Chronic social instability stress differentially affects the behavior and the transcriptome of the anterodorsal bed nucleus of the stria terminalis between male and female mice

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Abstract

Stress can be broken down into systemic and processive stressors with processive stressors requiring higher limbic processing. These are also often called social stressors as they require an understanding of social dynamics as opposed to physical based stressors. This differing of processing necessitates we study both phenomena. Additionally, sex is an important aspect of stress research as men and women show differing responses to stress and mood disorder development. To study this, we used a chronic social instability stress (CSIS) paradigm to stress male and female mice. This paradigm is approximately 7-weeks long and involves changing the cage mates of a mouse every 3 days so stable social dynamics cannot form. Afterwards, one cohort was used for avoidance behavior testing using the open field test, the elevated plus maze, the light/dark box emergence test, and the novelty suppressed feeding test. A second cohort was used for bulk RNA-Sequencing of the anterodorsal bed nucleus of the stria terminalis which is a limbic structure known to be related to chronic stress signaling. In the behavior assays, CSIS caused the females to be less avoidant, while the males became more avoidant. Additionally, we found that a low estrogen state in the females caused them to be less avoidant than in a high estrogen state. In the transcriptome, we found major differences between the males and females with the males expressing more genes related to transcription whereas the females expressed more genes related to synaptic transmission. We also found that the transcriptome in the males is more sensitive to the stress than the females. In summary, we have found how social stress is differentially regulated between males and females and how this may be related to the development of stress-related behavioral changes.

1. Introduction

When studying chronic stress, most research has been done with physical stressors, i.e. stressors that are direct affronts to the physiology or homeostatic function. These types of stressors typically activate the fight-or-flight response to ready the body for immediate action to protect itself. This can be studied in animal models through multiple paradigms, including but not limited to chronic variable mild stress [1], chronic restraint stress [2], and chronic foot shock [3]. Researchers have even modeled pure chronic stress outside of context through techniques such as chronic corticosterone administration [4] or chronic corticotropin releasing hormone (CRH/F) administration [5].

However, physical stressors are not the only type of stressors that an organism will face. Stress, can further be categorized into multiple different modalities, with a common delineation being stressors that require limbic processing, called processive stress, and stressors that do not, called system stress [6]. Another axis is social versus physical stressors, which closely align with the ideas of system and processive stressors wherein social stress requires higher cognitive processing whereas many physical stressors do not and instead directly activate the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system. For example, a meta-analysis of functional imaging of the human brain under physical versus social stress found that physical stress more preferentially activates the insula, cingulate cortex, and ventral striatum while social stress more preferentially activates the temporal gyrus and preferentially deactivates the dorsal striatum [7]. This suggested that physical stressors strongly activate the fight-or flight response while social stressors strongly activate mood regulation and motivation. We wanted to explore how physical stressors differentiate from social stressors in the central stress response by continuing previous

research that we had done with a chronic stress paradigm that was more physically-based [8] but now with a socially-based stress paradigm.

Social stress has historically been difficult to model in animal studies. This is due to social stress typically requiring understanding of complex concepts or complex social dynamics, as in situations such as financial stress, occupational stress, or relationship stress. One of the most common social stress paradigm is social defeat stress, which relies on an aggressive male to dominate a submissive mouse [9]; however this paradigm has a significant limitation in that it is not effective in females as neither males nor females will fight an intruder female mouse [10]. Though other techniques have been developed to allow for study of social defeat in females [10, 11], these still require the primary stressor to be an attack from an aggressive male which can be seen as equally both physical and social stress. For this study, we opted to use a chronic social instability stress paradigm as it was shown to be effective in both males and females [12] and may be seen as more ethnologically relevant that other social stress paradigms like total social isolation.

The bed nucleus of the stria terminalis (BNST) is a limbic region of the brain that is selectively activated by lo0ng-context stressors [13]. As such, this region of the brain is important for chronic stress signaling and is often called the “extended amygdala.” However, the BNST is an extremely heterogeneous region, being able to be split into multiple subnuclei, each of which can have multiple different types of neurons [14]. We were specifically interested in the anterodorsal (ad) nuclei of the BNST as it has been shown to have connections to both the prefrontal cortex as well as the paraventricular hypothalamus, suggesting that is modulates both behavior and stress signaling [15, 16]. Additionally, the BNST has been shown to be sexually dimorphic in its morphology as well as expression of certain important signaling and hormone related proteins [17]. Our own research has found that not only is the transcriptome of the adBNST

different between males and females, but that the transcriptome is differentially affected by stress according to sex [8, 18]. This makes the region extremely compelling to study when considering the sex difference in the development of stress-related mood disorders.

Overall, we were seeking to understand how social stressors uniquely and specifically affect the adBNST and how that may lead to behavioral changes. We hypothesized that the social stress would result in a general increase in avoidant behaviors but that this would be differential by sex and further differential by hormone status as seen in our previous publication [8]. We also hypothesized that the adBNST transcriptome would have genes that are differentially expressed to sex and stress condition with males being more sensitive as was previously shown [18].

2. Materials & Methods

2.1 Animals

All procedures were in accordance with National Institutes of Health standards and approved by the Rutgers Institutional Animal Care and Use Committee and agree with ARRIVE's guidelines for reporting animal research. Mice used for behavior and RNA-sequencing cohorts were adult male and female C57BL/6J mice that were bred in house. Mice were housed in a temperature and humidity-controlled room (22°C, 30-70% humidity), on a 12/12 h light/dark cycle, and provided food and water ad libitum. Control or stressed conditions started at 7-10 weeks of age. Due to the nature of the CSIS schedule, sample sizes were n=15 mice for each experimental group which allowed for detection of affects with at least power = 0.8 at $\alpha = 0.05$.

2.2 Chronic Social Instability Stress Paradigm

The chronic social instability stress (CSIS) paradigm lasts for over 7 weeks and involves frequent changes in cage mates [12]. For this paradigm, every 3 days the mice are trio housed with novel cage mates, only allowing repeat cage conformations every 2 weeks to allow enough time for extinction of possible conditioning to the cage mates. For this, mice are taken out of their current cage, identity of the mice are verified, their new cage is determined. This is repeated for every mouse until all mice are with 2 novel cage mates. This frequent changing ensures that stable social hierarchies cannot be established. Additionally, the control mice are also trio housed but are not ever exposed to novel mice, but cage changes are performed on them every three days to match the CSIS counterparts so that the only difference between the two groups in the social dynamics.

2.3 Corticosterone Assay & Body Weight

Corticosterone (CORT) levels in the serum were analyzed as previously described [8]. Briefly, blood was collected at 3 timepoints in the behavior cohort. Blood was collected prior to stress via submandibular bleeds, after stress but before behavior via submandibular bleeds, and after behavior at termination via trunk blood after decapitation. CORT concentrations were then analyzed via a commercially available sandwich ELISA kit #RTC002R; BioVendor). The lower limit for detection in this kit is 6.1 ng/ml with an intra-assay coefficient of variation between 5.9% and 8.9% and an inter-assay coefficient of variation between 7.2% and 7.5%. A logarithmic regression was then used to convert the absorbance to concentrations in ng/ml. A second analysis was done in terminal blood in which males were excluded, and females were separated by estrous stage Body weight (BW) was measured weekly and cumulative % BW was calculated.

2.4 Behavior Assays

Behavior was conducted over 8 days, alternating days between male and female testing so that mice were not affected by the scent of pheromones in the testing chambers. The tests conducted were the open field test (OFT), the elevated plus maze (EPM), the light/dark box emergence test (LDB), and the novelty suppressed feeding (NSF) test in that order. Vaginal cytology was performed on all females immediately after testing to check estrous stage. All tests were conducted as previously described [8].

2.4.1. OFT

The OFT tests avoidant behaviors by testing a mouse's willingness to explore an open arena which would be perceived as a dangerous to a prey animal. The OFT is conducted in an opaque plexiglass box with an open top (40 cm long x 40 cm wide x 40 cm tall). The mouse is allowed to freely explore the arena for 10 minutes and the entrances and amount of time spent in different zones is recorded. A mouse that spends more time in the perimeter and corners suggests a depressive-like increase in avoidant behaviors whereas a mouse that explores the center is less avoidant.

2.4.2 EPM

The EPM also tests avoidant behavior by giving the mouse a choice to explore either a perceived safe environment or risky one. EPM is conducted in a opaque plexiglass plus=shaped maze where two arms are closed by walls on three sides and the other two arms have no walls. Arms are 30cm long x 5 cm wide and connected at a 5 cm square center. The walls surrounding the closed arms are 15 cm tall with open tops. For this test, we analyzed the amount of time spent and the number of entries into the different arms. A mouse that spends more time in the closed arms is more avoidant than a mouse that is willing to explore the open arms.

2.4.3 LDB

The LDB tests uses the same arena as the OFT, but an insert is placed inside to cover half the arena in darkness. This tests the mouse's willingness to leave the perceived safety of darkness and explore lightened areas. The insert is an opaque box (20 cm long x 40 cm wide x 40 cm tall) with a closed top and a hole at the bottom to serve as an entryway between light and dark areas. For this test, we measured the amount of time as the number of entries between light and dark zones. A mouse that spends more time in the dark is considered more avoidant.

2.4.4 NSF

The NSF is the only tests that does not strictly measure avoidance behaviors. For this test, mice are fasted for 24 hours prior, then there are two trials. For the first trial mice are placed in a novel arena with a food pellet secured at the center. The mouse then must make a decision if they're going to stay in the safety of the edges or move to the center to obtain the pellet. The second trial but the mouse is similar to the first but is conducted in their home cage instead. This tests the interplay between avoidance and motivation. We analyze the latency to approach and eat the pellet in both arenas, the likelihood to eat in each arena, and the body weight changes due to fasting. A mouse that takes longer or does not eat the pellet at all is seen as more avoidant than one that quickly goes to the pellet.

2.5 RNA Sequencing

For RNA-Sequencing, mice were euthanized via rapid decapitation with ketamine. The brain was then dissected out and the sliced for later microdissection which isolated the anterodorsal (ad)

BNST. These samples were placed in RNAlater (Invitrogen) solution for storage. Prior to RNA extraction, three biological replicates were polled to account for interindividual differences resulting in an n=5 for each experimental group. RNA was extracted using the RNAqueous Micro Isolation kit (Invitrogen; AM1931). RNA was then checked for concentration and integrity with an Agilent 2100 Bioanalyzer. Any sample with an RNA integrity number less than 8 was discarded. Samples were then sent to the J.P. Sulzberger Columbia Genome Center at Columbia University (New York, NY) for library preparation and sequencing. Library preparation was performed with the TruSeq Stranded mRNA Library Prep Kit (Illumina) and pooled libraries were sequenced on the Illumina NovaSeq 6000 with 100 bp paired-end reads at a 40 million read depth. The resulting reads were quality trimmed with the FastX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) with a minimum quality score of 20.0. They were then aligned to the mm10 reference genome using STAR and the NCBI RefSeq mm10 gene annotation. Reads were counted with FeatureCounts [19, 20]. Data was then imported to R and differential expression was analyzed using DESeq2 with the following design parameters: (design ~ sex + condition + sex:condition) [21]. Principle component analyses (PCA) were also generated by PCAExplorer to check for outliers [22]. A. full list of all differentially expressed genes according to comparison can be found in the supplemental materials. Gene ontology terms were analyzed by clusterprofiler.

2.6 Statistical Analyses

All parameters unless otherwise specified were analyzed using Prism (GraphPad Prism, Version 10, Dotmatics) and used a p-value or adjusted p-value of <0.05 for significance. CORT assays and behavior were tested for outliers using the Grubbs test and then was analyzed by two-ANOVA. BW was analyzed by a repeated measures ANOVA. Behavior and CORT were first analyzed

comparing treatment and sex. A second analysis followed that excluded males and separated females by estrous stage into proestrus and estrus (P/E) which represented a high estrogenic, hormonal flux state and metestrus and diestrus (D) which represented a low estrogenic, stable state. The resulting variables were then treatment and estrous stage. For the CORT levels, this was only done in terminal blood. Probability to eat in the novel arena and home arena were analyzed by Kaplan-Meier survival curves. RNA-Sequencing was analyzed via DESeq2 in R. A table of sample sizes and excluded data, including outliers and censored data, can be found in the supplemental materials.

3. Results

3.1 BW & CORT

The CSIS paradigm only significantly impacted body weight in the males used for RNA-sequencing. For the RNA-sequencing cohorts, control males gained 16.69 ± 0.6 % of their BW and stressed males gained 23.16 ± 1.78 % over the course of the 7-week paradigm (fig. 1a). This was a significant effect of treatment as the stressed males gained more weight than the controls ($p = 0.0064$, $F(1, 28) = 8.704$). Female controls gained 15.13% ± 1.2 % while stressed females gained 17.4% ± 1.06 % (fig. 1a). There were no differences in the females. The behavior cohorts had a less drastic change in their BW, possibly due to the behavior cohorts being on average slightly older than the RNA-Sequencing cohorts. The control males in the behavior cohort gained 6.61 ± 1.33 % while stressed males gained 8.46 ± 2.26 % (fig. 1b). Female controls gained 8.25 ± 0.96 % while stressed females gained 7.75 ± 1.98 % (fig. 1b). Neither the males nor females of the behavior cohorts were different.

For CORT assay there was no effect of stress on the concentration of CORT in the serum. For the pre-stress blood, control males had an average of 50.63 ± 9.55 ng/ml, stressed males had an average of 84.28 ± 12.15 ng/ml, control females had an average of 78.25 ± 5.7 ng/ml, stressed females had an average of 71.72 ± 7.34 ng/ml (fig. 1c). There were no significant differences in treatment or sex in pre-stress serum. For post-stress, control males had 41.25 ± 5.37 ng/ml, stressed males had 53.5 ± 5.57 ng/ml, control females had 71.33 ± 6.13 ng/ml, and stressed females had 74.85 ± 8.59 ng/ml (fig. 1c). In the post-stress blood, there was no effect of treatment but there was an effect of sex in which females had higher CORT levels ($p = 0.0043$, $F(1, 34) = 9.377$). For terminal blood, control males had 65.16 ±18.42 ng/ml, stressed males had 78.78 ± 15.28 ng/ml, control females had 68.61 ± 7.4 ng/ml, and stressed females had 48.15 ± 6.19 ng/ml (fig. 1c). There was effect of sex or treatment on the terminal serum concentrations.

3.3 Behavior Assays

3.3.1 OFT

In our previous publication, the CVMS paradigm resulted in a general increase in avoidance of all mice that were stressed and an increase in avoidance in female mice compared to males regardless of treatment [8]. For our socially-based stress paradigm, CSIS, we similar but distinct effects on behavior and in relation to sex. We found no effects of stress or sex on the number of entries into the 20cm center or the amount of time spent in the 20cm center or perimeter (fig. 3a, d, and e). In the number of entries into the perimeter, we see a main effect of treatment wherein stressed animals had fewer entries ($F(1, 82) = 4.782$, $p = 0.0316$) (fig. 3b). This suggests a reduction in exploratory behavior but does not indicate effects on avoidance. However, we do see effects on avoidance when looking at percent time spent in the corners and 10cm center. In the corners, there were

pairwise effects when comparing the control males to their stressed and female counterparts as they spent more time in the center ($p = 0.0257$, $p = 0.0045$, respectively) (fig. 3c). Interestingly, we see a main interaction effect where it appears as if CSIS is affecting the behavior in the males and females differently such that the stress appeared to increase avoidant behaviors in males, but decreased it in females ($F (1, 79) = 7.104$, $p = 0.0093$) (fig. 3c). This interaction effect is repeated in the amount of time spent in the corners ($F (1, 82) = 28.79$, $p < 0.0001$) and is supported by pairwise comparisons that show control males spent less time in the corners compared to stressed males ($p = 0.0008$) but that control females spent more time in the corners compared to controls ($p < 0.0001$) (fig. 3f). The is also matched by sex effects wherein the control males spent more time in the center than control females ($p < 0.0001$) and stressed males spent less time in the center than stressed females ($p = 0.0022$) (fig. 3f).

When excluding males and separating females by ovarian stage, we see effects of both stress and hormone status on avoidant behaviors. We do not see significant effects in the number of entries into the 20cm or perimeter suggesting that this type of stress does not affect exploration in the OFT (fig. 3g and h). In the amount of time spent in the 10cm center, we see an effect of estrous stage where the D (diestrous) females were more willing to spend time in the center overall ($F (1, 53) = 4.276$, $p = 0.0436$) which is supported by a significant pairwise comparison between P/E (proetrous/estrous) stressed females and D stressed females ($p = 0.0286$) (fig. 3i). In the 20cm center, there was an increase in time spent in stressed D females in comparison to their P/E counterparts ($p = 0.0478$) (fig. 3j). In the perimeter, it appears as if only the diestrus females are sensitive to the stress as we see pairwise differences in the D group where stressed mice spent less time there in comparison to controls ($p = 0.0151$) and D stressed females spent less time there than P/E counterparts ($p = 0.0455$), this is also supported by a main interaction effect ($F (1, 53) = 5.195$,

p = 0.0267) (fig. 3k). For corner time, we see a main effect of stress in both groups where the stress decreased the time spent in the corners ($F(1, 54) = 21.20$, $p < 0.0001$), which is supported by pairwise comparisons between P/E control and P/E stressed mice (p = 0.0048), and D control and D stressed mice (p = 0.0008) (fig. 3l). This suggests that hormone status can affect how mice respond to stressful situations and that potentially social stress leads to a decrease in avoidant behaviors in female mice.

3.3.2 EPM

We see much fewer effects in the EPM. In the male/female comparisons, we do not see any effects on the number of entries into the open arms or the amount of time spent in the open arms, closed arms, or ends of the open arms (fig. 4a, d, e, and f). We do see a main effect of stress on the number of entries into the closed arms in which stressed animals had fewer entries ($F(1, 70) = 3.997$, $p = 0.0495$) (fig. 4b), suggesting a decrease in exploratory behavior. There was also a main sex effect in the number of entries into the ends of the open arms where females were more willing to enter ($F(1, 66) = 6.248$, $p = 0.0149$) (fig. 4c). This holds true when excluding the males and comparing by estrous stage. There were no significant differences in the entries to the closed arms or open arms ends, or amount of time spent in the open arms, closed arms or open arms ends (fig. 4 h, i, j, k, and l). There were effects in the entries into the open arms where stressed P/E females had fewer entries into the open arms compared to controls (p = 0.0246) and an interaction effect ($F(1, 46) = 5.491$, $p = 0.0245$) (fig, 4g).

3.3.3 LDB

In the LDB, we only see effects in the females but not the males. We found no effects of sex or stress on the amount of time spent in the light zone (fig. 5c). We did find effects of stress in the females in the number of entries into the light zone ($p = 0.0456$) , number of entries into the transition zone ($p = 0.0375$), and amount of time spent in the transition zone ($p = 0.0443$) (fig. 5a, b, and d). Stress caused a reduction in all these aspects, suggesting an increase in avoidant and exploratory behaviors. There were no effects in the males. Within the females, we see both effects of treatment and estrous stage in the LDB. In the number of entries into the light zone, we see stressed P/E females had fewer entries compared to controls ($p = 0.0074$) (fig. 5e). In the number of entries into the transition zone, we see significant effects where the control P/E females had more entries than both their stressed ($p = 0.0056$) and their D ($p = 0.0463$) (fig. 5f). We see a main effect of treatment on the amount of time spent in the light zone where stressed females spent less time there ($F (1, 54) = 6.378$, $p = 0.0145$) (fig. 5g). There was also a pairwise effect between the control P/E females and their stressed counterparts ($p = 0.0426$) (fig. 5g). We can also see a main treatment effect in the amount of time spent in the transition zone ($F (1, 54) = 4.672$, $p = 0.0351$) (fig. 5h). This suggests that, in contrast to the OFT, that social stress increases avoidant behaviors in females in the LBD.

3.3.4 NSF

In the NSF, we again see both treatment and sex effects. For NSF, we tracked the amount of weight lost during the food deprivation. We found effects in the control females where they lost more weight than their male ($p = 0.0026$) and stressed ($p = 0.0002$) counterparts which also resulted in an interaction effect ($F (1, 83) = 11.83$, $p = 0.0009$) (fig. 6a). In the latency to eat in the novel arena, we see a similar interaction effect as we see in the OFT. We observed that stress caused a

lower latency to eat in males ($p = 0.0242$) but a higher latency in females ($p = 0.0175$) as well as stressed females having a higher latency than stressed males ($p = 0.0001$) resulting in an interaction effect ($F (1, 59) = 10.43$, $p = 0.0020$) (fig. 6b). This is supported by the survival curves which show significant differences in every pairwise comparison. Male controls were less likely to eat than the stressed ($p = 0.0015$) and female ($p = 0.0459$) counterparts (fig. 6c). Additionally, stressed females were less likely to eat than both their control ($p = 0.0208$) and male ($p < 0.0001$) counterparts (fig. 6c). This again suggests that male and female mice respond to chronic social stress in a distinct manner. We did not find any effects on the amount of pellet eaten or the latency to eat in the home cage (fig. 6d and e). However, we found a main sex effect in the probability to eat in the home cage where females were overall less likely to eat ($p = 0.0088$).

When excluding the males, we found effects of stress but not estrous stage. In terms of weight loss, we see a main effect of treatment on the females where the stressed mice loss less weight than the controls ($F (1, 57) = 9.902$, $p = 0.0026$) along with a pairwise effect in the P/E females ($p = 0.0041$) (fig. 6f), suggesting that social stress is making the body protective against weight change. In the latency to eat in the novel arena, we see a main treatment effect where stressed mice had higher latencies ($F (1, 40) = 8.829$, $p = 0.0050$) along with a pairwise effect in the D females ($p = 0.0062$) (fig. 6g). In the survival curve for the novel arena, we see a treatment effect where stressed animals were more likely to eat ($p = 0.0208$) (fig. 6h). There were no effects on the amount of pellet eaten, the latency to eat in the home cage, or the probability to eat in the home cage (fig. 6i, j, and k).

3.2 RNA-Sequencing

In the PCA for the RNA-sequencing data, no data points fell outside the 95% confidence oval showing no outliers (fig. 2a). We can also see that the males and the females are separating out strongly and within sexes the males are separating by treatment, but the females are not (fig. 2a). We found 4162 genes that were differentially expressed in the adBNST according to sex (fig. 2b). 2027 genes are male biased and 2135 are female biased. Important male biased genes include: *Greb1* (log2FC = -2.0408, adj. p < 0.0001), a regulator of estrogen signaling [23]; *Tac2* (log2FC = -0.9777, adj. p = 0.0004), neurokinin B and involved in fear learning and stress [24]; *Mc4r* (log2FC = -0.6527, adj. p = 0.0013), melanocortin 4 receptor and responds to adrenocorticotropin and MSH hormones [25]; *Htr1a* (log2FC = -0.6395, adj. p = 0.0005), serotonin receptor [26]; *Adra2a* (log2FC = -0.5208, adj. p = 0.0005), adrenergic receptor [27]; *Crh* (log2FC = -0.5179, adj. p = 0.0035); *Slc1a6* (log2FC = -0.4621, adj. p = 0.0370), glutamate transporter [28]; and *Gabra3* (log2FC = 0.6295, adj. p < 0.0001), GABA A receptor subunit and is implicated in depression-like behaviors in mice [29]. Important female biased genes include: *Scn4b* (log2FC = 1.5514, adj. p < 0.0001), voltage-gated sodium channels [30]; *Drd2* (log2FC = 1.0257, adj. p = 0.0004) and *Drd1* (log2FC = 0.8513, adj. p = 0.0018), dopamine receptors [31]; *Adora2a* (log2FC = 1.1594, adj. p = 0.0002), adenosine receptor implicated in anxiety [32]; *Camk2n1* (log2FC = 0.7756822, adj. p <0.0001) an inhibitor of CaMKII signaling pathway [33]; *Syt2* (log2FC = 1.2392, adj. p < 0.0001) and *Syt6* (log2FC = 0.7613, adj. p = 0.0053), synaptic vesicle exocytosis [34]; *Inhba* (log2FC = 0.6513, p = 0.0171), a subunit of both inhibin and activin [35]; *Tac1* (log2FC = 0.6117, adj. p = 0.0068), a precursor to substance P and neurokinin A [36]; and *Synpo* (log2FC = 0.5510, adj. p < 0.0001), regulator of synaptic transmission [37]. When looking at the gene ontology, we see that male biased genes are related to RNA processing and protein transportation, whereas female biased genes are related to regulation of the synapse and neurogenesis (fig. 4e and f).

When comparing the males, 171 genes were found to be differentially expressed due to stress (fig. 2c). 59 genes were downregulated due to stress and 112 were upregulated due to stress. Of those affected by stress in males, important ones include: *Hspa1a* (log2FC = 1.4305 adj. p = 0.0049), a heat shock protein and the most upregulated gene [38]; *Sgk1* (log2FC = 0.4428, adj. p = 0.0395), a glucocorticoid induced kinase [39]; *Synj2bp* (log2FC = 0.1675, adj. p = 0.0363), a regulator of activin signaling [40]; *Mcl1* (log2FC = 0.2932, adj p < 0.0001), a regulator of apoptosis [41]; and *Hsf2* (log2FC = 0.1974, adj. p = 0.0040), a heat shock transcription factor important for brain development [42]. There were no gene ontologies significantly altered due to stress. In females, only 44 genes were found to be affected by CSIS with 12 genes downregulated and 32 upregulated. Important stress sensitive genes in the females include: *Bbc3* (log2FC = -0.4071, p = 0.0318), a positive regulator of apoptosis [43]; *P2ry13* (log2FC = -0.3213, adj. p = 0.0324), a purinergic receptor that mediates the acetylcholine post-synapse [44]; *Stx1b* (log2FC = -0.1026, adj. p = 0.0386), a protein related to synaptic transmission [45]; *Hsf2* (log2FC = 0.2048, adj. p = 0.0090); and *Sgk1* (log2FC = 0.8584, adj. p < 0.0001). No gene ontologies were significant in the females. This suggests that the adBNST transcriptome in male mice is more sensitive to social stressors.

4. Discussion

We were seeking to understand how social stress leads to neurophysiological changes that result in behavior changes which resemble mood disorders in humans. This project is seeking to understand how mood disorders develop and how that may be different between different types of stressors. We first analyzed the mice by physiological markers of stress, CORT concentration in

the serum and body weight gain, followed by behavior assays or RNA-Sequencing of the adBNST. This has shown how stress can still have effects on the mice regardless of the presence of physiological markers.

Initial analysis of the BW and CORT concentrations may suggest that CSIS is ineffective in females and has limited effectiveness in the males. CSIS reduced BW gain in the males but not the females, but this was only recorded in one of the cohorts. While BW is often a good predictor of stress in rodents, it is not always accurate and inhibited weight gain is seen most often ins stress paradigms that use more extreme stressors [46]. It is also potentially concerning that we do not find an increase in CORT concentrations after the stress paradigms. Chronic stress should results in an increased basal level of CORT; however, several studies have found that CORT returns to pre-stress levels after approximately 5 weeks [47]. Our paradigm takes place over approximately 7 weeks which may explain why we do not observe effects of stress on CORT.

In our previous publication, we found that a chronic variable mild stress paradigm increased avoidant behaviors in general, However, in females, the sensitivity was dependent on estrous stage as only P/E females were sensitive to the chronic stress, in part, due to D females exhibiting more avoidant without stress [8]. CSIS, however, resulted in differing effects between males and females. Particularly in the OFT and NSF it appears as if the social stress is causing the males to be more avoidant, as expected, but is causing the females to be less avoidant. This suggests that the social stress is in some way protective to the females or alternatively reduces inhibition in them. Few studies have been able to be conducted on the influence of stress on risk taking behavior [48]. This is likely due to it being difficult to model in both humans and animals. Some studies have found that stress's effects on risk-taking in humans is sex-biased with men increasing risk-taking behavior after stress but women decreasing it [49, 50]. If we assume that the

behavioral patterns in the OFT and NSF represent an increase in risk-taking behaviors in the female, this could suggest another avenue by which to explore stress and sex. However, this stands in contrast to previous research that showed that CSIS should be similarly effective in males and females [12]. This may be due to the different analysis styles as they analyzed distance traveled in a region in the OFT, whereas we analyzed by amount of time spent in different regions. These are different aspects of behavior and cannot be directly compared.

When analyzing by estrous cycle, we find that hormone status does affect avoidant behaviors. Previously, we found that CVMS resulted in a very distinct effect where only P/E females appeared sensitive to stress because D females are generally more avoidant regardless of treatment [8]. We find more mixed results with the CSIS where females are sensitive to some aspects, regardless of estrous stage, and that the diestrous females were less avoidant than proestrous/estrous females. This suggests that systemic and processive stress are processed and influenced differently by ovarian hormone production, wherein estrogens can both increase or decrease stressors depending on the conditions of the stress and mouse. This is interesting as estrogens are thought to generally decrease depressive-like symptoms in cisgender women [51]. For example, pre-menstrual dysphoric disorder (PMDD) is a disorder very similar major depressive disorder; however, it only occurs during the transition from the follicular phase to the luteal phase of the menstrual cycle which has high estrogen levels preceding a significant drop in etsrogen [52]. Recently, research in people experiencing perimenopause has found that stress sensitivity in terms of mood is increased by the fluctuations in steroid concentrations and not just in the absence of steroids [53-55]. This is potentially a difference between rodents and humans, or something that needs to be explored further in both rodents and humans.

Considering the adBNST transcriptome, we found a large difference between the control males and control females which implies a strong sex-associated variability in the adBNST. Regarding the gene ontology, it appears as if the male adBNST is primed for modifications in the transcriptome, whereas the female adBNST is primed for synaptic transmission and reorganization. The male gene ontology is quite interesting considering the males display many more genes that are affected by stress than the females, which is also similar to results we found in our transcriptomic data from a recent CVMS experiment [18]. In the females, the gene ontology implies a region that is prepared for synaptic transmissions. Further electrophysiological experiments would provide insight into how signaling may differ between males and females. In our previous research, we had found that chronic stress suppressed the M-current in male adBNST CRH+ neurons but suppressed excitatory post-synaptic potentials in the female adBNST CRH+ neurons [18]. This closely aligns with this gene ontology as the M-current is an intrinsic property that would be affected by transcription, whereas EPSCs are extrinsic and would be affected by synaptic signaling.

For both sexes, it may be important to understand how stress is affecting synaptic activity as both show DEGs that are related to synaptic transmission, like *Synj2bp* in males or *Stx1b* in females. Previous research suggests that in the BNST, chronic stress can significantly alter the synapse, causing some cells, typically the glutamatergic cells, to undergo long-term depression while other cells, typically GABAergic, may have increased synaptic activity [56]. Additionally, apoptotic pathways may need to be investigated more as both males and females showed increased expression of heat-shock and apoptotic proteins, such as *Hsf2* in males and females, *Hspa1a* in males, and *Bbc3* in females. This may also be another avenue to explore the sexual differentiation of the BNST as the heat-shock proteins appear to be upregulated in the stressed males, whereas

they are downregulated in the stressed females. This could provide further insight into why the males and females are responding differently to the stress.

Overall, our study demonstrates that social stress induces different behavioral responses between male and female mice that may be due to the variability in the transcriptomic response to social stress in the adBNST. We have recently demonstrated that chemogenetic activation of CRH neurons in the adBNST reduces motivation and avoidance behaviors with subtle variability across the sexes (Maita et al 2023). Our current study found that adBNST CRH expression is greater in males than females. However, these studies require further exploration as females were seemingly resistant to the social stressors in terms of avoidance behaviors. Future studies will characterize the influence of CSIS on motivational behaviors controlled by adBNST CRH neurons and will characterize the mechanisms underlying sex variability in the adBNST. Further studies will be conducted that will test the electrophysiological properties of adBNST CRH neurons after social stress, testing the strength of the M-current and the excitatory post-synaptic potentials. In summary, we have demonstrated avenues by which stress is differentially regulated between the male and female adBNST and this may be related to the sex-related differences in the diagnoses and prognoses of stress-related mood disorders.

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References

1. Simpkiss, J.L. and D.P. Devine, *Responses of the HPA axis after chronic variable stress: effects of novel and familiar stressors.* Neuroendocrinology Letters, 2003. **24**(1/2): p. 97-103.
2. Watanabe, Y., E. Gould, and B.S. McEwen, *Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons.* Brain Research, 1992. **588**(2): p. 341-345.
3. Kinn Rød, A., et al., *Long-term effects of footshock and social defeat on anxiety-like behaviours in rats: relationships to pre-stressor plasma corticosterone concentration.* Stress, 2012. **15**(6): p. 658-670.
4. Woolley, C.S., E. Gould, and B.S. McEwen, *Exposure to excess glucocorticoids alters dendritic morphology of adult hippocampal pyramidal neurons.* Brain Res, 1990. **531**(1-2): p. 225-31.
5. Krahn, D.D., B.A. Gosnell, and M.J. Majchrzak, *The anorectic effects of CRH and restraint stress decrease with repeated exposures.* Biological psychiatry, 1990. **27**(10): p. 1094-1102.
6. Herman, J.P. and W.E. Cullinan, *Neurocircuitry of stress: central control of the hypothalamo–pituitary–adrenocortical axis.* Trends in Neurosciences, 1997. **20**(2): p. 78-84.
7. Kogler, L., et al., *Psychosocial versus physiological stress - Meta-analyses on deactivations and activations of the neural correlates of stress reactions.* Neuroimage, 2015. **119**: p. 235-51.
8. Degroat, T.J., et al., *Chronic stress and its effects on behavior, RNA expression of the bed nucleus of the stria terminalis, and the M-current of NPY neurons.* Psychoneuroendocrinology, 2024. **161**.
9. Golden, S.A., et al., *A standardized protocol for repeated social defeat stress in mice.* Nature Protocols, 2011. **6**(8): p. 1183-1191.
10. Harris, A.Z., et al., *A novel method for chronic social defeat stress in female mice.* Neuropsychopharmacology, 2018. **43**(6): p. 1276-1283.
11. Takahashi, A., et al., *Establishment of a repeated social defeat stress model in female mice.* Scientific reports, 2017. **7**(1): p. 12838.
12. Yohn, C.N., et al., *Social instability is an effective chronic stress paradigm for both male and female mice.* Neuropharmacology, 2019. **160**: p. 107780.
13. Perez-Villalba, A., et al., *The effect of long context exposure on cued conditioning and c-fos expression in the rat forebrain.* Behavioural Brain Research, 2005. **161**(2): p. 263-275.
14. Beyeler, A. and J. Dabrowska, *Neuronal diversity of the amygdala and the bed nucleus of the stria terminalis.* Handb Behav Neurosci, 2020. **26**: p. 63-100.
15. Xie, M., Y. Xiong, and H. Wang, *The regulative role and mechanism of BNST in anxiety disorder.* Frontiers in Psychiatry, 2024. **15**: p. 1437476.
16. Gegenhuber, B., et al., *Gene regulation by gonadal hormone receptors underlies brain sex differences.* Nature, 2022. **606**(7912): p. 153-159.
17. Allen, L.S. and R.A. Gorski, *Sex difference in the bed nucleus of the stria terminalis of the human brain.* Journal of Comparative Neurology, 1990. **302**(4): p. 697-706.
18. Degroat, T.J., et al., *Chronic variable mild stress alters the transcriptome and signaling properties of the anterodorsal bed nuceleus of the stria terminalis in a sex-dependent manner.* bioRxiv, 2024: p. 2024.11. 11.623087.

19. Dobin, A., et al., *STAR: ultrafast universal RNA-seq aligner.* Bioinformatics, 2012. **29**(1): p. 15-21.
20. Liao, Y., G.K. Smyth, and W. Shi, *featureCounts: an efficient general purpose program for assigning sequence reads to genomic features.* Bioinformatics, 2013. **30**(7): p. 923-930.
21. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.* Genome Biology, 2014. **15**(12): p. 550.
22. Marini, F. and H. Binder, *pcaExplorer: an R/Bioconductor package for interacting with RNA-seq principal components.* BMC Bioinformatics, 2019. **20**(1): p. 331.
23. Mohammed, H., et al., *Endogenous Purification Reveals GREB1 as a Key Estrogen Receptor Regulatory Factor.* Cell Reports, 2013. **3**(2): p. 342-349.
24. Al Abed, A.S., N.J. Reynolds, and N. Dehorter, *A Second Wave for the Neurokinin Tac2 Pathway in Brain Research.* Biological Psychiatry, 2021. **90**(3): p. 156-164.
25. Wei, R., et al., *MC4R in Central and Peripheral Systems.* Advanced Biology, 2023. **7**(9): p. 2300035.
26. Mekli, K., et al., *The HTR1A and HTR1B receptor genes influence stress-related information processing.* European Neuropsychopharmacology, 2011. **21**(1): p. 129-139.
27. Kurnik, D., et al., *Variations in the α2A-adrenergic receptor gene and their functional effects.* Clinical Pharmacology & Therapeutics, 2006. **79**(3): p. 173-185.
28. Kanai, Y. and M.A. Hediger, *The glutamate/neutral amino acid transporter family SLC1: molecular, physiological and pharmacological aspects.* Pflügers Archiv, 2004. **447**(5): p. 469-479.
29. Miller, B.H., et al., *Quantitative trait locus analysis identifies Gabra3 as a regulator of behavioral despair in mice.* Mammalian Genome, 2010. **21**: p. 247-257.
30. Ji, X., et al., *The Sodium Channel β4 Auxiliary Subunit Selectively Controls Long-Term Depression in Core Nucleus Accumbens Medium Spiny Neurons.* Front Cell Neurosci, 2017. **11**: p. 17.
31. Zou, Y.-F., et al., *Association of DRD2 gene polymorphisms with mood disorders: A meta-analysis.* Journal of Affective Disorders, 2012. **136**(3): p. 229-237.
32. Hohoff, C., et al., *ADORA2A variation and adenosine A1 receptor availability in the human brain with a focus on anxiety-related brain regions: modulation by ADORA1 variation.* Translational Psychiatry, 2020. **10**(1): p. 406.
33. Saha, S., K. Datta, and P. Rangarajan, *Characterization of mouse neuronal Ca2+/calmodulin kinase II inhibitor alpha.* Brain Res, 2007. **1148**: p. 38-42.
34. Wolfes, A.C. and C. Dean, *The diversity of synaptotagmin isoforms.* Current Opinion in Neurobiology, 2020. **63**: p. 198-209.
35. Barton, D.E., et al., *Mapping of genes for inhibin subunits alpha, beta A, and beta B on human and mouse chromosomes and studies of jsd mice.* Genomics, 1989. **5**(1): p. 91-9.
36. Chiwakata, C., et al., *Tachykinin (substance-P) gene expression in Leydig cells of the human and mouse testis.* Endocrinology, 1991. **128**(5): p. 2441-8.
37. Zhang, X.L., et al., *Essential role for synaptopodin in dendritic spine plasticity of the developing hippocampus.* J Neurosci, 2013. **33**(30): p. 12510-8.
38. Hunt, C.R., et al., *Chromosomal localization of five murine HSP70 gene family members: Hsp70-1, Hsp70-2, Hsp70-3, Hsc70t, and Grp78.* Genomics, 1993. **16**(1): p. 193-8.
39. Schoenebeck, B., et al., *Sgk1, a cell survival response in neurodegenerative diseases.* Mol Cell Neurosci, 2005. **30**(2): p. 249-64.

40. Liu, Z.H., et al., *Characterization of isoforms of activin receptor-interacting protein 2 that augment activin signaling.* J Endocrinol, 2006. **189**(2): p. 409-21.
41. Reed, J.C., *Bcl-2 family proteins.* Oncogene, 1998. **17**(25): p. 3225-36.
42. Chang, Y., et al., *Role of heat-shock factor 2 in cerebral cortex formation and as a regulator of p35 expression.* Genes Dev, 2006. **20**(7): p. 836-47.
43. Han, J., et al., *Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals.* Proc Natl Acad Sci U S A, 2001. **98**(20): p. 11318-23.
44. Guarracino, J.F., et al., *P2Y13 receptors mediate presynaptic inhibition of acetylcholine release induced by adenine nucleotides at the mouse neuromuscular junction.* Neuroscience, 2016. **326**: p. 31-44.
45. Mishima, T., et al., *Syntaxin 1B regulates synaptic GABA release and extracellular GABA concentration, and is associated with temperature-dependent seizures.* J Neurochem, 2021. **156**(5): p. 604-613.
46. Rabasa, C. and S.L. Dickson, *Impact of stress on metabolism and energy balance.* Current Opinion in Behavioral Sciences, 2016. **9**: p. 71-77.
47. Pałucha-Poniewiera, A., et al., *The influence of the duration of chronic unpredictable mild stress on the behavioural responses of C57BL/6J mice.* Behavioural Pharmacology, 2020. **31**(6).
48. Porcelli, A.J. and M.R. Delgado, *Stress and decision making: effects on valuation, learning, and risk-taking.* Current Opinion in Behavioral Sciences, 2017. **14**: p. 33-39.
49. van den Bos, R., M. Harteveld, and H. Stoop, *Stress and decision-making in humans: Performance is related to cortisol reactivity, albeit differently in men and women.* Psychoneuroendocrinology, 2009. **34**(10): p. 1449-1458.
50. Lighthall, N.R., et al., *Gender differences in reward-related decision processing under stress.* Social cognitive and affective neuroscience, 2012. **7**(4): p. 476-484.
51. Payne, J.L., *The role of estrogen in mood disorders in women.* International Review of Psychiatry, 2003. **15**(3): p. 280-290.
52. Hofmeister, S. and S. Bodden, *Premenstrual syndrome and premenstrual dysphoric disorder.* American family physician, 2016. **94**(3): p. 236-240.
53. Joffe, H., et al., *Impact of Estradiol Variability and Progesterone on Mood in Perimenopausal Women With Depressive Symptoms.* The Journal of Clinical Endocrinology & Metabolism, 2019. **105**(3): p. e642-e650.
54. Süss, H., et al., *Estradiol and progesterone as resilience markers? – Findings from the Swiss Perimenopause Study.* Psychoneuroendocrinology, 2021. **127**: p. 105177.
55. Eisenlohr-Moul, T., et al., *Temporal dynamics of neurobehavioral hormone sensitivity in a scaled-down experimental model of early pregnancy and parturition.* Neuropsychopharmacology, 2024. **49**(2): p. 414-421.
56. Maita, I., T.A. Roepke, and B.A. Samuels, *Chronic stress-induced synaptic changes to corticotropin-releasing factor-signaling in the bed nucleus of the stria terminalis.* Frontiers in Behavioral Neuroscience, 2022. **16**: p. 903782.

Table 1. Top 25 most significant male-biased genes that are not Y-linked ordered by adjusted p-value.

Table 2. Top 25 most significant female-biased genes that are not ordered by adjusted p-value.

Table 3. Top 25 most significant stress-sensitive genes in males ordered by adjusted p-value. A positive log2FC represents an upregulation due to stress. A negative log2FC represents a downregulation due to stress.

Table 4. Top 25 most significant stress-sensitive genes in females ordered by adjusted p-value. A positive log2FC represents an upregulation due to stress. A negative log2FC represents a downregulation due to stress.

Figure 1. Body weight tracking of the mice used for the (A) RNA-Sequencing and (B) behavior cohorts during the CSIS protocol. (C) Concentration of corticosterone in the serum of CSIS-exposed or control mice in the behavior cohorts. Data are presented as mean +/- the SEM and analyzed by a two-way ANOVA with Sidak post-hoc comparison. (* = 0.05-0.01, ** = 0.01-0.001)

Figure 2. 10-minute Open Field Test. (A) Distance traveled. (B) Number of entries into the 20cm center. (C) Number of entries into the perimeter. (D) Percent time spent in the 10cm center. (E) Percent time spent in the 20cm center. (F) Percent time spent in the perimeter. (G) Distance traveled with males excluded and females separated by estrous stage. (H) Number of entries into the 20cm center with males excluded and females separated by estrous stage. (I) Number of entries into the

perimeter with males excluded and females separated by estrous stage. (j) Percent time spent in the 10cm center with males excluded and females separated by estrous stage. (K) Percent time spent in the 20cm center with males excluded and females separated by estrous stage. (L) Percent time spent in the perimeter with males excluded and females separated by estrous stage. Data are presented at mean +/- SEM and analyzed by two-way ANOVA with Šidák post-hoc comparisons (* = 0.05-0.01, ** = 0.01-0.001, *** = 0.001-0.0001, **** < 0.0001).

Figure 3. Elevated Plus Maze. (A) Distance traveled. (B) Percent time spent in open arms. (C) Number of entries into open arms. (D) Amount of time spent at the ends of the open arms. (E) Percent time spent in the closed arms. (F) Number of entries into the closed arms. (G) Distance traveled with males excluded and females separated by estrous stage. (H) Percent time spent in open arms with males excluded and females separated by estrous stage. (I) Number of entries into open arms with males excluded and females separated by estrous stage. (J) Amount of time spent at the ends of the open arms with males excluded and females separated by estrous stage. (K) Percent time spent in the closed arms with males excluded and females separated by estrous stage. (L) Number of entries into the closed arms with males excluded and females separated by estrous stage. Data are presented as mean +/- SEM and analyzed by two-way ANOVA with Šidák post-hoc comparisons (* = 0.05-0.01).

Figure 4. Light/Dark Box Emergence Test. (A) Distance traveled in the light and transition zones. (B) Percent time spent in the light zone. (C) Number of entries into the light zone. (D) Number of stretch attend postures while in the transition zone. (E) Percent time spent in the transition zone. (F) Number of entries into the transition zone. (G) Distance traveled in the light and transition

zones with males excluded and females separated by estrous stage. (H) Percent time spent in the light zone with males excluded and females separated by estrous stage. (I) Number of entries into the light zone with males excluded and females separated by estrous stage. (J) Number of stretch attend postures while in the transition zone with males excluded and females separated by estrous stage. (K) Percent time spent in the transition zone. (L) Number of entries into the transition zone with males excluded and females separated by estrous stage. Data are presented as mean +/- SEM and analyzed by two-way ANOVA with Šidák post-hoc comparisons. (* = 0.05-0.01, ** = 0.01-0.001).

Figure 5. Novelty Suppressed Feeding Test. (A) Amount of weight loss during the 24-h fasting period as a percent of total body weight. (B) Latency to eat in the novel arena. (C) Kaplan-Meier survival curve for the probability to eat in the novel arena. (D) Amount of pellet eaten during test. (E) Latency to eat in the home cage. (F) Kaplan-Meier survival curve for the probability to eat in the home cage. (G) Amount of weight loss during the 24-h fasting period as a percent of total body weight with males excluded and females separated by estrous stage. (H) Latency to eat in the novel arena with males excluded and females separated by estrous stage. (I) Kaplan-Meier survival curve for the probability to eat in the novel arena with males excluded and females separated by estrous stage. (J) Amount of pellet eaten during the test with males excluded and females separated by estrous stage. (K) Latency to eat in the home cage with males excluded and females separated by estrous stage. (L) Kaplan-Meier survival curve for the probability to eat in the home cage with males excluded and females separated by estrous stage. Data are presented as mean +/- SEM. A, B, C, D, G, H, I, and J analyzed by two-way ANOVA with Šidák post-hoc comparisons. E, F, K,

and L analyzed by Log-rank test (* = 0.05-0.01, ** = 0.01-0.001, *** = 0.001-0.0001, **** < 0.0001).

Figure 6. RNA-Sequencing. (A) Principal component analysis (PCA), with ovals representing 95% confidence. (B) Volcano plot comparing control males to control females. A negative fold change represents a male biased gene, and a positive fold change represents a female biased gene. (C) Volcano plot comparing control males to stressed males. A positive fold change represents an upregulation due to stress, and a negative fold change represents a downregulation due to stress. (D) Volcano plot comparing control females and stressed females. A positive fold change represents an upregulation due to stress, and a negative fold change represents a downregulation due to stress. For all volcano plots, a red dot is a significantly differentially expressed gene. (E) Gene ontology for male biased genes when comparing controls. (F) Gene ontology for female biased genes when comparing controls. PCA generated with PCAExplorer, differentially expressed genes analyzed by DESeq2, Gene Ontology analyzed by clusterProfiler.

Table 1.

Gene	log2FC	Adj. P	Chr	Description
Cpne7	-0.747695449	<0.0001	8	copine VII
Plekha7	-0.686338962	<0.0001	7	pleckstrin homology domain containing, family A member 7
Miat	-0.952846153	<0.0001	5	myocardial infarction associated transcript
Sdk2	-0.671151912	<0.0001	11	sidekick cell adhesion molecule 2
N4bp2	-0.69991721	<0.0001	5	NEDD4 binding protein 2
Map3k15	-1.163576395	<0.001	X	mitogen-activated protein kinase kinase kinase 15
Cfap44	-1.045914724	<0.0001	16	cilia and flagella associated protein 44
Ift140	-0.287709946	<0.0001	17	intraflagellar transport 140
Lrguk	-0.596810676	<0.0001	6	leucine-rich repeats and guanylate kinase domain containing
Cfap46	-0.869137704	<0.0001	7	cilia and flagella associated protein 46
Adcy6	-0.702829742	<0.0001	15	adenylate cyclase 6
Cfap251	-0.871820827	<0.0001	5	cilia and flagella associated protein 251
Magel2	-0.77206369	<0.0001	7	MAGE family member L2
Morn1	-0.477301751	<0.0001	4	MORN repeat containing 1
Sytl4	-1.964529824	<0.0001	X	synaptogamin-like 4
Cfap54	-0.864668144	<0.0001	10	cilia and flagella associated protein 54
Slit2	-0.484634548	<0.0001	5	slit guidance ligand 2
Nek10	-0.604449817	<0.0001	14	NIMA-related kinase 10
Fat1	-0.450186736	<0.0001	8	FAT atypical cadherin 1
Zim1	-0.958551243	<0.0001	7	zinc finger, imprinted 1
Scml4	-0.792229718	<0.0001	10	Scm polycomb group protein like 4
Zfp57	-0.75910857	<0.0001	17	zinc finger protein 57
Tub	-0.337969252	<0.0001	7	TUB bipartite transcription factor
Megf6	-0.615353477	<0.0001	4	multiple EGF-like-domains 6
Rabl2	-0.426072979	<0.0001	15	RAB, member RAS oncogene family-like 2

Table 2.

Gene	log2FC	Adj. P	Chr	Description
Xist	10.25628147	<0.0001	X	inactive X specific transcripts
Ddx3x	0.433775769	<0.0001	X	DEAD box helicase 3, X-linked
Kdm6a	0.459777653	<0.0001	X	lysine (K)-specific demethylase 6A
Eif2s3x	0.637129142	<0.0001	X	eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked
Fndc5	0.566591619	<0.0001	4	fibronectin type III domain containing 5
Syt2	1.239232042	<0.0001	1	synaptotagmin II
Sgpp2	0.780963942	<0.0001	1	sphingosine-1-phosphate phosphatase 2
Lynx1	0.554342889	<0.0001	15	Ly6/neurotoxin 1
Hcn2	0.598690551	<0.0001	10	hyperpolarization-activated, cyclic nucleotide-gated K+ 2
Klf10	0.808464379	<0.0001	15	Kruppel-like transcription factor 10
Rcan2	0.282721415	<0.0001	17	regulator of calcineurin 2
Map6d1	0.519422892	<0.0001	16	MAP6 domain containing 1
Ahcyl1	0.201031687	<0.0001	3	S-adenosylhomocysteine hydrolase-like 1
Unc13c	0.821666289	<0.0001	9	unc-13 homolog C
Reep1	0.311958224	<0.0001	6	receptor accessory protein 1
Tmcc1	0.257195029	<0.0001	6	transmembrane and coiled coil domains 1
Adora1	0.442957583	<0.0001	1	adenosine A1 receptor
Pcdhga6	0.556600811	<0.0001	18	protocadherin gamma subfamily A, 6
Cplx1	0.576827171	<0.0001	5	complexin 1
Rgs4	0.995009559	<0.0001	1	regulator of G-protein signaling 4
Gask1b	1.370700569	<0.0001	3	golgi associated kinase 1B
Snap25	0.262722925	<0.0001	2	synaptosomal-associated protein 25
Panx2	0.342352	<0.0001	15	pannexin 2
Lrtm2	0.549880353	<0.0001	6	leucine-rich repeats and transmembrane domains 2
Prima1	0.766476034	<0.0001	12	proline rich membrane anchor 1

Table 3.

Gene	log2FC	Adj. P	Chr	Description
Mcl1	0.29315796	<0.0001	3	myeloid cell leukemia sequence 1
Zcchc2	0.40997949	<0.0001	1	zinc finger, CCHC domain containing 2
Nr4a3	0.92059439	<0.0001	4	nuclear receptor subfamily 4, group A, member 3
P2ry13	-0.5503298	<0.0001	3	purinergic receptor P2Y, G-protein coupled 13
Usp53	0.36735142	<0.0001	3	ubiquitin specific peptidase 53
Tent4a	0.2464837	<0.0001	13	terminal nucleotidyltransferase 4A
Sik1	0.53441266	<0.0001	17	salt inducible kinase 1
Tent5a	0.72376085	<0.0001	9	terminal nucleotidyltransferase 5A
Rbm12b2	0.38537654	<0.0001	4	RNA binding motif protein 12 B2
Lig4	0.3286736	<0.0001	8	ligase IV, DNA, ATP-dependent
Frat1	-0.3910229	<0.0001	19	frequently rearranged in advanced T cell
Jun	0.40278466	<0.0001	4	jun proto-oncogene
Pprc1	0.35270586	<0.0001	19	peroxisome proliferative activated receptor, gamma, coactivator-related 1
Tent4b	0.23199183	0.0003	8	terminal nucleotidyltransferase 4B
Ppp1r3c	0.28014915	0.0003	19	protein phosphatase 1, regulatory subunit 3C
Tsc22d2	0.26271039	0.0003	3	TSC22 domain family, member 2
Zfp800	0.32696821	0.0003	6	zinc finger protein 800
Apold1	0.75953157	0.0003	6	apolipoprotein L domain containing 1
Prdm2	0.16909497	0.0004	4	PR domain containing 2, with ZNF domain
Utp14b	0.38800557	0.0005	1	UTP14B small subunit processome component
Rasa2	0.22333564	0.0009	9	RAS p21 protein activator 2
Sec24a	0.31814465	0.0012	11	SEC24 homolog A, COPII coat complex component
Lats1	0.19101254	0.0014	10	large tumor suppressor
Dedd2	0.43703269	0.0014	7	death effector domain-containing DNA binding protein 2
Spty2d1	0.29510554	0.0015	7	SPT2 chromatin protein domain containing 1

Table 4.

Gene	log2FC	Adj. P	Chr	Description
Apold1	1.27914249	<0.0001	6	apolipoprotein L domain containing 1
Nr4a3	0.98620785	<0.0001	4	nuclear receptor subfamily 4, group A, member 3
Sgk1	0.85838096	<0.0001	10	serum/glucocorticoid regulated kinase 1
Fosl2	0.95050426	<0.0001	5	fos-like antigen 2
Apcdd1	-0.356759	<0.0001	18	adenomatosis polyposis coli down-regulated 1
Tsc22d3	0.49272078	0.0001	X	TSC22 domain family, member 3
Ppp1r3c	0.29428128	0.0002	19	protein phosphatase 1, regulatory subunit 3C
Tiparp	0.5703888	0.0004	3	TCDD-inducible poly(ADP-ribose) polymerase
Ip6k2	0.16488918	0.0008	9	inositol hexaphosphate kinase 2
Trib1	0.39920351	0.0008	15	tribbles pseudokinase 1
Nfkbia	0.48993003	0.0009	12	NFKB inhibitor alpha
Pdk4	0.51809851	0.0020	6	pyruvate dehydrogenase kinase, isoenzyme 4
Trp53inp1	0.34999847	0.0035	4	transformation related protein 53 inducible nuclear protein 1
Plekhf1	0.81147742	0.0035	7	pleckstrin homology and FYVE domain containing 1
Arl4d	0.61557252	0.0064	11	ADP-ribosylation factor-like 4D
Rasd1	0.71913532	0.0064	11	RAS, dexamethasone-induced 1
Plekho2	-0.3594931	0.0082	15	pleckstrin homology domain containing O 2
Zfp667	0.2521297	0.0082	7	zinc finger protein 667
Slc25a25	0.25485917	0.0082	2	solute carrier family 25 member 25
Rrp8	0.34254908	0.0082	7	ribosomal RNA processing 8
Sik1	0.42188588	0.0082	17	salt inducible kinase 1
Sap30	0.53102235	0.0082	8	sin3 associated polypeptide
Arrdc2	0.55131092	0.0083	8	arrestin domain containing 2
Hsf2	0.20479394	0.0090	10	heat shock factor 2
Mat2a	0.19012732	0.0090	6	methionine adenosyltransferase 2A

Figure 1.

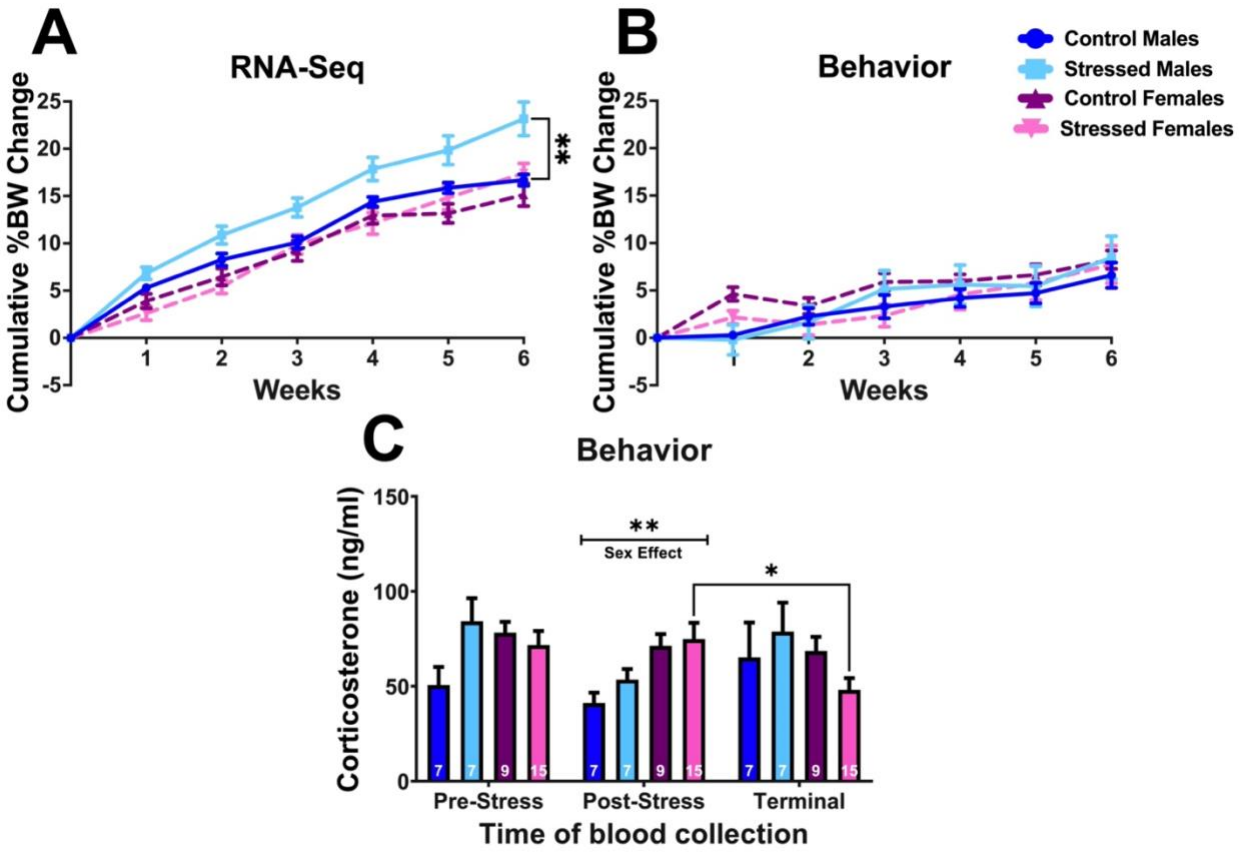

Figure 2.

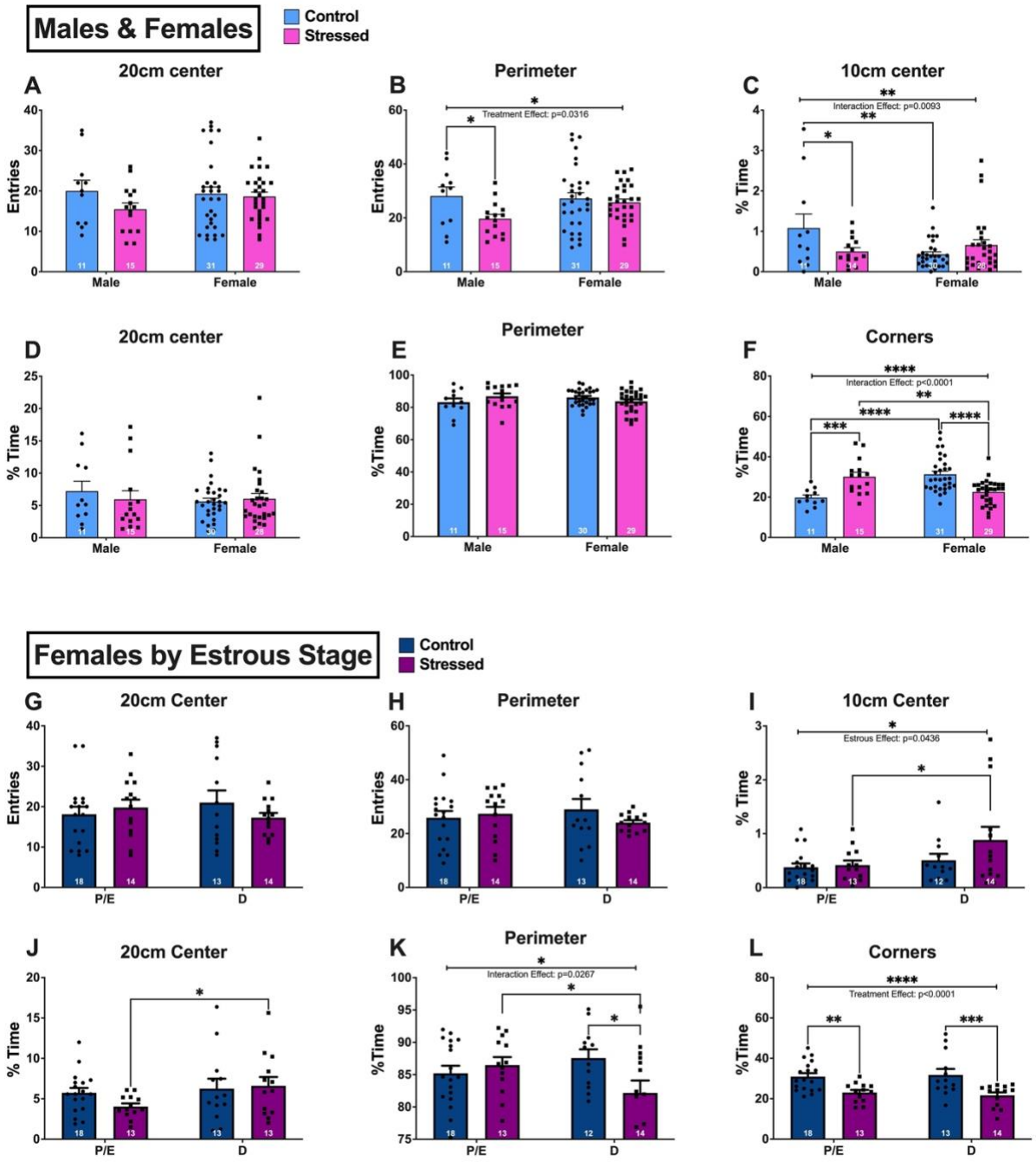

Figure 3.

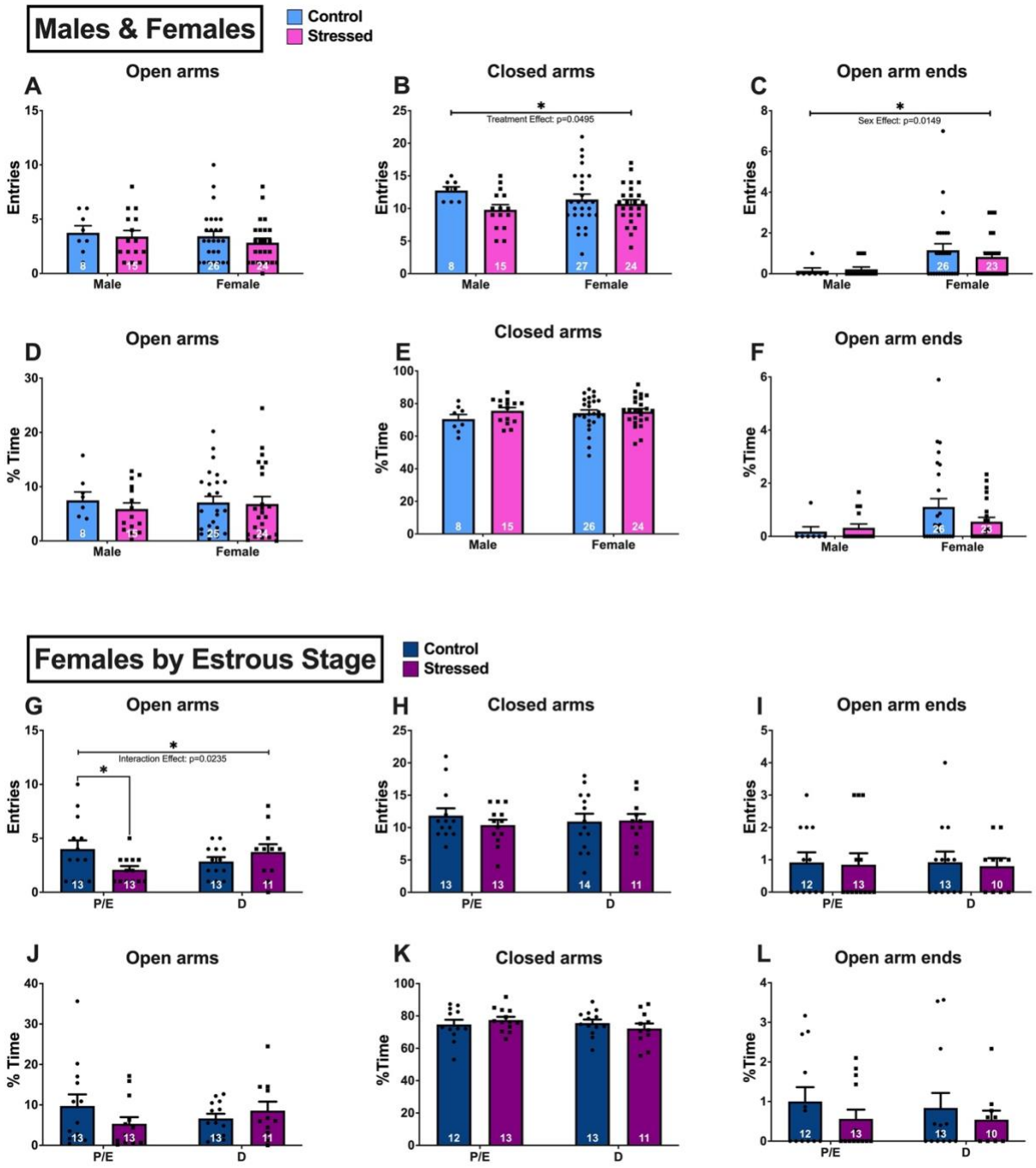

Figure 4.

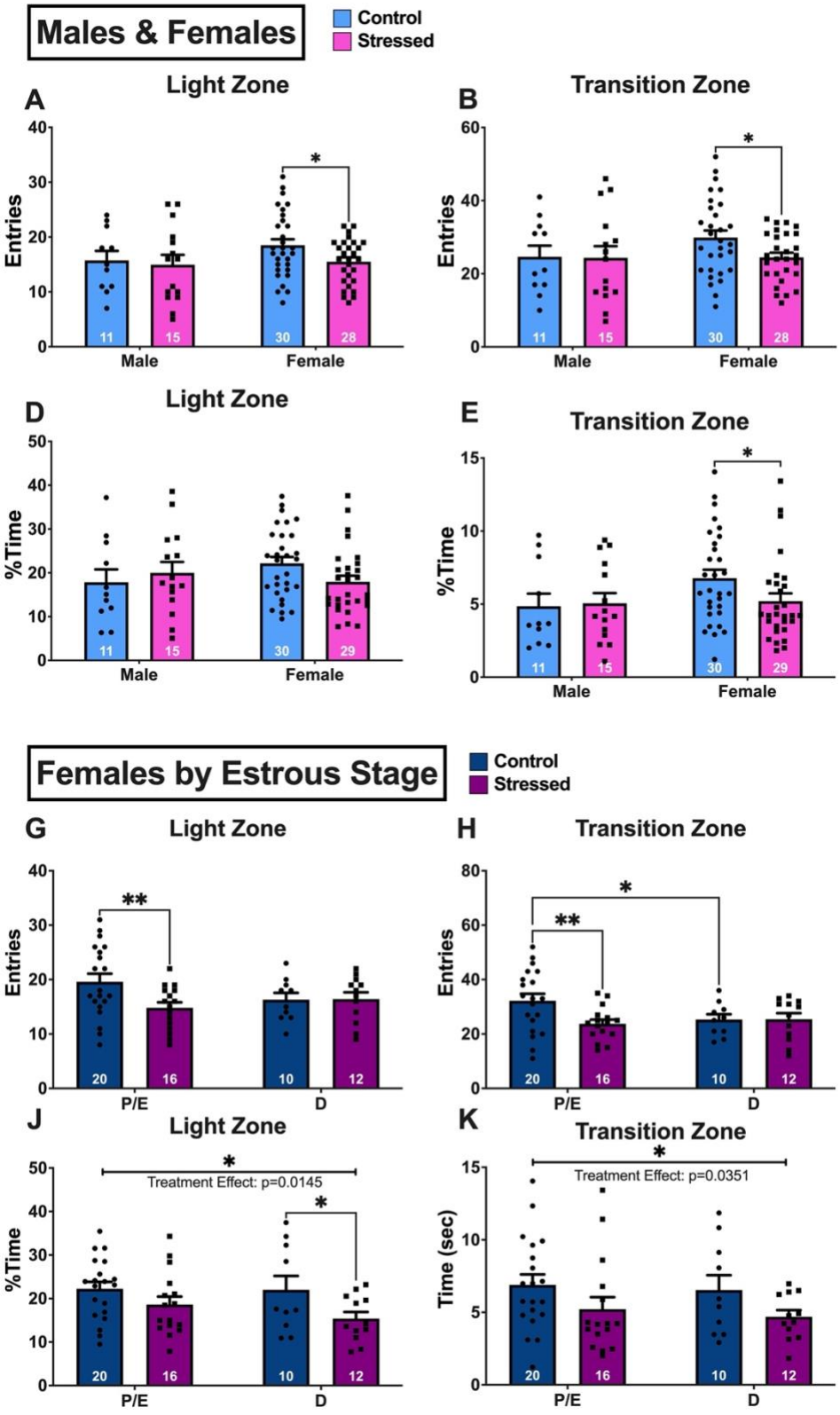

Figure 5.

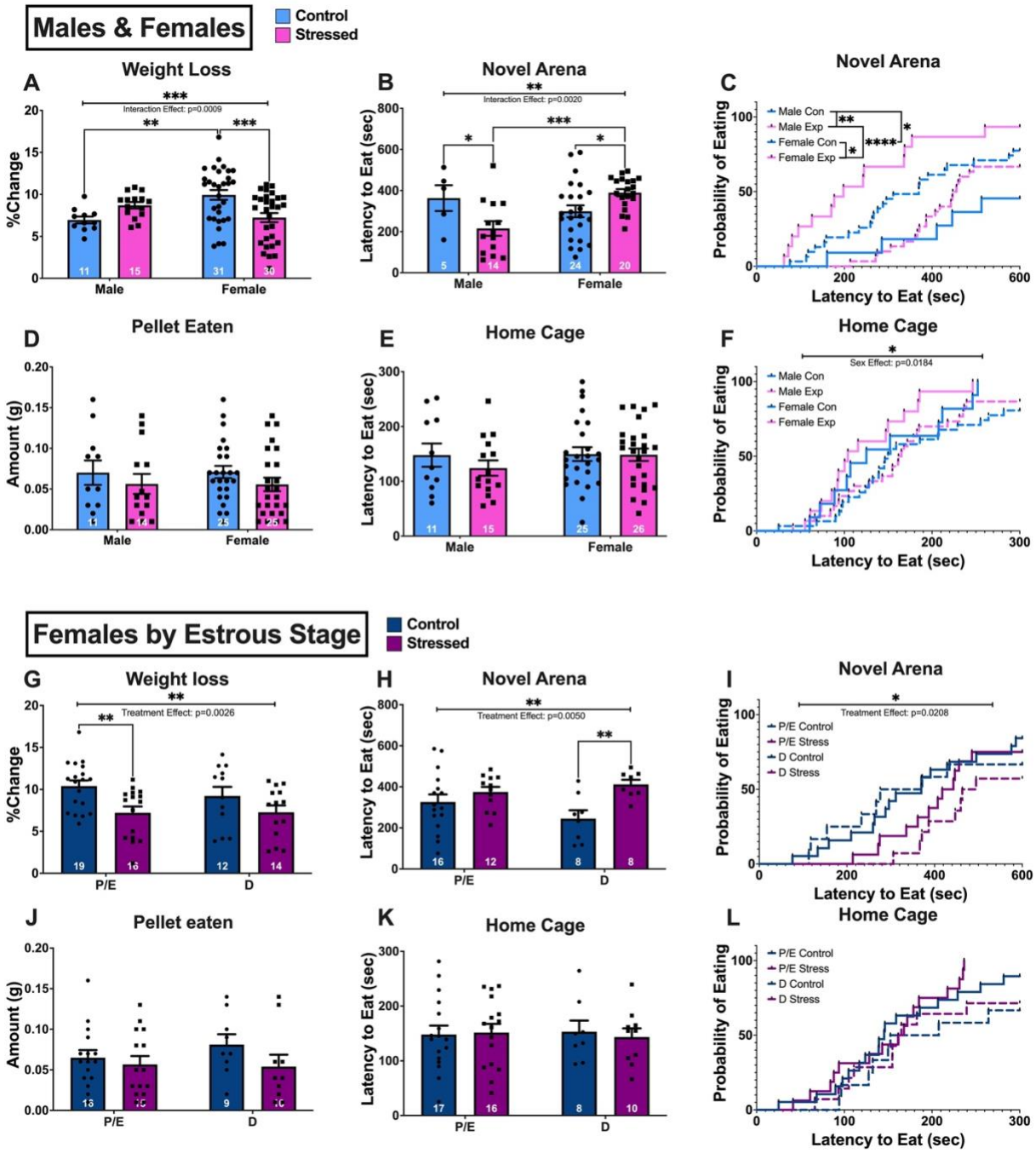

Figure 6.