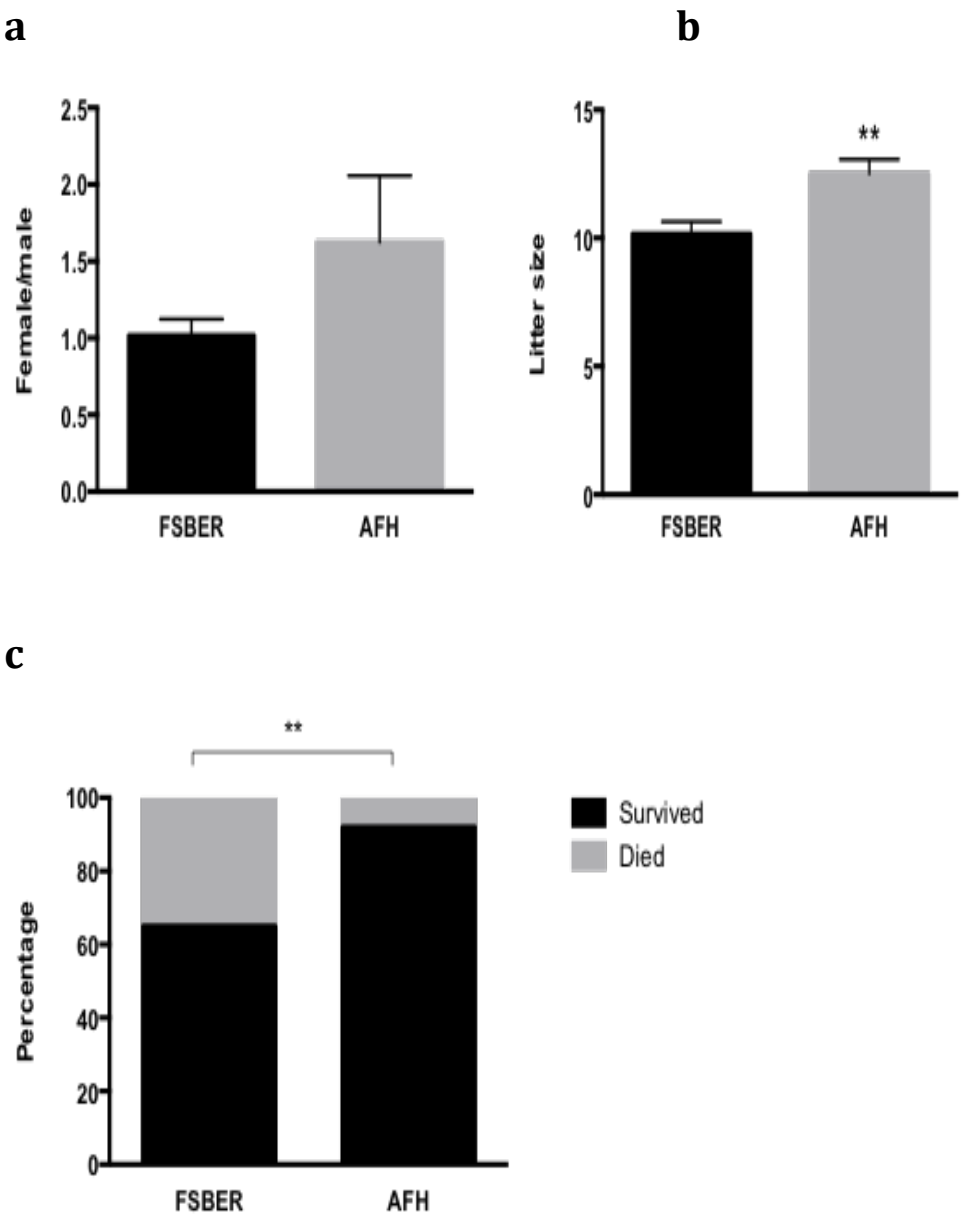


Figure 1.5

FSBER conditions are associated with decreased fitness. (a) Mean (± SEM) ratio of female to male pups (Mann-Whitney: $p = 0.2053$) (b) Mean (± SEM) number of pups at day of weaning (unpaired t-test: $p = 0.0029$) (c) Mean (± SEM) percent of offspring survival in female offspring in enclosures and AFH conditions (Fischer's exact test: $p < 0.0050$).

Chapter 2

Semi-natural environmental conditions influence stress reactivity and behavior in adult female rats.

INTRODUCTION

Glucocorticoids are the principle hormonal mediator of allostasis and the stress response in all vertebrate mammals. Specifically, glucocorticoids (cortisol in primates and corticosterone in rodents) aid in the immediate stress response by, among other processes, increasing the availability of energy to tissues involved in coping with the stressor and inhibiting energetically expensive processes that do not aid in immediate survival. Glucocorticoids, as key regulators of energy and locomotion, are thought to play an adaptive role in maintaining physiological and behavioral stability in response to every day challenges as well as to "emergency life-history stages" (McEwen and Wingfield, 2003).

Early life experiences, specifically during the fetal, neonatal, and infant environments, can have long-term effects on brain function and development that can be both enduring and irreversible. Organization of neural structures involved in the stress response can begin as early as *in utero* (Dupouy *et al.,* 1975; Zarrow *et al.,* 1970), although in most altricial species, the majority of neuroendocrine development occurs in the postnatal period (Dent *et al.,* 1999; Schmidt, 2002; Liu *et al.*, 1997; Weaver *et al.,* 2004). In rodents and primates, prolonged periods of maternal separation early in life have pronounced effects on the offspring's neuroendocrine and fear responses to stress (Levine, 1985; Plotsky and Meaney, 1993; Suomi, 1997) whereas brief periods of separation can have the opposite effects (Levine *et al.,* 1957; Lyons and Parker, 2007; Macri and Wurbel, 2006). In rats, naturally occurring variation in postnatal maternal licking and grooming (L/G) mediate behavioral and endocrine responses to stress in offspring (Caldji *et al.,* 1998; Liu, 2000; Francis, 2000). Specifically, pups that receive high levels of licking are resistant to stressors as adults (Liu *et al.,* 1997; Caldji *et al.,* 2000), are behaviorally more likely to explore and interact in new environments (Francis *et al.,* 1999; Caldji *et al.,* 1998), and are more likely to show decreased anxiety and learned helplessness (Caldji *et al.,* 1998; Francis *et al.,* 1999). These effects are correlated with increased hippocampal GR gene expression throughout life, and are seemingly permanent, as the level of GR mRNA transcripts is a direct result of the methylation status of the GR promoter (Weaver *et al.,* 2004). In controlled laboratory conditions GR expression is behaviorally transmitted, as these effects are reversed by postnatal cross-fostering to a dam with a different L/G phenotype (Francis, 1999)

Investigating stress axis development and stress behavior in a controlled environment is necessary for elucidating the physiological and behavioral processes behind individual variation; but it is equally necessary to determine how individual stress responses change in response to a fluctuating environment. Although some current research has tried to incorporate environment into the laboratory maternal care paradigm, such as housing animals in environmental enrichment (group housing, novelty, and exercise) at critical developmental periods (Bredy *et al.,* 2003, 2004; Champagne and Meaney, 2007; Francis *et al.,* 2002), housing or exposing animals to stressful stimuli at critical developmental periods (Bagot *et al.,* 2009;

Champagne *et al.,* 2008) or housing animals in environmental enrichment with subsequent exposure to stress (Welberg *et al.,* 2006); very little is known regarding how maternal care and stress physiology interact in the offspring of free-living populations of rats. Although these experiments are an important first step, they involve paradigms that the animal would be very unlikely to experience outside of the laboratory environment. Studying individual stress responses in the environment in which they evolved in is critical to understanding "normal" HPA functioning and elucidating the adaptive value of intra-specific variations in response. As Calhoun (1962) stated, "…without a knowledge of the biology of the experimental subject in its native haunts, one cannot hope to achieve effectiveness in experimental design or even to rephrase the hypothesis appropriate to the experimental subject".

The female rat is a highly social animal. In semi-natural conditions and at low population densities, females, unlike males, communally nest, typically sharing a burrow with up to six maternally related females and their juvenile offspring (Calhoun 1961; Barnett, 1975). In the present study, we attempted to simulate some of the salient features of a typical female inhabited burrow system, to test if ecologically relevant stimuli will produce responses to stress compared to laboratory animals. We also evaluated if variations in maternal care in semi-naturalistic conditions have long lasting effects on individual differences to stress that are different compared to laboratory females. If maternal care has a significant influence on the adult HPA response to stress in free-living animals, then females receiving lower maternal L/G will have prolonged responses during recovery from a stressor and increased anxious and depressive behavior as adults compared to female offspring receiving higher L/G. Alternatively, if maternal care is not correlated to pup CORT levels or behavior, than factors other than maternal care influence individual responses to stress in adult females living in more naturalistic conditions. Because maternal L/G is not known to affect basal CORT or CORT levels during stress, we did not expect these measures to be correlated to L/G in semi-natural conditions. However, because semi-natural conditions will likely be experienced as different from laboratory paradigms of enrichment or impoverishment, we expected to see marked changes in basal and peak CORT in response to stress depending on if animals are housed in cages or enclosures at the field station.

METHODS

Animals and housing

Laboratory controls

Female offspring (AFH, n = 35) born in our animal facility were pair-housed in same- sex and same-litter cages (20 x 20 x 32 cm), in standardized temperature (22 °C) and humidity on a 12h light/12hr dark cycle. A subset of offspring was

phenotyped for the maternal care they received from PND (n = 15). All females were left undisturbed until stress/recovery testing in adulthood. Another cohort (AFH, n = 31) of age matched females ordered from Charles River were same sex pair housed in standard animal facility conditions and left undisturbed until behavioral testing.

Semi-natural (FSBER) conditions

Female dams that had been bred in our animal facility and phenotyped for maternal care (n = 20), were pair housed with their previous cage mate and transferred to the Field Station for Behavioral and Ecological Research (FSBER). After a forty-day habituation period, all females were individually placed in new cages and bred with "stud" males for six days. Dams were exposed to ambient light, temperature, and humidity while at FSBER, as well as exposure to sights, sounds and smells from outside the enclosure. On gestational day 18, females (n = 8) that gained more than 90 grams and that had been previously phenotyped as higher or lower lickers, were removed from cages and placed in semi-natural enclosures (2.4 x 3.1 x 2.7m). Each room had five walls, three solid walls and two constructed of 6.35mm mesh hardware cloth. The larger of the mesh walls was open to an outdoor walkway and thus allowed for seasonally dependent environmental conditions. The upper half of the smaller wall, which faced onto an inner hallway, was 50% mesh /50% observation window allowing for unobtrusive observations. In an attempt to simulate the hidden burrow system, all rooms contained black nest boxes located on 0.6m x 0.6m platforms accessible by 1.2m ramps. Ambient conditions were monitored and ranged from -6°C to 32°C, humidity was approximately 70-85%, and lighting ranged from 10-15 hours per day. Dams were left alone in both enclosures and cages until pups were weaned at PND 30, a weaning date that more closely approximates when wild rats wean their litters. At PND 30, males were removed and dams sacrificed. Because female rats typically live with closely related females in free-living populations, females born in enclosures (FSF, n = 36) were kept in their same-sex litter groups (approx. 3-6 pups per enclosure). Females born in cages (FSC n = 30) were same sex pair housed with a littermate and kept in cages and exposed to ambient environmental conditions. Female pups were left undisturbed, except for regular animal husbandry and weekly weighing until stress/recovery and behavioral testing at 6 months old of age. All rats had *ad libitum* access to food and water throughout the experiment.

Maternal behavior

Maternal behavior was scored from PND 1 through PND 6. Each day consisted of five hours of observations starting one hour before sunrise and one hour after sunrise, noon, and one hour before sunset and 24:00 h. Within each observational period, the behavior of each mother was scored every 2 min (60 observations/period × 5 periods/day = 300 observations/mother/day = 1800 observations per mother). The

following behaviors were scored- mother out of contact with pups, licking and grooming (both body and anogenital licking), and self-grooming on or off the nest.

HPA response to restraint stress

Restraint stress and recovery was performed between 08:00 h and 12:00 h on adult AFH females and at sunrise to 12pm on FSF and FSC females. Basal CORT was collected in all rats between 8am-9am in AFH and at sunrise to an hour after sunrise at the FSBER. Blood was collected from the tail vein after a positive vaginal smear indicating estrous was confirmed. All basal samples were easily collected within 60sec after removal from the cage. Blood samples were also sequentially collected 15min after the start of restraint (time 0), and at 30, 60, 90 minutes from the end of restraint (Meaney et al., 1989). All blood samples were collected into microcentrifuge tubes and stored on ice until the end of the experiment. Samples were centrifuged at 4°C and plasma extracted and stored at -20°C until assayed. In order to decrease intra and inter-assay error, the same individual performed all stress testing. Only animals that were later confirmed to be in estrous were included in analysis (FSF n=21, FSC n=15, AFH n=31), although all animals were tested.

Corticosterone ELISA

CORT was quantified using commercially available free plasma CORT kit (Assay Designs, Inc.). All procedures were completed according to the manufacturer's instructions except a modification to 1:20 in plasma to buffer concentration.-a modification that allowed for samples to fall within the linear portion of the standard curve. Intra-assay variation was less than 8% and inter-asssay variation was less than 9%. All samples were run in duplicate. Statistical analysis was conducted using the average of duplicates. Values are reported as μg/dl.

Offspring behavioral testing

Light/Dark Box

FSF (n = 26), FSC (n = 24) and AFH (n = 20) females were removed from their home cage or enclosure and placed head first into the dark portion of a two chambered box, half opaque black Plexiglas and half clear Plexiglas (100cm x 50cm x20cm). Recording started immediately after placing the rat into the entrance of the dark box and lasted five min. During the five minute test the following behaviors were quantified: latency to exit the dark box with all four paws out, total time spent in the light box, and escape from the testing apparatus. The first two are basic measures of anxiety (rats are nocturnal and neophobic) and escape behavior was a unique behavior observed in virtually all offspring housed in naturalistic conditions in previous pilot studies. All rats were tested between 12:00 h-14:00 h on the day of

estrous, any rat whose estrous did not fall in the 5 day cycle was tested but not included in the analysis. Sample sizes above reflect the animals included in analysis.

Forced swim

Animals were removed from their cage, AFH (n = 16) and FSC, (n = 20) or enclosure, FSBER, (n = 17) and were placed in a Plexiglas cylinder (46cm high x 20cm diameter) of water (30 cm) at (25 ± 1 °C) for 15min on day one. After the 15min testing period, animals were dried and placed back in their cage or enclosure. After a 24 hr recovery period, animals were retested. Recording lasted 5min and started immediately when animals were placed in water. The following behaviors were scored: total time spent immobile, total time spent actively climbing the sides of the tank, and total time spent diving/escaping (Porsolt et al., 1977). All rats were tested between 12:00 h-14:00 h on the day of estrous, any rat whose estrous did not fall in the five day cycle was tested but not included in the analysis. Sample sizes above reflect the animals included in analysis.

Statistical analysis

Two-way ANOVA with repeated measures and Tukey-H multiple comparisons were used to analyze total CORT differences between environmental conditions and housing at all time points. Non-parametric Kruskal-Wallis tests followed by Dunn's test for multiple comparisons were used to compare CORT profiles and stress behavior between populations. Non-parametric Spearman's correlation was used to compare maternal care and maternal CORT to offspring CORT. Pearson's correlation was used to compare AFH maternal care with AFH female offspring.

RESULTS

Basal corticosterone

FSF and FSC basal CORT concentrations were significantly higher compared to AFH ($p < 0.0005$ in each case, Dunn's test, Figure 2.1a,b) but significantly different to each other. Basal CORT levels (mean ± SD) in FSF females (51 ± 47 μg/dl, n = 21) and FSC females (40 ± 35 μg/dl, n = 16) were six times higher than average basal CORT in female Long-Evans rats of comparable age (Sapolsky, 1992) and 10 times higher than basal in ARH females (5 ±4μg/dl, n = 25) (Figure 2.1a,b). Furthermore, resting basal in semi-natural conditions is 5 times as high as what is generally considered to be the basal range in laboratory housed animals-defined as <10 ug/100ml during the circadian trough (Dallman *et al.,* 1987). We did not measure the effects of maternal care on AFH CORT basal concentrations. Spearman's correlation showed no significant relationship between maternal care received at the FSBER and offspring basal levels in either FSF or FSC offspring.

Corticosterone during a stressor

Because basal levels were significantly different, we compared the amount of CORT released during a stressor by magnitude and as a percent increase from basal. FSF (Mean ± SEM) (72% ± 5) and FSC (68% ± 4) released significantly less CORT in response to restraint stress compared to AFH (89% ± 2) females ($p < 0.005$ in each case, Dunn's test, Figure 2.2b) as well as in order of magnitude FSF (10 ± 3), FSC (5 ± 1) and AFH (28 ± 5)(($p < 0.005$ in each case, Dunn's test, Figure 2.2a). Peak stress values FSF and FSC were not significantly different. Spearman's correlation showed no significant effect of maternal peak CORT and maternal care on peak stress in FSF, FSC, and AFH.

Recovery from a stressor

The ratio of stress/basal CORT level did not significantly differ between FSF and AFH, whereas FSC had a significantly prolonged secretion of CORT ($p \leq 0.05$ in each case, Dunn's multiple comparisons, Figure 2.2c). CORT levels at post stress (recovery) at the FSBER were positively correlated CORT recovery at 30 and 60min of FSF (Spearman's correlation: $p = 0.0214$, $p = 0.0097$). There was a significant negative relationship between AFH maternal L/G and AFH female's recovery at 60min (Pearson's correlation: $p = 0.0287$). FSF, FSC maternal licking did not significantly predict FSF recovery levels at any time point.

Light and dark box in female offspring

To explore the relationship between stress related behavior and environment, we investigated how semi-natural conditions affected anxiety behavior in the light and dark box and if maternal care mediated these effects. Analysis showed that FSF females had a significantly shorter latency to exit the dark box compared to FSC and AFH ($p < 0.005$, Dunn's test, Figure 2.4a) and spent significantly more time in the light portion of the box compared to FSC and AFH females ($p < 0.05$ compared to FSC and AFH females, Dunn's test, Figure 2.4b). FSF escape behavior was significantly different from FSC and ARH (Chi-square: $p < 0.0001$). All FSF rats were able to jump the sides of the box in almost every testing apparatus, so to prevent excluding this behavior from the study the box was placed on a small table. Rats could easily climb back into the box after climbing out and rats were only excluded from the study if they jumped off the table. There was no significant effect of CORT, maternal CORT, or maternal behavior on either latency to exit, the amount of time spent in the light box, or escape behavior in FSF or FSC females.

Forced swim in female offspring

To assess how environment and maternal care affected anti-depressive behavior we tested all females in the forced swim. Analysis showed that FSF females spent significantly more time climbing compared to AFH females ($p < 0.05$, Dunn's test, Figure 2.5 a) and diving compared to AFH and FSC ($p < 0.05$ in each case, Dunn's test,

Figure 2.5b). There was no significant difference between groups in amount of time spent immobilized. There was no significant effect of maternal behaviors or maternal CORT on immobility, climbing, or diving in the forced swim.

DISCUSSION

There is strong experimental evidence that maternal L/G plays a mediating role in neonatal HPA responses to stress. This study assessed whether maternal care is a significant contributor to individual offspring stress responses in naturalistic environments. We found that maternal care did not predict adult offspring CORT values during basal state, stress exposure or recovery period. Maternal care also did not predict behavior in semi-natural conditions regardless of whether the animals were housed in cages or enclosures. Our data also show that stress profiles in animals housed in cages at the FSBER, enclosures at the FSBER, and cages in animal facility conditions significantly differed from each other, irrespective of maternal care received, suggesting that variability between housing has a main influence on adult offspring stress responses. However, we do not know if maternal care is a significant mediator of to the neonate or adolescent stress response in semi-natural conditions and, because we did not test which factors contributed to the variability within group, we are unable to determine what is mainly mediating this variability, although preliminary data suggest maternal CORT is likely a contributor.

There was a pronounced increase in baseline CORT levels at the FSBER compared to AFH females, irrespective of housing in cages or enclosures. Interestingly, females born in the animal facility and moved to field conditions had significantly less CORT at baseline when tested after months of housing at the FSBER, compared to adult females born at the field station. Early life can have long lasting effects on the brain (Herrenkohl, 1986; Weinstock, 1997) and our results suggest that adult baseline CORT could originate in early life and persist into adulthood. There is evidence in the literature, however, to suggest it is unlikely baseline levels are permanently determined in the brain, as there is evidence that female basal CORT can be lowered by removal of the ovaries in adult females (Kitay, 1963).

High basal concentrations at the FSBER mirror studies comparing GC concentrations between captive and free-living populations of animals. For example, studies comparing field and lab stress data found CORT was higher in free-living animals compared to animals housed in laboratory conditions in rodents (Kunzl and Sachser, 1999; Woodruff *et al.,* 2010) and in captive birds (Romero and Wingfield, 1998; Romero *et al.,* 2008). Studies comparing adrenal weights, a contentious proxy for GC production, between free-living rats (*Rattus norvegicus)* and laboratory rats found free-living animals had larger adrenals by weight (Nichols, 1950; Rogers and Richter, 1948).

High basal GC's, typically framed as detrimental in experimental conditions, can be an adaptive response to an animal that experiences many predictable acute

stressors. The "cort-activity" hypothesis argues that challenging environments cause a concurrent increase in baseline CORT and an increase in individual fitness by increasing the animals coping mechanisms (Bonier *et al.,* 2009). Specifically, increasing GC's increase available energy and have been correlated to increased locomotors activity and anti-predator behavior (Breuner and Hahn, 2003; Cote *et al.*, 2006), as well as increased foraging and provisioning behavior (Love *et al*., 2004), and most recently, increased CORT increased neurogenesis and activation (Kirby *et al.,* 2012). Increased baseline CORT also allows the animal to react immediately to an acute stressor. Sapolsky *et al.,* (2000) maintains that because many naturalistic stressors start and finish within seconds, long before the genomic effects of GC's can manifest, the effects of basal concentrations are more important in naturalistic populations of animals than are the effects of stress induced GC's. As I mentioned in chapter one, despite the potential short term adaptive effects of high basal GC's, the "wear and tear" (allostatic load) of prolonged exposure to GC's in challenging environments could be a cause of the higher mortality and morbidity in animals housed in FSBER conditions (Sapolsky, 1992; McEwen and Gianaros, 2010), and potentially of individual fitness (Bonier *et al.,* 2009).

When differences in basal levels were controlled, field station conditions, irrespective of housing, resulted in significantly decreased responses to an acute stressor compared to AFH females. Rodents in the free-living populations, like many animals, lead very stress filled lives. Most free-living animals are exposed to temperature fluctuations, food scarcity and real or perceived predation, and yet breed and survive (Boonstra, 2013). Rats housed in FSBER semi-natural enclosures are exposed to physiological stress (temperature fluctuations) as well as psychosocial stressors (intraspecific competition, exposure to predators) as well as conditions that are likely enriching and result in eustress (the ability to escape into dark nest boxes, voluntary exercise, novelty, sociality). Drastically increasing CORT levels relative to baseline during mild to moderate stress would be energetically costly. Experimental evidence has found that animals exposed to mild prenatal stress blunt their adult stress responsiveness (Levine *et al.,* 1967; Sapolsky and Meaney, 1986; Welberg, 2006), findings that suggest critical developmental periods can program (organize) the brain to be less responsive to hormonal exposure later in life. Whereas mild stress can "inoculate" young animals from stress later in life (Lyons and Parker, 2009), severe stress has the opposite effect and can elevate stress responses (Rots *et al.,* 1996), suggesting FSF and predictably AFH females did not experience early life as overwhelmingly stressful, although as I'll discuss further, FSC profiles imply they might be chronically stressed.

Experimental conditions show that higher levels of maternal L/G increase GR gene expression in the hippocampus of pups. It is not known whether variation in maternal L/G is a primary mediator of offspring HPA development in free-living populations. We did not find a significant correlation between maternal care and HPA function or behavior in FSF females, either in magnitude or direction. We did find significant differences between housing and environmental conditions, suggesting these factors significantly influence adult stress physiology and behavior

in the female rat. This result does not imply maternal care does not influence HPA functioning, however. Recent studies that examine the effects of early life programming on development found that low levels of early maternal care can be reversed or compensated by postnatal environmental experience. For example, postnatal interventions can reverse the effects of prenatal stress on stress reactivity and behavior (Maccari *et al.,* 1995; Vallee et al., 1997; Weinstock, 1997) and peripubertal rearing in environmental enrichment can change HPA reactivity to a stressor in animals that were previously phenotyped as having high CORT responses to a stressor (Francis *et al.,* 2002; Champagne and Meaney, 2007). Therefore it is possible that HPA reactivity could be programmed by maternal care in semi-natural environments but the effects on HPA responses and behavior are later masked by enrichment. These findings may explain why FSF females that are exposed to enrichment as well stressors recover faster from a stressor than FSC females housed in cages, irrespective of the L/G they received. We tested females in adulthood, so we do not know how influential maternal L/G is in younger rats at the FSBER.

Although housing clearly had a significant effect on adult CORT profiles, FSF CORT recovery levels were positively correlated to their dams CORT recovery levels, and surprisingly, maternal recovery concentration was significantly positively correlated to basal and peak stress CORT levels in FSC females, a finding not supported in laboratory studies (Meaney, 1998). These findings suggest that some aspect of the maternal environment alone, or in conjunction with maternal care, mediates adult offspring CORT concentrations. As I discussed in chapter one, dams had a prolonged stress response at the FSBER, suggesting that females were experiencing field station conditions as challenging and possibly distressful. The mechanism by which maternal prenatal GC's are transmitted to offspring is unclear, but experimental findings show that maternal exposure to glucocorticoids has powerful programming effects on offspring (Barbazanges *et al.,* 1996; Welberg and Seckl, 2001). For example, pregnant rats treated with synthetic CORT have offspring with increased serum CORT (Liu, 2012), as well as adult offspring with impaired in negative feedback in the HPA axis, as a result of decreased GR's (Barbazanges *et al.,* 1996). The effects of prenatal stress on the HPA axis (see Welberg and Seckl for a review, 2008) can be partially reversed in a rat postnatal handling paradigm (Wakshlk and Weinstock, 1990; Smythe, 1994) and by adoption (Smythe, 1994).

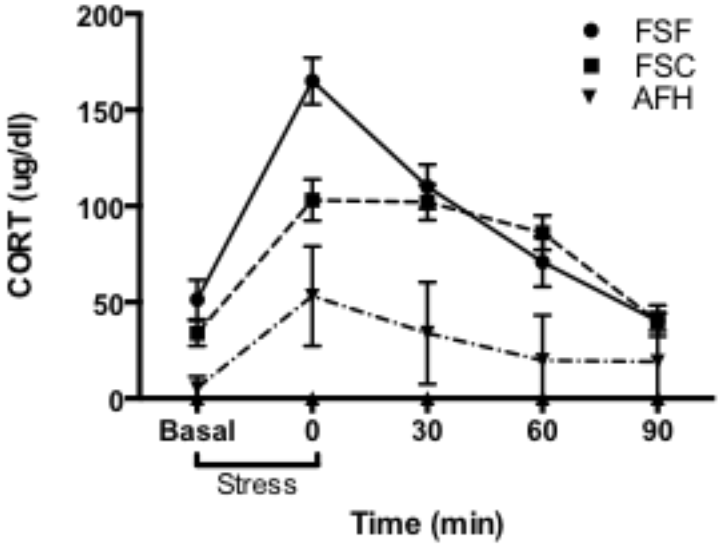
Behaviorally, FSF females were more resilient to mild stressors, compared to FSC and AFH females, irrespective of the maternal care they received. A rare paper looking at the effects of maternal environment on wild-type (F1) offspring behavior found that wild type rats had shorter latencies to activity and increased total activity, and these behaviors were not dependent on maternal behavioral phenotypes (Price, 1973). There is some evidence that 5-HT(1a) receptor binding in hippocampal tissue is a critical feature of anti-depressive behavior (Pineyro and Blier, 1999). Environmental enrichment can increase 5-HT receptor binding in the hippocampus (Rasmuson *et al.,* 1998), and if enclosures are enriching, and CORT recovery data from this study suggests they are, than FSF's decreased anxious and anti-depressive

behavior could be a result of increased 5-HT(1a) binding. Somewhat surprisingly, these effects can occur despite elevated basal glucocorticoids. Perhaps counterintuitive, basal GC's can be elevated in response to stressful (distress) and enriching environments (eustress), but have opposing effects on brain plasticity and mood behavior. For example, enriched environments as well as running increase neurogenesis (van Pragg *et al.,* 1999) and exercise and environment enrichment elevate GC's (Droste *et al.,* 2003) but increase cognition and responses to stress (Duman et al., 2008; Kirby et al., 2013) A recent study found that social defeat and environmental enrichment both increased GC's but they had opposing effects on behavior and cell survival, and that GC's were necessary for these effects to manifest (Lehmann *et al.,* 2013).

Alternatively, FSF females' could be exhibiting active avoidance behavior. The vast majority of FSF females were able to escape the light/dark box apparatus and, if given enough time, the Morris Water Maze (Chapter 3). Diving behavior in the forced swim, classically interpreted as escape behavior, was also pronounced in some FSF females. Their unique ability to escape was likely due to increased baseline CORT which is associated with increased locomotors activity and anti-predator behavior (Breuner and Hahn, 2003; Cote, 2006) as well as their access to vertical space (they were often seen climbing the screens and along pipes located on the ceiling) and darkened elevated cage boxes that allowed for escape. Previous studies also found that behaviors typically interpreted as exploratory and anxiolytic might actually be better interpreted as escape behavior (Boisser and Simon 1969; Roy and Chapillon 2004). Regardless of how the behavior is interpreted, environmental variation clearly affected behavioral phenotypes and these affects were not mainly dependent on maternal care.

Results from this study found significant differences between groups in behavior and stress physiology, as well as, differences within groups that were mediated by maternal care in laboratory conditions but not in semi-natural environments, irrespective of whether the females were housed in cages at the field station or enclosures. Preliminary data suggest that, unlike laboratory findings, the early maternal CORT environment might be partly mediating individual differences in stress responses at the field station, as well as factors unknown to us. In addition to these findings, we also observed that semi-natural enclosures had a pronounced affect on baseline GC's and decreased responses to stress, and while these profiles are defined as distressful in a laboratory context, might be better thought of as profiles falling within normal HPA functioning in rats living in heterogeneous environments.

a



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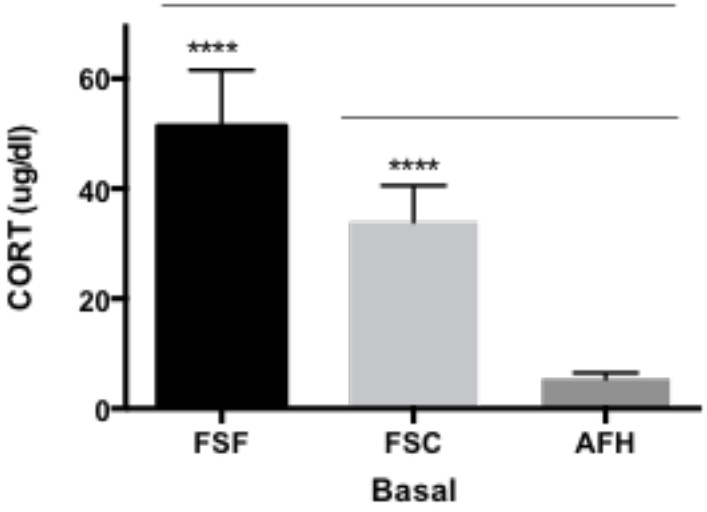


Figure 2.1
(a) Mean CORT (± SEM) CORT restraint stress/recovery. (b) Mean (± SEM) baseline CORT. FSF and FSC females had significantly higher baseline CORT than AFH adult females. **** $p \leq 0.00001$

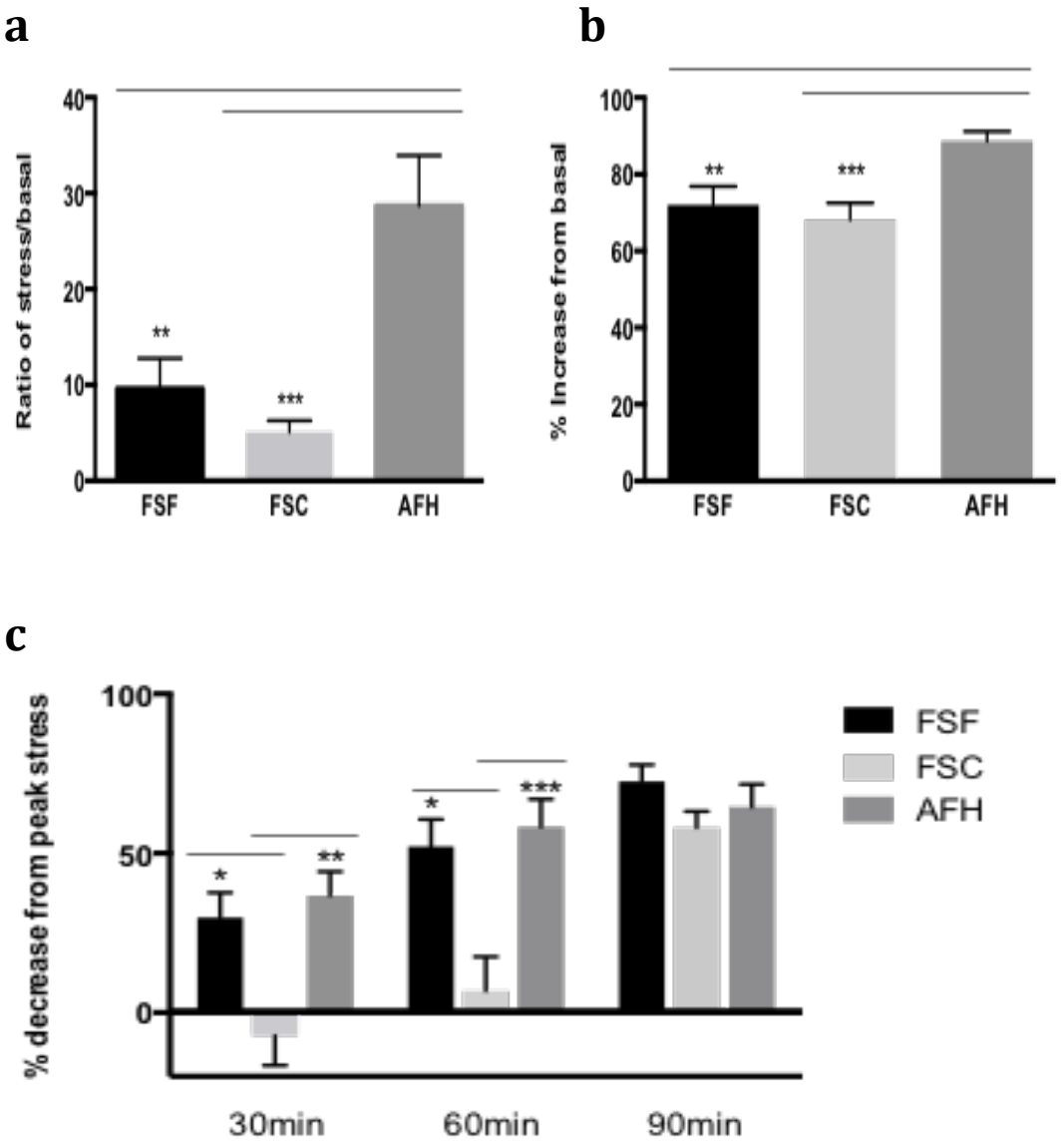


Figure 2.2
(a) Mean (±SEM) Order of magnitude of increase from baseline to peak stress. FSF and FSC had significantly less CORT compared to AFH. (b) Mean (± SEM) percent increase from baseline to peak stress. FSF and FSC secreted significantly less CORT in relation to their baseline compared to AFH. (c) Mean (± SEM) percent decrease from peak CORT at 30min, 60min and 90min. AFH had a significantly prolonged recovery at 30 and 60min. ** $p \leq 0.001$, *** $p \leq 0.0001$.

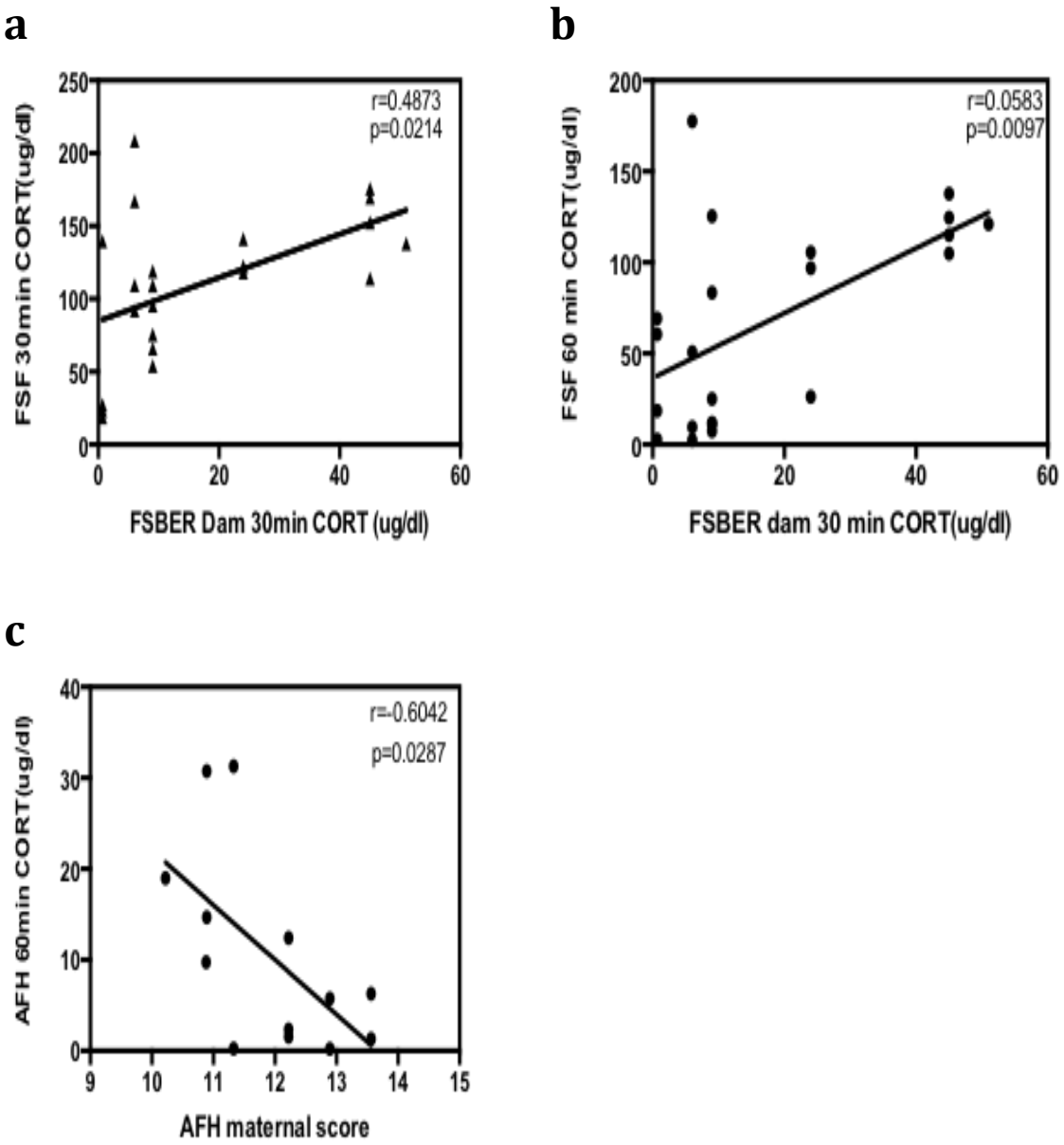


Figure 2.3
(a), (b) Linear regression of individual FSBER maternal CORT at 30min versus FSF adult CORT at 30min, 60min. Dam CORT at 30min is positively correlated to FSF CORT at 30 and 60min. (c) Linear regression for a subset of AFH dams and AFH offspring, maternal L/G is negatively correlated with AFH 60min CORT values.

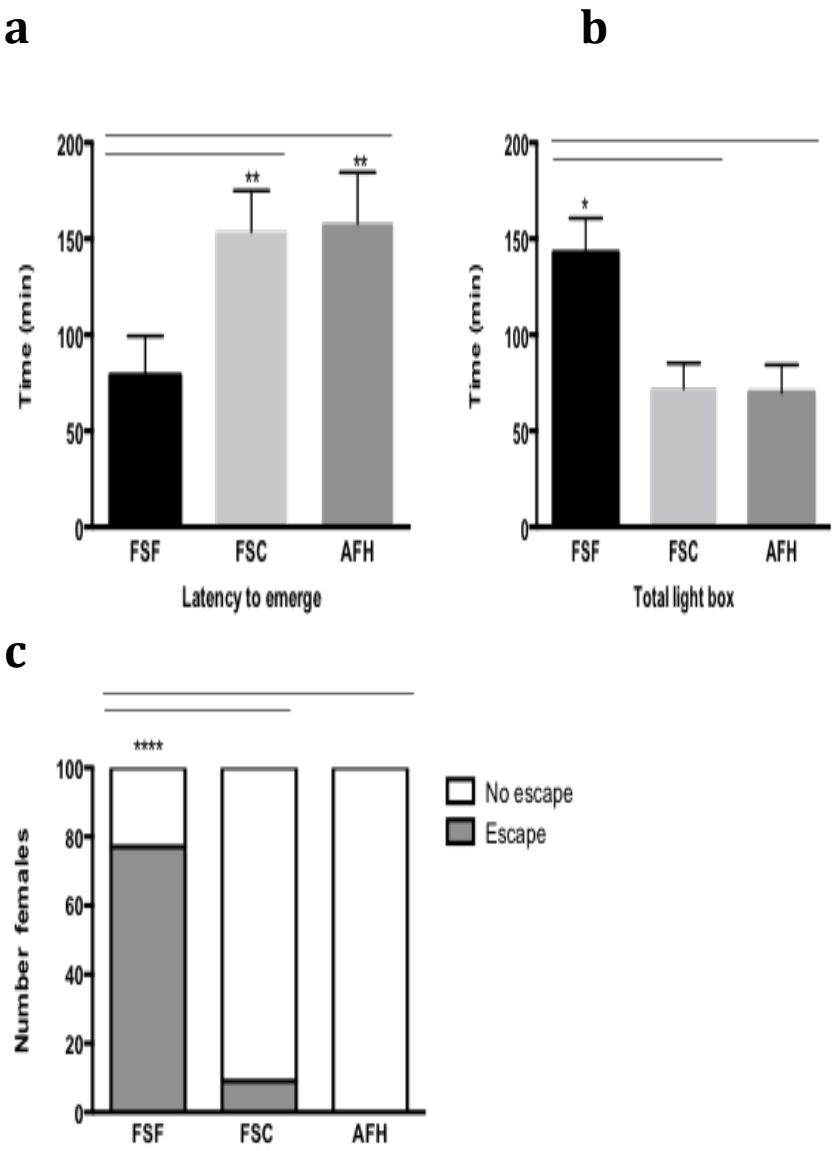


Figure 2.4
(a) Mean (± SEM) latency time to emerge from the dark box. FSF emerged from the dark sooner than FSC and AFH females. (b) Mean (± SEM) total time spent in or on top of the light box. FSF spent significantly more time out of the dark box than FSC and AFH females. (c) Total number of animals that escaped from the testing apparatus. Significantly more FSF females escaped. * $p \leq 0.01$, ** $p \leq 0.001$, **** $p \leq 0.00001$

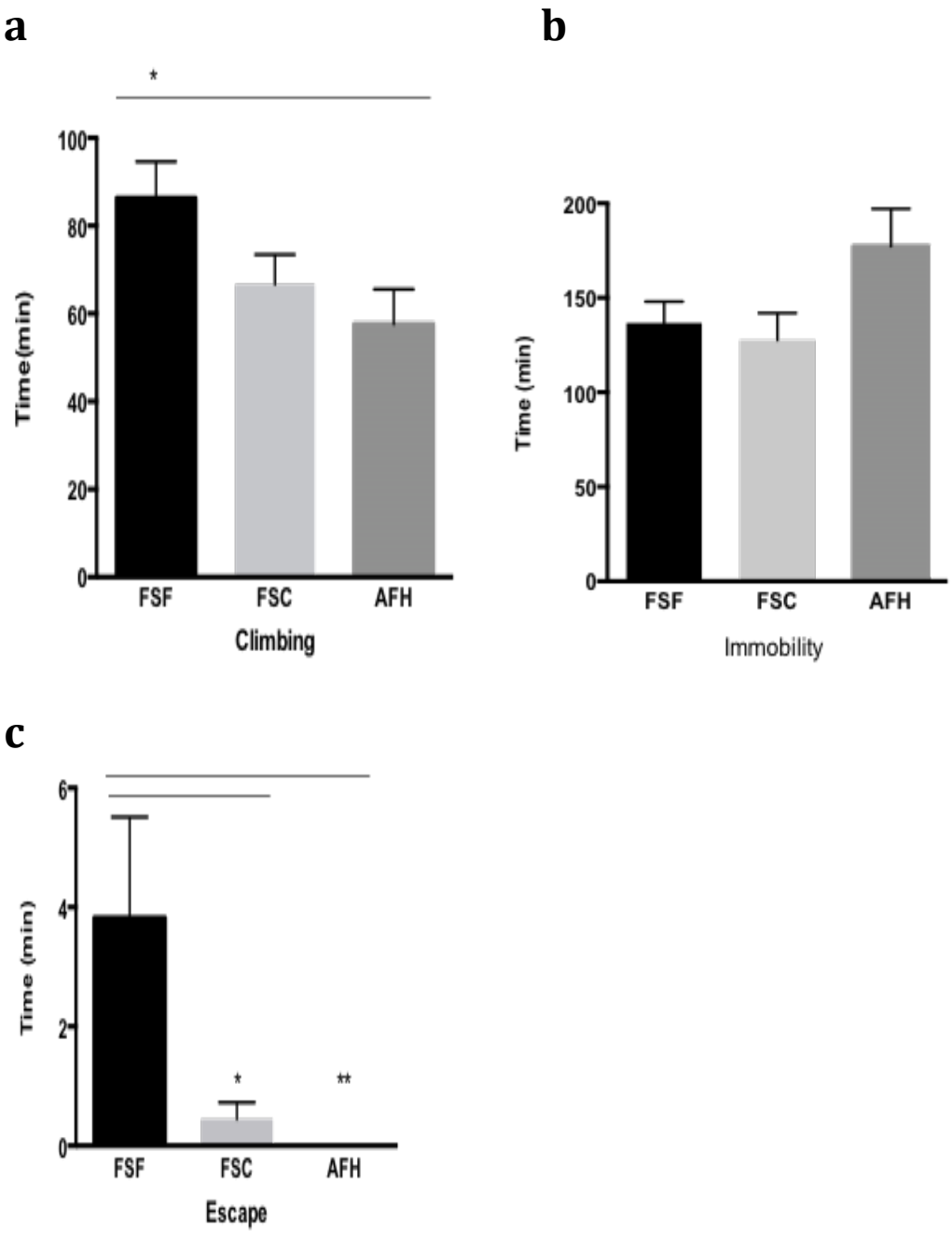


Figure 2.5
(a) Mean (SEM) time spent immobile and actively climbing the sides of the tank. AF FSF females spent more time climbing immobile was not significantly different between groups. (b) Mean (SEM) time spent actively diving. FSF females spent more time diving than FSC and AFH animals. * p≤ 0.01 ** p≤ 0.001

Chapter 3

Semi-natural environmental conditions influence adult hippocampal proliferation and spatial learning in aged female rats.

INTRODUCTION

Neural stem cells are newly generated in the dentate gyrus (DG) of the hippocampus throughout the life of many mammals (Altman and Das, 1965; Gould *et al*., 1997; Ericksson *et al.,* 1998). These new cells appear to integrate into the existing neural network, possess characteristics similar to functional neurons, and are widely thought to play a role in memory, learning and behavior (van Pragg *et al.,* 2002; Abrrous *et al.,* 2005; Marin-Burgin, *et al.,* 2012). Specifically, evidence indicates that increasing or decreasing proliferation and survival of neural progenitor cells affects performance in hippocampal dependent spatial tasks as well as affecting anxious and depressive-like behaviors (van Praag *et al.,* 2005; Dalla et al., 2009; Synder *et al.,* 2011; Winocur 2006).

Numerous studies in the 1960's demonstrated that changing the housing of captive animals could alter gross brain morphology and chemistry (Bennett *et al.,* 1964; Hubel and Wiesel, 1964; Diamond *et al.,* 1964, 1985), dendritic length and density (Diamond *et al.,* 1976, 1987), increased hippocampal glia (Altman and Das, 1964; Walsh *et al.,* 1969), and increased higher order dendrites in the visual cortex (Holloway, 1966). Recent studies have focused on the ability of a variety of stimuli to increase or decrease neurogenesis in the dentate gyrus of the hippocampus. For example, housing animals in larger cages with toys, running wheels and multiple litter-mates increases neurogenesis in young mice (Kempermann *et al.,* 1997). Age (Kuhn *et al.,* 1996; Heine *et al.,* 2004) and stress (Mirescu and Gould, 2006; Kirby and Kaufer, 2009) related decreases in neurogenesis can be ameliorated by exposing older animals (Kempermann *et al.,* 1998; van Praag, 2005; Speisman *et al.,* 2013) or prenatally stressed (Lemaire, 2006) rats to environmental enrichment or voluntary exercise.

If relatively minor alterations in laboratory animal housing can dramatically affect brain architecture, it is reasonable to assume that free-living animals experiencing the inherent complexity of "real" life, have brains that are neuroanatomically different, if not dramatically different, than their captive relatives (reviewed by Calisi and Bentley, 2010). Evidence for anatomical variation in the brains of wild versus captive animals goes back at least as far as Darwin's (1874) observations that free-living rabbits have noticeably bigger brains than domesticated rabbits. Recent work indicates living in complex natural habitats is associated with dramatic structural changes in the brain, including increased neurogenesis in the DG, across a broad range of taxa (Barnea and Nottebohn, 1996; Boonstra *et al.,* 2001; Barnea, 2010; LaDage *et al.,* 2010; Dunlap *et al.,* 2010).

Even enriched laboratory conditions, probably the closest paradigm to natural conditions, is likely impoverished compared to the environment wild populations experience (Kempermann *et al.,* 1997; Nottebohm, 2002). Because captivity and its reduced environmental complexity can inhibit the recruitment of new neurons in

the brain, we examined cognition and neural proliferation and survival in aged females in two environments-semi-natural enclosures that exposed females to many salient features left out of enrichment paradigms (more three dimensional and spatial complexity, ambient environmental conditions, group housing with littermates, predator cues, dark nestboxes) and females pair-housed in standard animal facility conditions. Semi-natural females at the FSBER live in a complex environment and are exposed to stimuli that are enriching as well as stressful. How these factors interact to affect neurogenesis in rats outside of standard laboratory studies is relatively unknown. Frequent exposure to stress (intra-specific competition, perceived predation, and temperature fluctuations) could decrease neural proliferation and survival, or exposure to potentially enriching stimuli (voluntary exercise, dark nest boxes, social interactions) could compensate for the negative effects of stress resulting in no net change in neurogenesis. Alternatively, exposure to challenge, both positive and negative, could work to increase proliferation and survival.

METHODS

Laboratory animals

Juvenile Long-Evans female rats (Charles River) were pair-housed on a 12:00 h light dark cycle with lights on at 07:00 h. Animals were subject to regular animal facility husbandry. Testing for this study started when females were 13 months of age.

Field station animals

Adult female Long-Evans rats born at the Field Station for Behavioral and Ecological Research (FSBER) were housed in semi-natural enclosures (chapter two), with same-sex same litter mates (approximately 3-6 rats /enclosure) and exposed to ambient environmental conditions. Testing for this study started when females were 13 months of age.

Morris water maze

Spatial learning was assessed using the Morris water maze. The circular testing pool (1.5m diameter x 60cm depth) was filled with water maintained at 21°C to a depth of 40cm and made opaque by the addition of non-toxic white paint. A circular platform ($10cm^2$) was hidden 2cm below the surface of the water in the middle of one of four imaginary quadrants. Landmarks were placed on the wall around the pool. On day one of two, rats underwent 15 trials (after every third trial rats were given an approx. 15 min rest period), followed by a probe test in which the platform was removed, the platform was returned and females were tested on 3 more trials. A second probe trial was performed 24 hr later. For each trial, rats were allowed to swim until they reached the platform, if they did not reach the platform by 60 sec they were gently guided and placed on the platform and left there for 30 sec

reinforcement period. The starting position varied for each trial but remained consistent across all females. All trials began on day of estrous with the 24 h probe trial on the day of proestrous. All trials were videotaped for later analysis.

Brdu Injections

5-Bromo-2'-deoxyuridine (Brdu, Sigma) was dissolved in physiological saline. Rats were injected (intraperitonally, 50mg/kg × 5 days=250mg/kg) for 5 days to wash out estrous differences and 1 week after completing the Morris Water Maze.

Immunohistochemical staining

Rats were anesthetized with Euthasol euthanasia and transcardially perfused with ice cold 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS. Brains were post-fixed for 24 hr at 4°C, equilibrated in 30% sucrose in 0.1M PBS and stored at -20°C. Immunostaining was performed on a 1 in 6 series of free-floating 40um cryostat sections cut on the coronal plane.

Ki67 staining for cell proliferation was done for sections from a random subset of FSF (n=7) and AFH (n=7) females. Sections were rinsed in 0.1 m Tris-buffered saline (TBS), followed by 10min in 0.9% saline. Sections were antigen retrieved using 10mM citrate buffer, pH 8.0 at 80°C for 20min. All sections were incubated in blocking solution (1% normal donkey solution, 0.3% Triton-X 100 in TBS) for 30 min followed by overnight incubation at 4 °C on rotation in primary antibody (rabbit anti-Ki67 1:200 in blocking; Abcam, Cambridge, MA, USA). Sections were rinsed and transferred to secondary antibody (Cy3 anti-rabbit 1:500 in blocking; Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at room temperature. Sections were mounted on gelatin-coated slides and coverslipped with Krystalon mounting medium (Harleco, Gibbstown, NJ, USA).

Triple labeling for cell fate analysis was done on sections from FSF females (n=7) and AFH females (n=7) 3 weeks after the last Brdu injection. Sections were rinsed in 0.1m TBS, followed by incubation 30min in blocking solution, followed by overnight incubation at 4°C on rotation in primary antibody (mouse anti-NeuN 1:200; chicken anti-GFAP 1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Sections were rinsed and incubated with secondary antibodies (Alexa 647 anti-mouse 1:500; Cy3 anti-chicken 1:500; Jackson ImmunoResearch) for 2 hr at room temp on rotation. All sections were then washed and incubated in 4% paraformaldehyde for 10 min, rinsed and incubated in 0.9% saline solution for 10min followed and denatured in 2 N HCl for 30min @ 37°C. Sections were washed and incubated in blocking solution for 30min (3% normal donkey serum, 0.3% Triton-X 100 in TBS) followed by an overnight incubation in primary antibody against BrdU in blocking solution (rat anti-BrdU 1:500, Abcam; mouse anti-BrdU 1:500, BD Biosciences) at 4°C on rotation. The next day, sections were rinsed and incubated in secondary antibody (biotin anti-rat 1:500; Jackson ImmunoResearch) for 2 hr on at room temp on rotation

followed by a tertiary incubation (streptavidin-Alexa488 1:1,000; Jackson ImmunoResearch) for 1 h at room temperature. Sections were mounted on gelatin-coated slides and coverslipped with Krystalon mounting medium (Harleco, Gibbstown, NJ, USA).

Quantification

Ki67- and Brdu-positive cells were counted in the dorsal and ventral dentate gyrus, subgranular zone, and hilus using 20x air objective (Zeiss, Oberkocken, Germany). Immunopositive cells were counted throughout the entire dentate gyrus and hilus. GFAP positive cells were counted in the hilar region of the dentate gyrus. Size of the anatomical area sampled was calculated using Stereoinvestigator software (Microbrightfield, Wiliston, VT).

Statistical analysis

All data was analyzed for normalcy using a Shipiro-Wilk test, parametric tests were used depending on how data was distributed. A two way ANOVA with repeated measures followed by Sidak's multiple comparisons was used to analyze the trail latencies to reach the platform, and time spent in quadrant of interest. A Fischer's exact test was used to analyze the number of females to reach the platform on the first trial. All two group comparisons were analyzed using unpaired t-tests, either non-parametric for when distributions were not normal or unpaired t-tests with Welch's correction for when distributions were normal. $P \leq 0.05$ was considered significant.

RESULTS

Morris water maze

FSF females had significantly shorter latencies to reach the platform during trials 3-5 (2 way ANOVA, $p \leq 0.01$; Figure 3.1 a). There was a noticeable difference in the number of FSF females (38%) that initially reached the platform compared to AFH females (0%). Because we limited behavior to a 60sec time interval in the maze, the means were potentially inflated, so we quantified the number of animals in each group that reached the platform on the first trial. Using a Fischer's exact test we found significant differences in the number of FSF females to reach the platform versus AFH females (Trial 1, $p = 0.0407$, Figure 3.2 b). We found significant differences in initial trial strategies, such that FSF either quickly found the platform or spent significantly more time escape climbing the sides of the pool compared to AFH females (2 way ANOVA, trail 1, 3, Probe 0, Probe 24 $p \leq 0.01$; trial 2 $p \leq 0.0001$; Figure 3.2 a). Here, we define escape climbing as it is defined in the forced swim test-the entire animal is facing the side of the tank with both front paws breaking the water. There were no differences between the groups in latency to reach the platform or in time spent in the platform quadrant in either Probe 0 or Probe 24 hr

(Figure3.1b).

Neural progenitor cell proliferation

Semi-natural conditions significantly increased the number of cells immunopositive for the proliferation marker Ki67 in the dorsal (Mann Whitney, $p = 0.0256$) and ventral dentate gyrus (Unpaired t-test with Welch's correction, $p = 0.0008$) in FSF females. There were no significant differences in cells immunopositive for Ki67 in the hilus of either group. We found a significant decrease in the survival of cells immunopositive for BrdU.

Neural progenitor cell survival

Semi-natural and animal facility conditions did not differ in the amount of cells surviving after 3 weeks in either the dorsal (Mann-Whitney, $p > 0.9999$) or ventral (Mann-Whitney, $p = 0.3853$) hippopcampus. Because there were no differences in DG regions, we combined dorsal and ventral data. There were also no significant differences between NeuN-and GFAP-immunopositive cells.

DISCUSSION

Our analysis indicates that housing aged female rats in semi-natural enclosures is sufficient to increase proliferation of hippocampal progenitor cells compared to laboratory-maintained animals. This increase in proliferation is correlated with an increase in cognitive flexibility in the Morris water maze and an increase in anti-depressive behavior in the forced swim and the light and dark box as described in previous chapters. By extension, these findings suggest that free-living populations of rats that are exposed to stress that is not overwhelming, might also have increased rates of proliferation. Consistent with our results, a recent study found enhanced spatial memory and cognition, neurogenesis, and reduced neophobia associates with an increase in exposure to harsh environments in wild-caught birds (Roth *et al.,* 2011). This same group also found higher rates of neurogenesis and plasticity in hippocampal volume in wild-caught adult birds that increased along a gradient of environmental harshness (Roth, 2009). Taken together, along with results from the present study, these findings suggest that challenging environments might exert selective pressures on areas of the brain that enable higher cognition, an adaptive feature that could enable survival in harsh conditions.

Studies in free-living rodents, although few, have documented adult neurogenesis in wild yellow-necked wood mice (*A. flavicollis*), wood mice (*A. sylvaticus*), European pine voles (*M. subterraneus*), bank voles (*C. gladiolus*) (Amrein *et al.,* 2004), meadow voles (*M. pennsylvanicus*) (Galea and McEwen, 1999), and Eastern grey squirrels (*S. carolinensis*)(Lavenex *et al.,* 2000; Barker, 2005). The only study, to my knowledge, that has examined adult neurogenesis in free-living populations of brown Norway rats and compared the results to age-matched laboratory rat strains found no differences in proliferation or survival in free-living rats versus age matched Long-

Evans rats and only a juvenile increase in proliferation in wild Norway males versus Sprague-Dawley males (Epp, 2009). Caution should be used when generalizing findings from this study to other populations of free-living rats as the authors only caught males, 7 adults and 3 juveniles, within a relatively short period of time- an indication that the local population was high and that these males were likely subordinates excluded from safer food sources and potentially food stressed (Calhoun, 1962).

In the current study, we found increased progenitor proliferation in the ventral and dorsal DG. It is becoming increasing clear that these distinct regions in the hippocampus (ventral versus dorsal) are functionally separate structures (see Fanselow and Dong for review, 2010). Although the function of these regions and how adult neurogenesis fits into this puzzle is intensely debated, the dorsal region is likely connected to spatial memory whereas the ventral region is associated with emotion. The ventral hippocampus has been hypothesized to regulate anxiety by comparing current conditions to stored memories of past conditions the ventral hippocampus can creating conflict when these states are mismatched (Gray and McNaughton, 1983; McNaughton and Gray, 2000). In addition to this role, the inhibition of neurogenesis is also hypothesized to increase anxiety related behaviors (Revest *et al*., 2009). Following this logic, females from semi-natural enclosures, that were behaviorally less anxious and depressed but had high basal CORT (chapter two), could be resistant to stress on two fronts-they had increased proliferation that could serve as an adaptive buffer to stress and they experienced many more early life stressors thus exposing them to acute stress would not create conflict between current and expected conditions.

A series of studies examining the effects of early life stress on future stress responses in spider monkeys found exposure to early stress could decrease stress reactivity later in life. Specifically, they found that young monkeys separated from their natal group for brief periods of time early in life, conditions that evoked stress but not overwhelming distress, were behaviorally more resilient to mildly stressful behavioral tasks as adults. This result suggests that repeated exposure to acute stress can "inoculate" animals to future stress (Lyons and Parker, 2007). Another more recent paper that addressed the effects of a one-time acute stressor on proliferation, showed that acute stress increased proliferation and activation in the dorsal DG (Kirby *et al.,* 2013). Taken together, these findings suggest that animals living in challenging environments might benefit from exposure to stress.

In addition to increased proliferation in the ventral DG, we also found significantly more proliferation in the dorsal DG of FSF females. Newly born neurons in the dorsal DG are implicated in enhanced performance in spatial learning and memory in laboratory studies (Moser *et al*., 1995; Klur *et al.,* 2009). In order to examine whether adult neurogenesis is important for spatial memory in semi-natural conditions, we tested females in the Morris water maze, a classic test of spatial cognition in rodents (Morris, 1984). Similar to other biomedical testing apparatus used throughout this dissertation study, analyzing the Morris water maze using

traditional behavioral maze criteria was not as informative as interpreting these findings from an adaptive perspective. FSF females spent significantly more time trying to escape, as measured by the amount of time spent climbing the pool walls during initial trials and during both probes, and by actual escapes from the platform. FSF females had the ability to hide in dark nest boxes during stress in enclosures (e.g. they had stressor controllability), and in novel environments they might also attempt to find an escape before assessing the environment. It is interesting that this was the initial strategy in the majority of FSF females, although most reverted to the platform after unsuccessful attempts to escape, and they reverted back to climbing during the probe trails. Although FSF females, on average, had shorter latencies and paths to the platform during initial trials, it is clear that quantifying navigational paths and time in quadrants does not accurately capture the behavior of an animal trying to vertically escape the maze.

However, even if semi-natural conditions did not enhance spatial memory in FSF females, there is some evidence that neurogenesis might not play a critical role in spatial learning in all free-living rodents. A recent study looking at neurogenesis in long-lived red squirrels that stockpile food for survival during winter, found no differences in proliferation or survival between populations that store many small caches and populations that store food in one large stockpile. They also found a significant decrease in neurogenesis with age, although these squirrels were still able to stockpile food and survive, suggesting adult neurogenesis might not be important for spatial memory in this species (Johnson, 2010). However, in the black-capped chickadee, seasonal peaks in neurogenesis are correlated with increased food caching (Barnea and Nottebahm, 1994; Ladage *et al.,* 2010) and another study found inter-specific differences in neurogenesis in rodents that was correlated with the ability to stockpile food (Barker *et al.,* 2005)

Neurogenesis is age-dependent in all species examined to date, including laboratory mice, rats, and monkeys (Kuhn *et al.,* 1996; Gould, 1999) and free-living rodents (Barker, 2005; Epp, 2009; Johnson *et al.,* 2010). Environmental enrichment has been documented to ameliorate some of the effects of stress and age on neuronal proliferation and survival (Kempermann, 2002; Bredy, 2003; Mirochnic, 2009 although not entirely. Thus, even though semi-natural conditions seemingly improved some of the effects of aging on neurogenesis compared to AFH females, it is likely all females in this study have less proliferation in the DG than females.

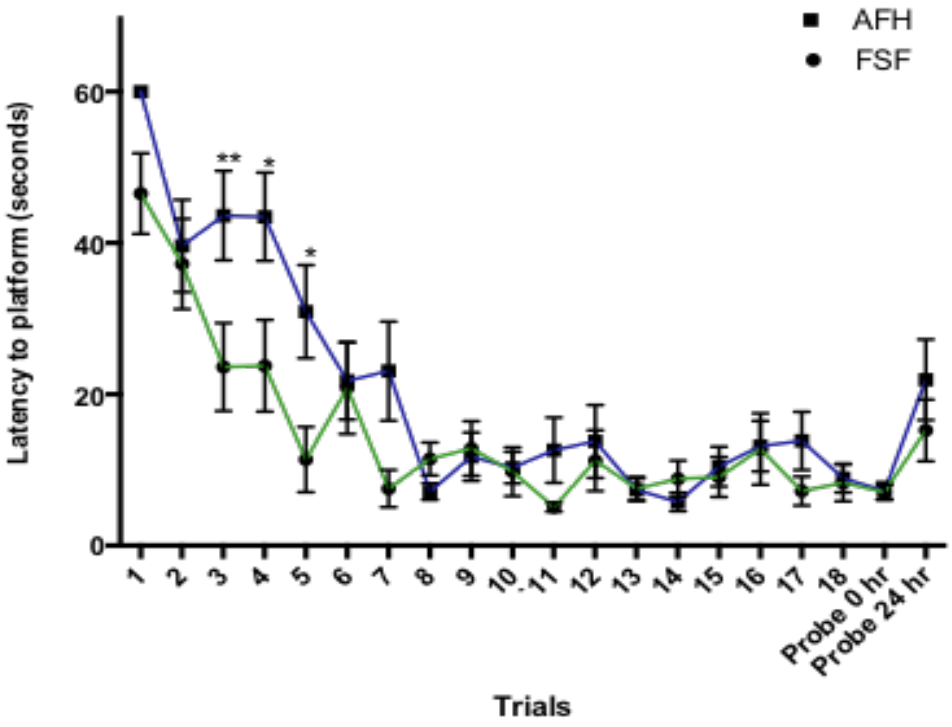
Somewhat surprising, we did not find significant differences in cell survival in FSF and AFH females. These findings suggest FSF females, in addition to higher proliferation, also had higher rates of cellular apoptosis. Greater numbers of cells immunopositive for Ki67 may provide an individual with larger reserves of proliferating cells that could be recruited during challenging conditions. Commensurate with our findings, Barker *et al.,* (2004) found that chipmunks and squirrels, species with different spatial use, have different rates of proliferation but not of survival and living in natural environment partially ameliorated age related decrease in cell proliferation (Barker, 2004). Taken together with our findings, this

study suggests that laboratory enrichment, which increases cell survival but not cell proliferation (Kempermann *et al.,* 1998), might not be an accurate paradigm for a complex environment that includes 'enrichment' as well as stress.

It is also possible that a single snapshot of adult neurogenesis does not give an accurate picture of neuron survival in an animal living in complex environments. If relatively mild stimuli can increase or decrease neurogenesis in experimental conditions (exercise, enrichment, acute versus chronic stress, sexual experience, diet), it is very possible that the complexity of natural environments (seasonality, differing reproductive conditions, varying predation pressures) cause proliferation to fluctuate more frequently. In support of this hypothesis, recent studies examining adult neurogenesis in free-living populations of animals found proliferation can vary with season in song control nuclei in birds (Lindsey and Tropepe, 2006) and between breeding versus non-breeding females (Galea and McEwen, 1999).

Phenotypic plasticity is thought to be an evolutionary adaptation to environmental variation. The current study suggests semi-natural environments increase proliferation and this could be associated with changes in spatial memory, learning and behavior. How neurogenesis adapts an animal to its environment is still largely unknown; future studies examining neurogenesis in free-living populations of animals may shed light on the functional significance of heightened hippocampal plasticity.

a



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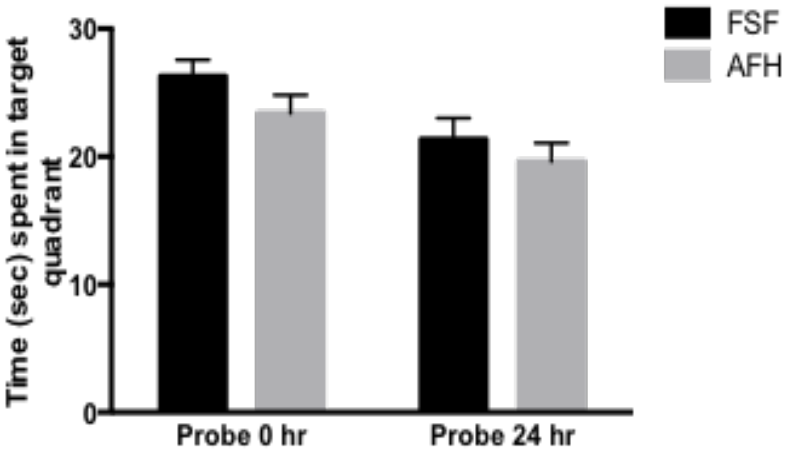
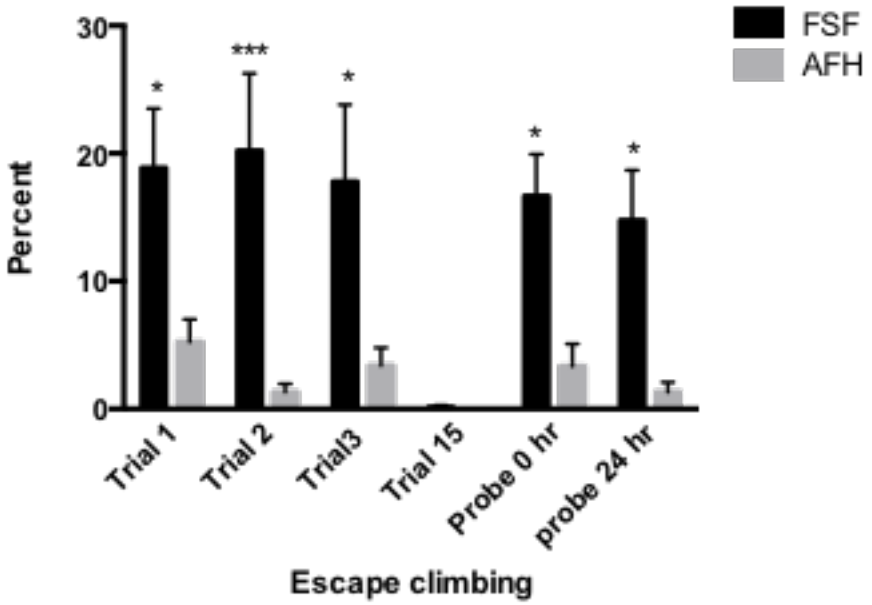


Figure 3.1
(a) Mean ± (SEM) latency in the Morris water maze to find the hidden platform during trials 1-18, Probe 0 hr and Probe 24 hr. FSF females had shorter latencies during trials 3.4.and 5. (b) Percentage of time spent swimming in the quadrant of interest.

a



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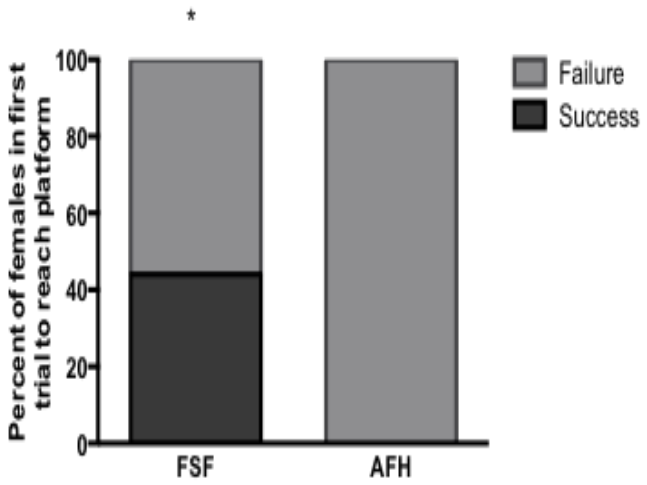
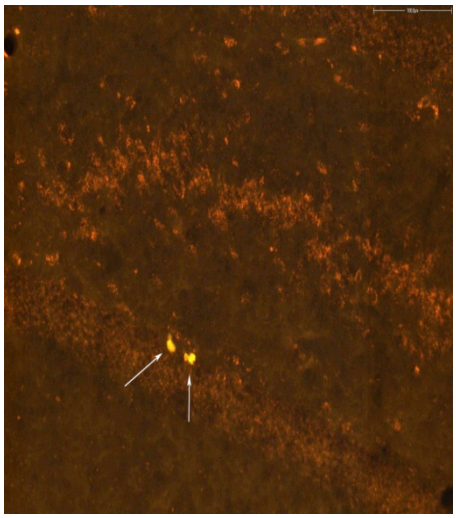
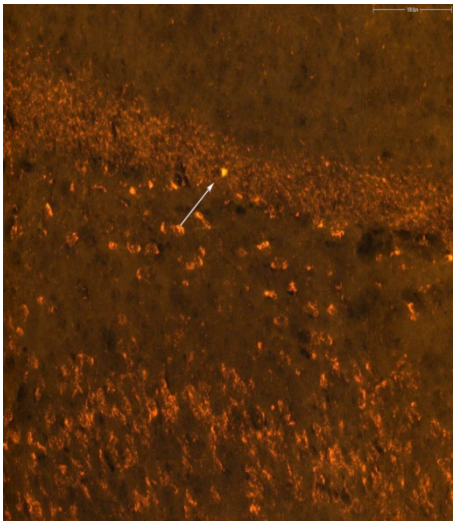


Figure 3.2
(a) Mean ± (SEM) time spent trying to escape from the pool. FSF females spent significantly more time trying to climb escape during trials 1-3 and probe 0 hr and probe 24 hr. (b) Mean ± (SEM) significantly more FSF females reached the platform during trial 1.

a



FSF females



AFH females

b

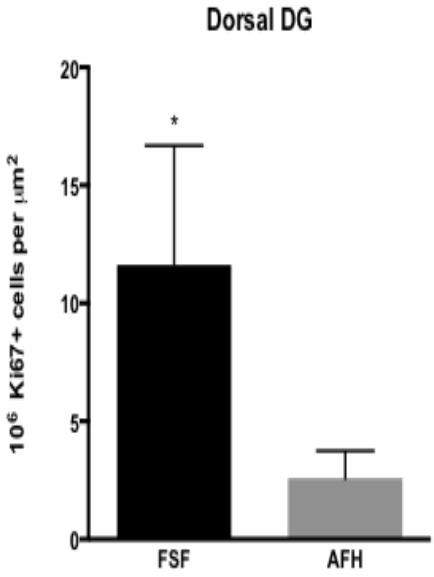
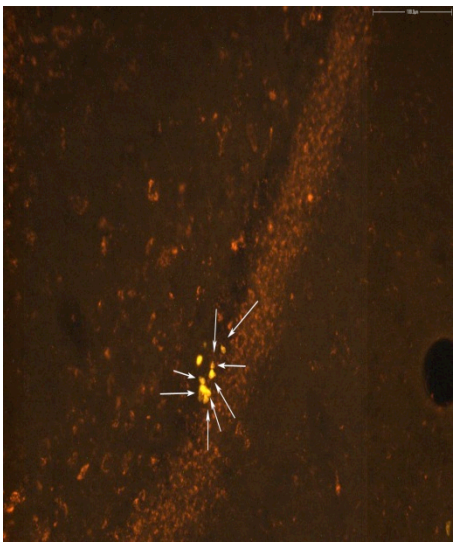
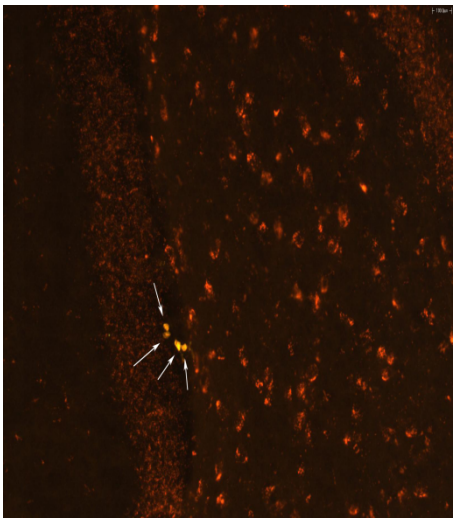


Figure 3.3
Semi-natural conditions increase adult cell proliferation in the dorsal DG. (a) Representative images of Ki67+ cells (white arrows) in the dorsal DG of FSF and AFH females. Scale bar is 100 um. (b) Mean ± (SEM) FSF females had significantly more Ki67 immunopositive cells in the dorsal DG.

a



FSF females



AFH females

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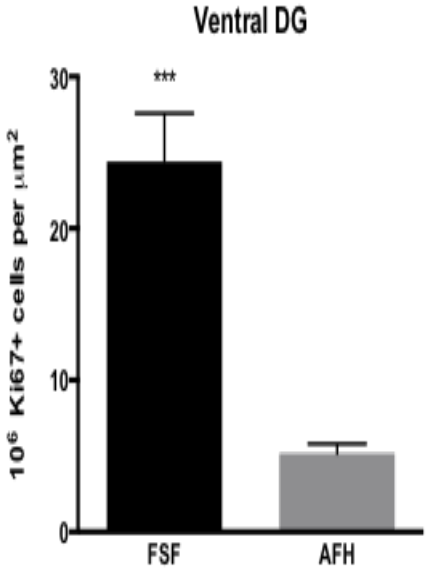

Figure 3.4Semi-natural conditions increase adult cell proliferation in the ventral DG. (a) Representative images of Ki67 + cells (white arrows) in the ventral DG of FSF and AFH females. Scale bar is 100 um. (b) Mean ± (SEM) FSF females had significantly more Ki67 immunopositive cells in the ventral DG.

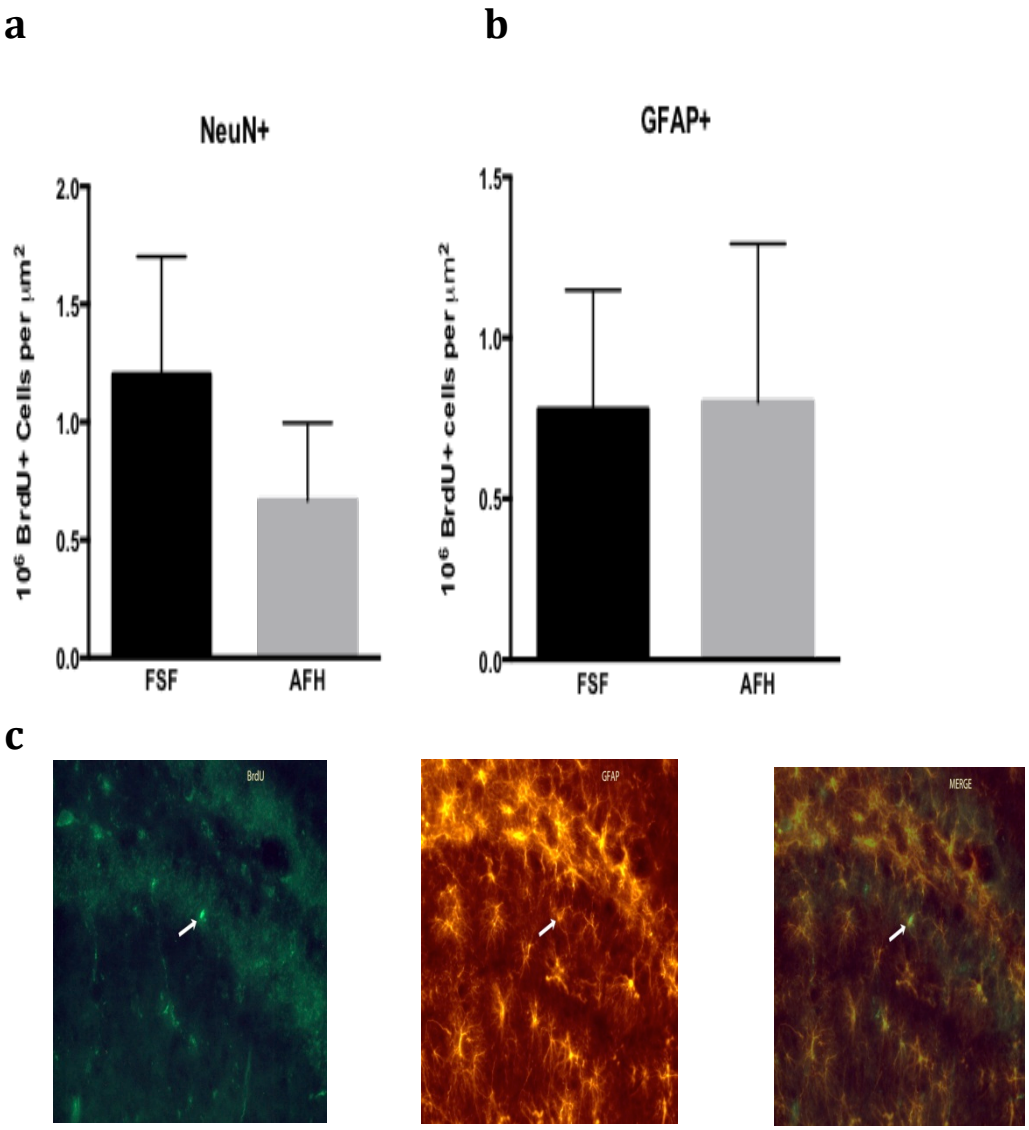

Figure 3.5
Semi-natural conditions do not increase cell survival in the dorsal or ventral DG. (a)(b) Mean ± (SEM) semi-natural conditions does not alter the number of BrdU+ cells co-expressing neuronal nuclei (NeuN) or glial fibrillary acidic protein (GFAP). (c) Representative images of Ki67+ cells (white arrows) in the ventral DG of FSF and AFH females. Scale bar is 100 um. (b) Mean ± (SEM)

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