

Effects of Social Status on Responses to Social Defeat Stress in Female Syrian Hamsters

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Abstract

Understanding the neurocircuitry and neuroendocrine responses that impart stress resilience is an early step toward developing more effective treatment options for patients who suffer from stress-induced psychopathologies. Social defeat models have been used to investigate the cellular mechanisms of stress vulnerability in male rodents, although limited research has been conducted in females. We have previously shown that male Syrian hamsters exhibit elevated social avoidance following acute social defeat stress. Interestingly, male hamsters with dominant social status exhibit elevated plasma testosterone and less defeat-induced social avoidance in comparison to subordinates and controls. The objective of this study was to investigate whether dominant female hamsters display resistance to social defeat stress similar to their male counterparts. Adult female hamsters were matched based on their estrous cycle and paired in 12 daily social encounters to establish dominance relationships. Blood was collected via retro-orbital eyebleed prior to their first dominance interaction, 15 min after their first dominance interaction, and 15 min after their twelfth dominance interaction. Animals were then subjected to 3, 5-min aggressive encounters with 3 separate resident aggressors, resulting in an acute social defeat experience. Twenty-four hours after social defeat stress, animals were placed in a neutral arena with an unfamiliar, restrained animal. Their approach and avoidance behavior toward this stimulus animal was then measured and quantified. While acute social defeat increased social avoidance in the social interaction test, social status did not differentially alter social avoidance or plasma concentrations of testosterone and progesterone. Collectively, these results indicate that the neuroendocrine mechanisms that regulate individual differences in stress-related behavior in male hamsters do not necessarily apply to females.

1. Introduction

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is an adaptive response to a stressor and ultimately dictates how an animal will behave in a potentially life threatening situation. Activation of this neuroendocrine system initiates a cascade of neurochemical and hormonal activity which disrupts homeostasis and leads to profound effects on an organism's physiology and behavior. Altogether, activation of the HPA axis is the defining characteristic of the stress response. While the stress response is by nature an adaptive response, it can become maladaptive when stressors are severe, chronic, or unpredictable. In these situations, the stress response is exaggerated by stressors that are perceived as overwhelming, which can lead to long-term health complications (Korte et al., 2005).

Exposure to stressful life events is a strong risk factor for the development of psychopathologies such as post-traumatic stress disorder (PTSD), depression, anxiety disorders, and other mental illnesses; however, individual differences play a crucial role in modulating stress reactivity (Goh and Agius, 2010). In fact, only a small fraction of people exposed to a traumatic or stressful situation will develop stress-related mental illness (Ramchand et al., 2015). As such, a concerted effort is underway to better understand the individual differences which predispose some people to be resilient and others to be vulnerable to the harmful effects of trauma. A better understanding of the biological basis of individual differences in stress reactivity could lead to improved treatment options for patients who suffer from stress-related psychopathologies.

Syrian hamsters are a model organism for studies of stress and aggression (Huhman, 2006). Because these animals are solitary and territorial, social defeat stress is an ethologically relevant model to specifically investigate neurobiological mechanisms regulating the effects of social stress. Importantly, Syrian hamsters display profound changes in social behavior following

a single exposure to social defeat stress. These behavioral changes, which are marked by increased social avoidance and decreased territorial aggression towards a smaller, nonaggressive intruder, are collectively termed the conditioned defeat response. Interestingly, female hamsters are less susceptible to conditioned defeat than males, although the specific mechanisms that underlie this resilience remain unknown (Huhman et al., 2003).

Previous studies using male hamsters have shown that social dominance status modulates the degree to which animals display the conditioned defeat response. This finding indicates that dominance relationships influence subsequent stress reactivity. Specifically, animals with dominant social status display less defeat-induced social avoidance compared to subordinates and controls (Morrison et al., 2011; Morrison et al., 2014). This reduced conditioned defeat response in dominant animals is accompanied by an increase in neural activity in the ventromedial prefrontal cortex (Morrison et al., 2012). Additionally, dominant hamsters exhibit a surge in plasma testosterone 15 min following an initial dominance encounter and an increase in androgen receptor density in the medial amygdala following 2 weeks of dominance encounters compared to subordinates and controls (Clinard et al., 2016). Furthermore, a pharmacological blockade of androgen receptors during the maintenance of dominance relationships prevents the increase in androgen receptor expressions in the medial amygdala and reinstates a normal conditioned defeat response in dominant hamsters (Clinard, 2016). These findings suggest that the testosterone surge experienced by dominant male hamsters may upregulate androgen receptors in the medial amygdala and thereby reduce social avoidance following acute social defeat stress.

Despite the status-dependent differences in how male hamsters respond to social defeat stress, little is known about how social status may modulate the effects of social defeat in females. The objective of this study was to investigate whether dominant female hamsters

display resistance to social defeat stress similar to their male counterparts. Specifically, we hypothesized that dominant females would display increased plasma testosterone levels following an initial dominance encounter and decreased social avoidance following social defeat.

2. Methods

2.1 Animals

Subjects were female Syrian hamsters (age 10-13 weeks) born in our breeding colony that was established with male and female animals purchased from Charles River Laboratories (Wilmington, MA). Animals were kept on a 14:10 h light:dark cycle, and all behavioral measures were conducted during the first four hours of the dark cycle, between 1300 and 1700 h. Animals were housed in 12cm x 27cm x 16cm cages with corncob bedding, cotton nesting, and wire mesh tops. Food and water were available *ad libitum*. Animals were singly housed 3-6 days before the first day of behavioral testing to promote territorial behavior. Animals were divided into six groups in a 2X3 factorial design. In regard to social status, they were either dominants, subordinates, or controls (i.e. had no social status). In regard to stress exposure, approximately half of the subjects experienced acute social defeat while the others did not. All procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee.

2.2 Determination of Estrous Cycle

Female hamsters have a reliable 4-day estrous cycle which consists of two days of diestrus followed by one day of proestrus and one day of estrus. Beginning one week prior to the study, animals were monitored daily between 1230 and 1630 h for signs of estrus. During this procedure, animals were gently held on their backs while a cotton swab was placed against their vaginal area. When the cotton swab was slowly removed, the presence of a thin string of

vaginal discharge indicated that the animal was in estrus (Wise, 1974). Animals that did not demonstrate a reliable 4-day cycle were excluded from the study (n=5).

2.3 Establishment of Dominance Relationships

Animals with the same estrous cycle and similar weight were paired in 12 daily dyadic encounters over the course of 15 days (Fig. 1). In order to promote aggressive interactions in dyads, a resident-intruder model was utilized. One animal was placed in the home cage of the other animal for daily agonistic encounters. These encounters lasted for 10 min until a dominance relationship was established, after which the encounters were reduced to 5 min each day in an effort to minimize wounding. Although the initial selection as resident or intruder was arbitrary, the roles remained consistent throughout the study. Daily aggressive encounters began on the first day of diestrus and continued through proestrus, but animals were not exposed to one another on the day of estrus. Previous studies indicate that female hamsters display very little aggression while in estrus (Solomon et al., 2007), so dyadic encounters were skipped on the day of estrus to decrease the opportunity for dominance relationships to reverse. Dominant or subordinate social status was defined by a consistent display of aggressive or submissive behavior, respectively, as described in the Syrian hamster ethogram (Albers et al., 2002). Dominant animals displayed aggressive behavior marked by chasing, attacking, biting, and side aggressive posture, while subordinate animals displayed submissive and defensive behavior marked by fleeing, avoiding, head flagging, stretch attends, and raised tails. Animals that did not form stable dominant/subordinate relationships were excluded from the study. Control animals remained in their home cages with no social interaction until social defeat stress.

2.4 Blood Collection and Hormone Assay

At three separate time points (immediately before day 1 dyadic encounters, 15 min after day 1 dyadic encounters, and 15 min after day 12 dyadic encounters), animals were anesthetized with 4% isoflurane and blood was collected via retro-orbital eyebleed. Blood samples were centrifuged for 15 min at 4400 rpm, after which the plasma layer was pipetted off the top of the sample and stored at -80°C until further testing. After all blood samples were collected, the plasma was treated with an ether extraction procedure. Testosterone and progesterone were then assayed with a commercial ELISA kit according to the manufacturer's protocol (Cayman Chemical). Samples were analyzed in duplicates with 50µl of plasma per well. Intra-assay reliability was 5.6% within a single plate, and inter-assay reliability was 9.0% between plates.

2.5 Acute Social Defeat Stress

Forty-eight hours after the twelfth and final dyadic encounter, animals experienced an acute social defeat stress when they were in the diestrus I phase of their cycle. Similar to the daily dominance encounters, the social defeat procedure utilized a resident-intruder model. Subjects were placed in the home cage of a larger, more aggressive female hamster for 3 separate 5-min social defeat sessions. Subjects were exposed to a different resident aggressor in each session and were given a 5-min rest in their home cage between sessions. We used breeder females as resident aggressors, and they quickly subjugated the smaller female subjects. Breeder females were screened for estrous and they were not used as resident aggressors on their estrous day. Control animals were exposed to the empty cages of 3 separate resident aggressors in a parallel fashion.

2.6 Social Interaction Testing

In order to assess stress-induced changes in social behavior, animals received a social interaction test 24 hours following social defeat stress when they were in the diestrus II phase of their cycle. First, subjects were introduced into a clean, neutral arena with a small, empty mesh box in the corner. They were allowed to freely explore the arena in a 5-min habituation period. Next, the subjects were briefly removed from the cage, the bedding was stirred to reduce the influence of chemical cues left from the habituation period, and the empty mesh box was replaced with one that held an unfamiliar, adult female hamster. Then, the subject was re-introduced into the arena, and her response to the novel animal was monitored. Both trials were digitally recorded and later scored for approach and avoidance behaviors.

The duration of time that the subject spent in different sections of the arena was measured, including the far side, near side, and interaction zone. Far side and near side were defined by a midline that split the arena in half with the mesh box contained in the near side. Additionally, the subject was considered to be in the interaction zone when it made physical contact with the mesh box or was positioned with its snout within 2 cm of the mesh box. In addition to these durations, the frequency of several behaviors was scored, including flee, avoid, stretch attend, head flag, flank mark, and self-groom. Flees and avoids were both defined by a rapid movement away from the stimulus animal with the distinction that flees covered at least half of the cage and avoids did not. A stretch-attend was recorded when the subject made a cautious approach marked by low body posture and attention toward the social target. Head flagging was characterized by bouts of quick rotations in head and shoulder position toward and away from the social target. Flank marking was defined by the subject rubbing its flank gland against the wall of the cage. Self-grooming was recorded when the subject voluntarily manipulated its fur with its hands or mouth.

2.7 Statistical Analysis

Changes in plasma testosterone and progesterone levels were analyzed using a 3x3 repeated measures ANOVA, followed by a one-way ANOVA for post-hoc analysis. Behavioral differences displayed during the social interaction test were analyzed using a two-way ANOVA, followed by Fisher's protected least significant difference (LSD) post-hoc test. All statistical tests were two-tailed, and the α level was set at $p \leq 0.05$.

3. Results

3.1 Maintenance of Dominance Relationships

Consistent with findings from studies with male hamsters, females formed stable dominance relationships. Of the 26 dyads that were initially paired together, 20 dyads Established and maintained a stable dominance relationship. In 3 of the 6 dyads that were excluded from analysis, at least one of the animals showed signs of estrus outside of a consistent 4-day cycle. These animals and their dyadic partners were excluded from analysis on the basis that the dyads were not estrous cycle-matched. Animals in the remaining 3 dyads switched their dominant/subordinate roles after the first four days of interaction and were excluded from analysis because they failed to establish a stable dominance relationship. Each of the 20 dyads that successfully completed the 12 daily encounters established a dominance relationship on the first day of interaction. Two of these dyads switched roles within the first 4 days of interactions, but because their status remained stable thereafter, they were included in subsequent analysis. This propensity to consistently establish dominant and subordinate roles during the first interact differs from studies conducted with males, who often take two or three days to establish dominance roles (Morrison et al., 2012; Clinard et al., 2016).

3.2 Plasma Hormone Levels

There was a main effect of time on plasma progesterone levels ($F = 71.1$; $df = 1,26$; $p < 0.001$), but no effect of social status ($F = 0.11$; $df = 2,29$; $p > 0.05$) and no significant interaction ($F = 0.62$; $df = 2,26$; $p > 0.05$) (Fig. 2a). Blood collection during the first day of dominance encounters occurred while females were in diestrus I, while the twelfth day of dominance encounters occurred during proestrus. There was a significant decrease in plasma progesterone levels from pre-encounter to post-encounter in the samples collected on day 1 ($p < 0.001$), followed by another significant decrease in the post-encounter samples collected on day 12 ($p < 0.001$). Overall, the estrous cycle stage produced a similar change in plasma progesterone levels in dominant, subordinate, and control animals.

Similarly, there was a main effect of time on plasma testosterone levels ($F = 12.7$; $df = 1,26$; $p = 0.001$), but no effect of social status ($F = 1.6$; $df = 2,26$; $p > 0.05$) and no significant interaction ($F = 0.57$; $df = 2,26$; $p > 0.05$) (Fig 2b). Specifically, plasma testosterone levels were significantly lower on day 1 post-encounter than they were on day 1 pre-encounter ($p = 0.016$) and on day 12 post-encounter ($p = 0.008$). The decrease in plasma testosterone on day 1 was observed in dominants, subordinates, and controls, even though controls did not experience agonistic encounters. Further planned comparisons indicated that subordinates exhibit a trend toward lower testosterone levels after the initial aggressive encounter compared to dominants ($F = 1.77$; $df = 30$; $p = 0.088$). Overall, changes in plasma testosterone levels were similar in dominants, subordinates, and controls.

3.3 Social Interaction Testing

There was a main effect of social defeat stress on time spent in the interaction zone during social interaction testing ($F = 36.2$; $df = 1,57$; $p < 0.001$), although there was no

significant effect of social status ($F = 0.57$, $df = 2,57$; $p > 0.05$) and no significant interaction ($F = 1.2$; $df = 2,57$; $p > 0.05$) (Fig. 3a). All animals who received an acute social defeat spent less time in the interaction zone when the social stimulus animal was present. Similarly, there was a main effect of social defeat stress on the social interaction ratio ($F = 26.9$; $df = 1,57$; $p < 0.001$), and no significant effect of social status ($F = 2.0$; $df = 2,57$; $p = 0.149$) or significant interaction ($F = 1.0$; $df = 2,57$; $p > 0.05$) (Fig. 3b). The social interaction ratio was greater than 1.0 for both dominant and subordinate subjects that experienced social defeat which indicates that they spent more time near the mesh box when it contained the social stimulus animal in comparison to when it was empty. Among defeated animals, we found a non-significant trend indicating that dominant animals had a greater social interaction ratio in comparison to controls ($p = 0.074$). Overall, acute social defeat led to a modest level of social avoidance in dominant, subordinate, and control hamsters.

While social status did not significantly alter behavioral responses to social defeat stress, residency status has been known to influence aggressive behavior and the effects of winning (Fuxjager et al., 2009). When defeated subjects were categorized according to residency status during their daily encounters, and we found that resident and intruders did not significantly differ in time spent in the interaction zone during social interaction testing ($t = 1.09$; $df = 22$; $p = 0.286$). Additionally, we recorded very low rates of submissive, defensive, and displacement behaviors during social interaction testing (e.g. flees/flights, avoids, stretch attends, head flagging, flank marking, and grooming). Many subjects in each experimental group did not display these behaviors, and the frequency of most of these behaviors was less than 1.5 per 5-min test. However, flank marking was the most common behavior and was displayed with a frequency of 3.2 per 5-min test. Because behavioral frequencies were low and no significant

differences emerged with regard to social status or social defeat exposure, these behaviors were not investigated further.

4. Discussion

Our results demonstrate that female Syrian hamsters display increased levels of social avoidance following acute social defeat stress in a manner similar to males, but this response to stress is not modulated by dominant social status. Specifically, males display a surge in plasma testosterone after winning a dyadic encounter, and after maintaining dominant status for two weeks, they exhibit less defeat-induced social avoidance compared to subordinates and controls (Clinard et al., 2016). In contrast, we found that female hamsters do not exhibit elevated plasma testosterone or progesterone after winning an initial dyadic encounter. Also, the maintenance of dominance relationships for 15 days does not lead to differences in defeat-induced social avoidance in dominants and subordinates. Taken together, these results indicate that sex differences exist in the effects of social status on behavioral and neuroendocrine responses to acute social defeat stress.

While social status did not influence plasma hormone levels, there was an effect of time on both testosterone and progesterone. Progesterone was lowest at post-encounter day 12, which occurred early during the active period on the day of proestrus. This finding is consistent with prior studies on the cyclical expression of progesterone, which reported a nadir during early proestrus in female hamsters (Saidapur and Greenwald, 1978) and rats (Sato et al., 2016; Pawluski et al., 2009). Progesterone also declined from pre-encounter to post-encounter on day 1, which occurred approximately 30 min apart. While progesterone levels decrease from diestrus 1 to diestrus 2 (Saidapur and Greenwald, 1978), it seems unlikely that progesterone could significantly decrease in 30 min. Testosterone levels also showed a significant decrease

from pre-encounter to post-encounter on day 1, despite evidence suggesting testosterone levels should be stable during diestrus I (Saidapur and Greenwald, 1978). Together, these findings indicate that the drop in progesterone and testosterone levels from pre-encounter to post-encounter could be the result of blood collection and associated anesthesia. However, repeated blood collection and anesthesia do not produce a similar reduction of testosterone in male hamsters (Clinard et al., 2016).

In females, steroid hormone concentrations do not selectively change after winning or losing an aggressive encounter. These findings are consistent with literature in humans showing that women do not exhibit elevated salivary testosterone after winning competitive interactions (Zilioli et al., 2014). However, ovarian hormones do fluctuate over the 4-day estrous cycle and this fluctuation is associated with changes in defeat-induced social avoidance (Solomon et al., 2007). Overall, defeat-induced social avoidance in male hamsters is modulated by hormone changes that occur after aggressive encounters, whereas defeat-induced social avoidance in females appears to be modulated by hormone fluctuations during the estrous cycle.

Little is known about the specific mechanisms that underlie behavioral responses to social defeat stress in female hamsters, although early studies indicated that female hamsters show less defeat-induced social avoidance than males. Huhman et al. (2003) found that 100% of male hamsters and only 28% of female hamsters displayed a conditioned defeat response toward a nonaggressive intruder one day following an acute social defeat. In fact, 44% of the female subjects displayed species-typical territorial aggression towards the nonaggressive intruder on the first day of social interaction testing. Furthermore, these stress-induced changes in behavior were much longer lasting in males than females. A majority of the males in this study continued to display a conditioned defeat response through the conclusion of the study 33 days after the social defeat stressor. Conversely, 100% of the females returned to normal

social behavior by the second trial of social interaction testing, which occurred 4 days following social defeat (Huhman et al., 2003).

One important distinction between the work of Huhman et al. (2003) and our study is the nature of the social target during social interaction testing. While they used a freely ambulating juvenile hamster in the subject's home cage, we used a restrained adult hamster in a neutral arena. Recent studies using a restrained adult animal for social interaction testing have shown similar levels of defeat-induced social avoidance in male and female hamsters (McCann et al., 2012; McCann et al., 2017). Consistent with these findings, we demonstrated that female hamsters will exhibit defeat-induced social avoidance when tested with a social target restrained in a mesh box. Moving forward, this testing method could be used to further investigate the mechanisms contributing to sex differences in behavioral and physiological responses to social defeat stress.

Looking forward, a few paths for further research emerge. First and foremost, male animals should be tested using the exact same procedure used in this study in order to make accurate comparisons between the sexes. Namely, the effect of having no dyadic encounters every fourth day and of using a restrained stimulus animal in a neutral arena instead of using a juvenile nonaggressive intruder in the subject animal's home cage should be investigated. Second, immunohistochemistry (IHC) should be performed on the brain tissue collected from this study to investigate status-dependent changes in androgen or estrogen alpha receptor densities in the medial amygdala. Future studies could explore sex differences in c-Fos expression in the ventromedial prefrontal cortex, medial amygdala, hippocampus, and other brain regions known to regulate responses to social defeat. Additionally, future research should focus on whether estradiol modulates behavioral responses to social defeat stress. While estradiol treatment reduces the amount of defeat-induced submissive behavior in

ovariectomized hamsters (Faruzzi et al., 2005), cycling hamsters display the highest levels of defeat-induced submissive behavior during proestrus, when estradiol levels peak (Solomon et al., 2007). These contradictory findings, coupled with the broader consensus that estradiol plays an essential role in developing the sexually dimorphic central processes that underlie the stress response (Gillies and McArthur, 2010), provide evidence that fluctuating estradiol levels may play a role in modulating stress reactivity in female Syrian hamsters.

In sum, we demonstrated that female hamsters display increased levels of social avoidance following exposure to an acute social defeat stress. Social status did not differentially alter plasma testosterone or plasma progesterone concentration or behavioral responses to social defeat. These results indicate that social status differentially modulates response to social defeat in male and female Syrian hamsters and that neuroendocrine mechanisms likely play a key role in this sex difference. These findings underscore the importance of further investigating sex differences in stress reactivity in order to better understand how men and women respond to and cope with stress. Improved understanding of the mechanisms underlying sex-differences in stress reactivity should lead to the development of better treatment options for patients who suffer from stress-related mental illness.

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7. Figures

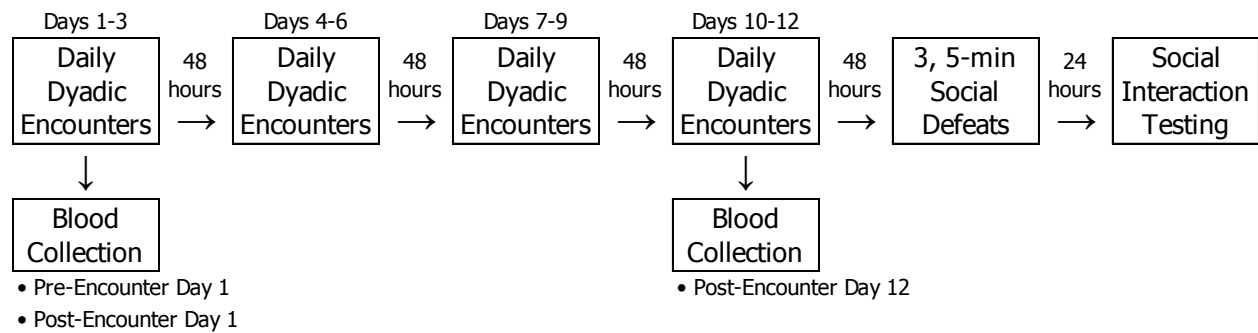
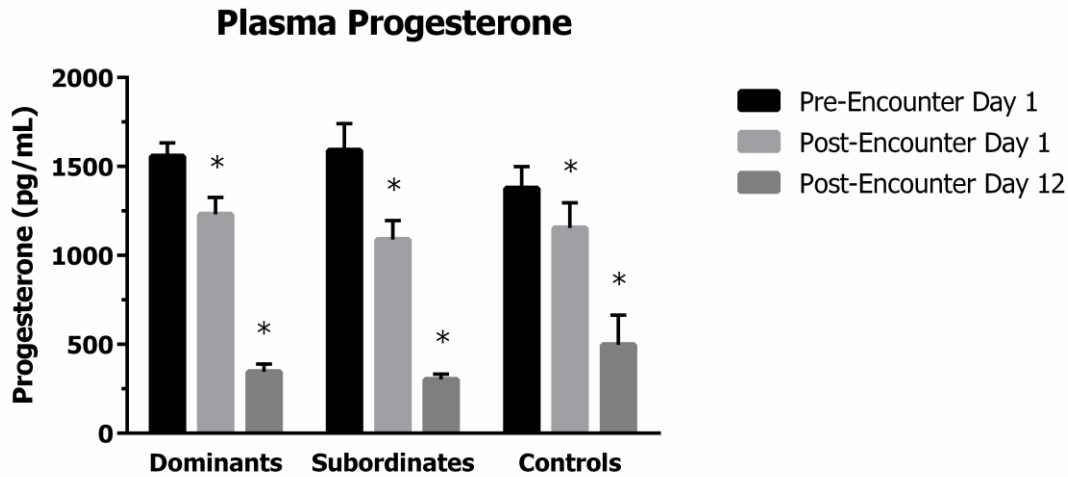


Figure 1. *Experimental design.* Animals were screened for estrus, cycle-matched with a weight-matched conspecific from a separate litter and paired in 12 daily dyadic encounters. Syrian hamsters have a reliable 4-day estrus cycle consisting of two days of diestrus, one day of proestrus, and one day of estrus. Dyadic encounters began on diestrus I and skipped the day of estrus. Initial dyadic encounters were 10-min in duration and then 5-min in duration after dominance relationships were established. Blood was collected immediately before the first encounter, 15 min after the first encounter, and 15 min after the twelfth encounter.

a)



b)

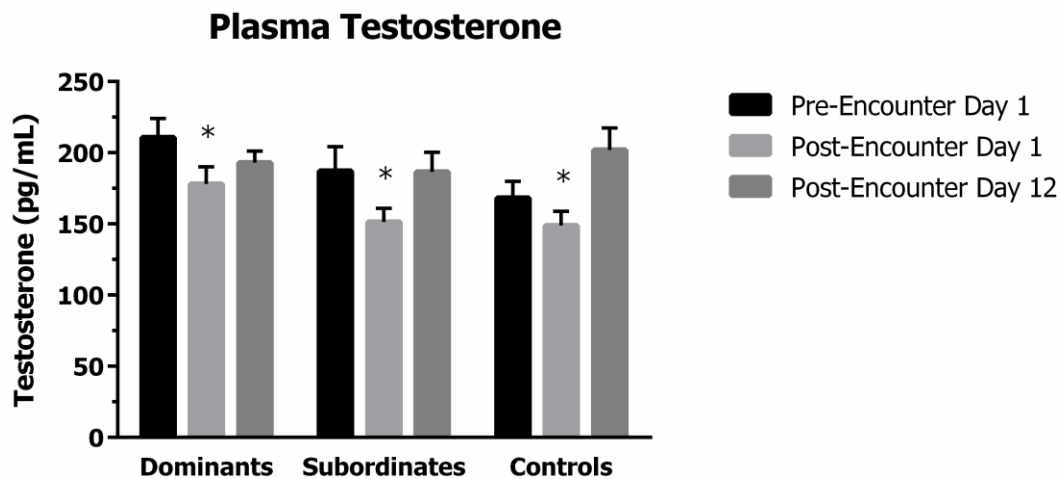
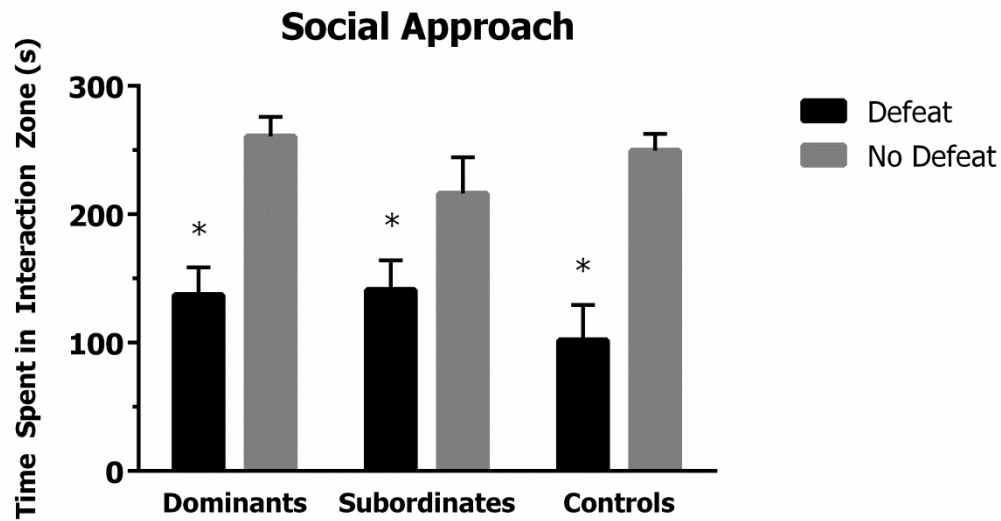


Figure 2. *Plasma hormone concentrations.* Plasma concentrations (mean $\pm$ SEM) of progesterone (a) and testosterone (b) immediately before the first dyadic encounter, 15 min after the first dyadic encounter, and 15 min after the twelfth dyadic encounter. All day 1 encounters were conducted when animals were in diestrus I, while all day 12 encounters were conducted when animals were in proestrus. Asterisk (*) indicates a significant difference compared to pre-encounter (day 1) and post-encounter (day 12) concentrations ($p < 0.05$). $n = 14-18$ for dominants and subordinates, and $n = 2-9$ for controls.

a)



b)

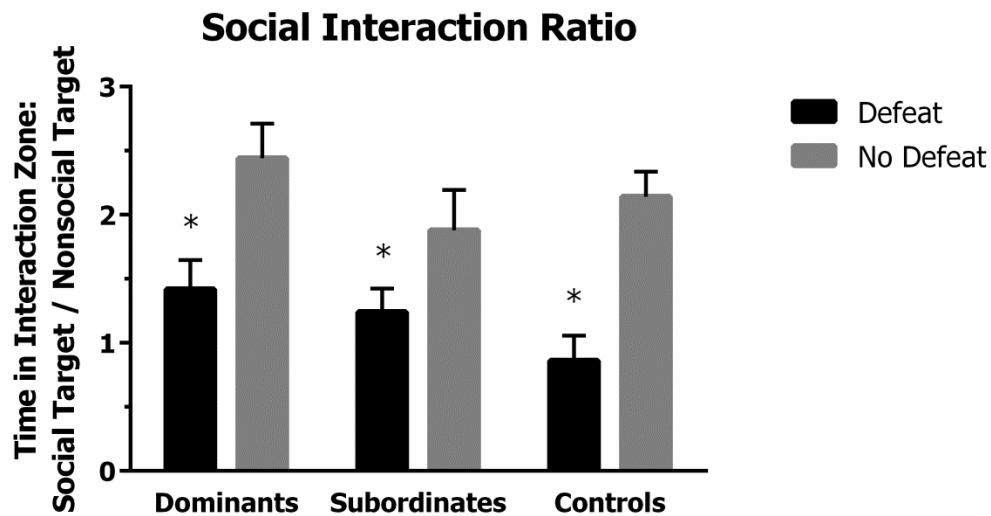


Figure 3. *Social interaction testing results.* Time (mean $\pm$ SEM) subjects spent in the interaction zone (a) and the ratio (mean $\pm$ SEM) of time subjects spent in the interaction zone with the full mesh box divided by the time spent in the interaction zone with the empty mesh box (b). Asterisk (*) indicates a significant difference compared to no defeat animals ($p < 0.05$). $n = 7-12$ per group.