Does Time Really Tell All?

The Effect of Circadian Rhythms on Emotional-like Behavior in Rats Selectively Bred to Model Mood Disorders

by

Rina R. Joshi

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Advisor: Dr. Megan Hastings Hagenauer

Author Note

Authors include:

Elyse L. Aurbach - Molecular, Behavioral, and Neuroscience Institute, Ann Arbor, MI

Megan Hastings Hagenauer - Molecular, Behavioral, and Neuroscience Institute, Ann Arbor, MI & Department of Psychology, University of Michigan, Ann Arbor, MI

Peter Blandino, Jr. - Molecular, Behavioral, and Neuroscience Institute, Ann Arbor, MI

Sarah Clinton - Molecular, Behavioral, and Neuroscience Institute, Ann Arbor, MI

Angela Koelsch - Molecular, Behavioral, and Neuroscience Institute, Ann Arbor, MI

Stanley J. Watson - Molecular, Behavioral, and Neuroscience Institute, Ann Arbor, MI

Huda Akil - Molecular, Behavioral, and Neuroscience Institute, Ann Arbor, MI

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Abstract

The circadian system is the body's internal clock that generates rhythms over a 24-hour time period. If not properly aligned, our circadian rhythms can affect our mood or emotional state. By studying models of mood disorders in rats, we can better understand the connections between mood and circadian rhythms. This study analyzed existing data on novelty-induced locomotor scores, a measure of anxiety-like behavior, in relationship to time of day in rats. The rats were selectively bred for 40 generations based on their locomotion behavior in a novel environment to amplify two lines of behavior: high-responders (bHRs or low anxiety-like behavior) or low-responders (bLRs or high anxiety-like behavior). Over ten scripts were manually written in MATLAB to analyze and sort 10,833 data points from both male and female bHRs and bLRs. The software R was used for the final linear regression analysis. This study found that phenotype and generation number were related to novelty-induced locomotion (Phenotype: $\beta = 1566.29$, $p < 2x10^{-16}$, Generation: $\beta = -6.4741$, $p < 0.0065$), whereas sex and time of day, independently, showed no significant relationship (Sex: $\beta = -35.7058$, $p = 0.2649$, Time of Day: $\beta = -1.5560$, $p = 0.8964$). However, there was a significant interaction between sex and phenotype ($\beta = 330.9929$, $p < 2.68x10^{-13}$). These results are important because they help to identify variables that could affect emotional behaviors. By understanding how these variables affect emotional behaviors, we may be able to tailor solutions for mood disorders, including depression and addiction, to individual characteristics.

Keywords: mood disorders, circadian systems, anxiety, depression, rats

The Effect of Circadian Rhythms on Emotional-like Behavior in Rats Selectively Bred to Model Mood Disorders

Our daily patterns of sleep, activity, and release of hormones are controlled by the circadian system. The circadian system is the body's endogenous or internal clock that generates rhythms within a 24-hour time period, or cycle length (Hagenauer & Bradley, 2013). If not properly aligned, our circadian rhythms can affect our mood or emotional state. Mood disorders, a broad category of illnesses that affect the underlying emotional state of a person, have a lifetime prevalence of 20.8% and range from major depressive disorder (MDD) to bipolar disorder (DSM-IV-TR). By studying models of mood disorders in rats we can further understand the connections between mood and circadian rhythms. With such a high prevalence rate and a strong impact on societal functioning, understanding the connection between circadian rhythms and mood disorders is essential for the promotion of communal welfare.

The circadian timekeeping system is responsive to daily time cues (such as sunlight), also known as zeitgebers, allowing the endogenous rhythm of our bodies to synchronize, or entrain, to a 24-hour solar day (Gorman & Lee, 2002, Hagenauer et al., 2013). One important aspect of circadian rhythms is their free-running period. The freerunning period, τ, is defined as the length of the daily cycle under constant conditions, or those lacking environmental cues. To entrain the cycle to a 24-hour solar day, circadian clocks reset in response to light or dark (Gorman et al., 2002). Therefore, the circadian clock will shift to an earlier or later phase in response to a realignment of the 24-hour light:dark cycle. For example, whenever an individual travels from New York to India, their clock will initially be out of phase with the local time in India. As the internal clock readjusts to environmental cues, the individual may experience symptoms of jet

lag, which are a result of desynchrony, or uncoupling, between behavioral and psychological rhythms (Gorman et al., 2002).

Research has shown that the brain region most closely associated with circadian rhythms is the suprachiasmatic nucleus (SCN), which is a tiny region of the hypothalamus that lies at the base of the brain (Hagenauer et al., 2013). Individual cells in the SCN express genes responsible for our internal clocks. These genes, associated with circadian rhythms, are collectively referred to as "clock genes." A few genes that make up this list are Bmal1, Clock, Period (Per1, Per2, Per3), and Cryptochrome (Cry1, Cry2) molecules. These genes generate transcription-and translation based feedback loops with a periodicity of roughly 24 hours (Dunlap, Loros, & DeCoursey, 2004). CLOCK and BMAL1 activate the transcription of a family of PER and CRY genes. PER and CRY are the negative elements, which encode proteins that block expression of PER and CRY and interfere with BMAL1 production (Dunlap et al., 2004).

Several studies have demonstrated the connections between circadian rhythms and emotional behavior in both humans and rats. In one very famous study, human subjects were isolated from all possible factors that could act as zeitgebers by being placed in an underground bunker for approximately 3-4 weeks. The men, most of whom were college students and eager to be isolated in order to study for final exams, were asked to lead "regular lives" while underground. Rectal temperature and urinary metabolite rhythms were measured throughout the experiment as a means for gauging the endogenous clocks. This experiment proved vital in learning more about circadian rhythms because it became evident that human bodies do not need to rely solely on the light/dark to create a rhythmic cycle. They will create a cycle endogenously that will be constantly repeated. In addition, this study showed how rhythmic functions that become out of phase, or desynchronized, have a major impact on mood, as seen in the journal

entries by several subjects. While in isolation, one subject had sleep rhythms with a very long mean period of 32.6 hours, but maintained a normal period for all other bodily functions of 24.7 hours. Therefore, the subject's functions drifted in and out of phase. He noted in his diary that he felt very 'fit' whenever all functions were in phase. However, the subject did not feel as 'fit', whenever his sleep was out of phase with other physiological rhythms (Aschoff, 1965). This illustrates that when circadian rhythms are aligned, the bodies' emotional state is content and healthy.

More recently, studies have shown that disruptions in circadian gene expression are characteristic of MDD (Edgar & McClung, 2013). It was found that MDD is accompanied by a desynchronization between clock gene rhythms in the brain and the day/night cycle, demonstrating that MDD patients have both time-shifted rhythms and disrupted regulation of cyclic genes (Edgar et al., 2013). Understanding the relationship between abnormal phasing of circadian rhythms and MDD could be the next step in finding the right treatment for readjusting the clock. Stabilization between endogenous clocks and zeitgebers is important for mood stabilization in both MDD patients and manic patients (Roybal et al., 2007). Therefore, it is vital in the study of circadian rhythms to understand the connection between mood disorders and the desynchronization of endogenous clocks in order to tailor the right treatments for individual patients.

Animal models have proven useful in studying the interactions between mood disorders and circadian rhythms due to the ability to control certain factors, such as genotype and stress exposure (Nestler & Hyman, 2010). For example, a study showed that disruption of the CLOCK gene in mice induces mania-like behavior. This mania-like behavior, was similar to human mania and included hyperactivity, decreased sleep, lowered depression-like behavior, and lower

anxiety (Roybal et al., 2007). For example, in the forced swim test, it was found that CLOCK mutant mice experienced reduced helpless-like behaviors, and in the learned helplessness test they showed fewer escape failures. Therefore, a mutation of the CLOCK gene causes increased mania-like behavior and decreased depressive-like behavior (Roybal et al., 2007).

Historically, mood and anxiety disorders have been difficult to model in animals because they have a complicated pathophysiology arising from both genetic and environmental factors. In animal models, individual tests are geared at looking at specific aspects of either anxiety-like or depressive-like behavior. In our lab, two lines of rats, “high responders” (bHRs) and “low responders” (bLRs), have been selectively bred for behavior in one such anxiety-like behavioral test. Characterizing these selectively bred strains of rats, both behaviorally and neurobiologically, gives clues to the genetics behind mood and anxiety disorders. High responders (bHRs) correspond to rats that show a high locomotor response to novelty and have low anxiety. Low responders (bLRs) refer to rats that show a low locomotor response to novelty and have high anxiety. Both extremes vary in several basal and maternal behaviors, such as the elevated plus-maze (which is a measure of anxiety-like behavior), forced swim test (which is a measure of depression-like behavior), addiction-like behavior, and maternal behavior during lactation (Stead et al., 2006).

bHR and bLR rats differ in circadian as well as emotional behavior. Similarly, bLR and bHR rats show differing rhythms in CLOCK gene expression (Kerman et al., 2011). It was found that bLR rats expressed an increase in CLOCK towards the evening within the SCN, whereas bHR expressed an increase in CLOCK towards the morning hours (Kerman et al., 2011). These differences could be caused by an alteration in the length of the circadian cycle (Kerman et al.,

2011). These studies indicate that the circadian functioning in rodents that are behaviorally low anxiety-like (bHR) are innately different from those that are high anxiety-like (bLR).

In addition to seeing bHR and bLR differences in the expression of CLOCK genes, behavioral data has shown differences in daily locomotor rhythms. Male rats were tested for diurnal rhythms in homecage locomotor scores. The homecage locomotor testing showed that bHRs were more active than bLRs. Most of the animals both bHR and bLR – were most active during the dark phase (nocturnal). However, the majority (80%) of bHR animals had their peak activity early in the dark phase (ZT2 and ZT18), whereas the bLRs either peaked really early (ZT2-ZT18) or really late (ZT18-ZT24) during the dark phase. This experiment indicated that bHR/bLR behavioral differences may represent a divergent circadian profile in addition to differences in baseline locomotor activity. Since previous studies in mice indicate that altering the CLOCK gene changes emotionality, these circadian differences could exacerbate emotional differences between the bLR/bHR animals (Kerman et al., 2011).

Similar to the differences in behavioral rhythms seen in bHR/bLR rats, human patients that are depressed have different behavioral rhythms than healthy individuals. Numerous studies conducted in humans have found that two independent mood variables, positive affect (PA) and negative affect (NA), vary with time of day (Nicolson, 2006). Positive affect is defined by enthusiasm, delight, and activeness, while negative affect is associated with feelings of sadness, distress, and disgust (Courtet & Olie, 2012). One study tested NA and PA separately to examine the prevalence of both variables in depressed and non-depressed individuals. They found that the average reported PA level was lower in depressed patients than healthy patients, but it followed a linear increase throughout the day in depressed individuals. In depressed patients, NA had increased diurnal variation and peaked in the morning and decreased over the course of the day

compared to healthy participants (Peeters, Berkhof, Delespaul, Rottenberg, & Nicolson, 2006). Similar results were found in another study, which tested categorically depressed patients and defined them either as highly depressed or lowly depressed. Highly depressed patients were defined as receiving a score of above 23 or above on the Center for Epidemiological Studies-Depression Scale (CES-D). The CES-D is a 20-question survey used to detect depression symptoms in subjects. The lowly depressed participants looked similar to healthy controls. Highly depressed patients had a low PA once awoken, which progressively increased until afternoon, where it began to decline (Murray, 2006). This pattern suggests that the circadian system is disrupted or misaligned greatly when the patient is in a depressive state (Murray, 2006). In another study that observed over 509 million Twitter messages, from 2.4 million people, in 84 countries, it was found that PA was high in the morning, then decreased mid – morning (right before work) and increased in the evening (end of work; Golder & Macy, 2011). However, the shape of the affective cycle was not simply due to the workday because it was similar on weekends, but with a two hour delay. This pattern shows that sleep and the biological clock are important in determining affect, regardless of environmental stress (Golder et al., 2011).

Not as many studies on circadian rhythms in mood and emotional behavior have been conducted in rats. One study examined sex differences in relation to circadian rhythms in rats with anxiety and depressive behavior, but there was a confounding light variable (Verma, Hellemans, Choi, Yu, & Weinberg, 2009). The rats were tested under white light during the day and red light at night. Since white light makes nocturnal rats more anxious, it is inconclusive whether the rhythms in depressive- and anxiety-like behaviors that they observed were endogenous or if they were a reaction to the lighting. In another experiment that tested rats using

the forced swim test during different times of the day was limited by the same light confound (Kelliher et al., 2000). In addition, another confounding variable was sex. In the experiment, only males were tested making it difficult to compare the differences between males and females. However, a study by Chaudhury and Colwell (2002) was performed under constant conditions. This experiment tested the effects of circadian rhythms on learning and memory in fear conditioned mice. The researchers compared whether the mice acquired conditioning better in the morning or night. It was found that performance and recall were greater for the mice that were conditioned during the day than night. Loss of conditioning was greater in the mice that were conditioned at night (Chaudhury et al., 2002).

Several gaps remain in the study of circadian rhythms and mood disorders in rats. Most importantly, none of the previous animal studies have analyzed an animal model of mood disorder to determine whether depressed or anxious animals have altered emotional rhythms that are similar to human depressed subject. Additionally, it is vital to not only further investigate the effects of mood disorders on circadian rhythms but to also analyze the differences between males and females. In a behavior study conducted by Kerman et al. (2012), only male rats were tested. However, the results should differ if both sexes were tested because males have different circadian rhythms than females. Furthermore, in humans, chronotype, or the phasing of the internal clock relative to the solar day, is dependent on both sex and age. Children are early chronotypes, meaning they wake early and sleep early. Their chronotype progressively gets later during pubertal development, with the maximum occurring around age 20. Typically females reach their peak earlier than males and are more morning-type overall. These differences between chronotypes of males and females continue until females reach menopause, after which the difference disappears (Roenneberg et al., 2004). In other species (hamsters, rats, mice,

humans, and degus), freerunning rhythms and stability of entrainment of males and females significantly differ. Females maintain a stable pattern of activity, while males recover from phase-shifts with ease. These sex differences owe to the fact that males and females have different steroid hormones being released from the gonads (Gorman et al., 2002). The removal of gonads, or activational hormones, eliminates the differences between males and females.

Knowing this information, we propose to examine differences between male and female bHRs and bLRs within daily rhythms in novelty-induced locomotor behavior (Kerman et al., 2010). The bHR/bLR rats that are in the lab have been selectively bred over 40 generations. Due to the in-breeding, the high responder traits and low responder traits have become even more extreme, making it easier to identify bHRs from bLRs. We hypothesize that as the generations of inbreeding progress, bLR locomotor scores will be low, no matter the time of day, and bHR locomotor scores will peak in the morning, decrease in the afternoon. We also expect to see higher sex differences amongst the bHRs, and not as much in the bLRs. We will include data from 29 generations in our analysis, with 10,833 data points being analyzed.

Method

All experiments were approved by the University Committee on the Use and Care of Animals at the University of Michigan and were conducted in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals, dictated by the National Research Council in 1996.

Animals

Several generations of male and female bHR and bLR Sprague Dawley rats were bred and acquired in-house. The original population began with purchasing 60 males and 60 females from three separate Charles River Laboratory breeding colonies. Upon arrival, all animals were

separated based on their locomotor response to novelty. Males and females with the highest and lowest scores were bred together to create the first generation of bHR and bLR lines. Each successive generation encompassed twelve litters, and we tested every adult male and female for novel locomotor response.

Breeding pairs of animals that were derived from separate colonies initially limited inbreeding. For every litter a viable or "best" female and male are chosen to breed for the next generation. In the chance that one female did not get pregnant, a second female who was also mated was chosen as a back up.

For breeding purposes, one male and one female were housed together for a single week. Females that were pregnant were pair housed until gestational day 18, after which they were singly housed again. All litters were culled (reduced) to 12 pups, 6 males and 6 females, if possible, on postnatal day 1 and then raised by the mother. More than 12 pups were never kept since the dam only has 11-12 teets to feed the pups. Animals were pair-housed and food and water were available ad libitum (Kerman et al., 2011). Behavioral testing for locomotor response to a novel environment took place when the rats were between 45-60 days old (P45 – P60). At this age the rats were still adolescents. The females would have started showing secondary sex characteristics and ovulating between P29-P36, and the males would just be starting to show some of their secondary sex characteristics between P37-P49 (Hagenauer et al., 2011). It is important to note that the onset of MDD is prevalent in late adolescence in humans, and therefore late adolescence is an appropriate time to examine circadian rhythms in emotional behavior in animals selectively-bred to model mood disorder (DSM-IV). Prior to testing, female animals were not screened to see if they were undergoing estrous during testing.

All male rats were housed in a 12:12 light - dark cycle (lights on at 6 a.m./Zeitgeber Time (ZT) 0, and lights off at 6 p.m./ZT12) from November through March. During this same time period, all females had lights on at 4 a.m. and lights off at 6 p.m. March through November, all males were kept on a 12:12 light-dark cycle, with a 7 a.m. lights on and 7 p.m. lights off policy, females had lights on at 5 a.m. and lights off at 7 p.m. The clock shifted for daylight savings time, which was accounted for through an automatic, computerized process.

Screening for Locomotor Response to Novelty

Animals from the bHR/bLR lines were all screened to analyze novelty induced locomotor activity. With no prior exposure to the testing site, "rats were individually placed in standard clear acrylic cages (43 cm x 21.5 cm, 25.5 cm high) equipped with infrared photocell emitters mounted 2.3 and 6.5 cm above the floor to record horizontal and rearing movement", which were placed in a separate location from the housing quarters (Kerman et al., 2011). Horizontal and rearing (vertical) movements were monitored in 5 min intervals over a period of 60 min via a computer (Kerman et al., 2011). Cumulative data collected after all 60 minutes encompassed both the horizontal and rearing scores for the total testing time. Up to 18 animals were tested simultaneously, with males and females being tested on separate days. All males and females from a single litter were tested at the same time, and if possible HR and LR bred pups were tested simultaneously (Stead et al., 2006). Rats were tested between the hours of 7 a.m. – 2 p.m., depending on the generation. Most generations were tested between 9 a.m. – 12 p.m.

Generation Selection

Based on our analysis of locomotor scores, divergence between the two-bred lines was not seen until generation 12 (for definition of divergence, see description in "Labeling LRs & HRs"). Therefore, data was discarded from generations 1-11 because bLRs were not clearly

distinguishable from bHRs. Generation 40 is the most recent generation from which colony data was collected. Therefore, this experiment contains data from 29 generations, specifically generations 12-40.

Importing All Data

Once locomotor data collection had finished, all spreadsheets were saved as separate excel files based on generation, sex, and litter. Manually, all folders were reorganized by generation number, then by sex, then by number of files present in each folder. In total, there were 29 generations, with approximately 12 excel files in each folder (separated by sex), and approximately 200 data points in each excel file. A MATLAB code was created to compile all of the separate excel files into one cell array, where all the data points could be accessed. The code inserted all of the raw data that was necessary, such as the locomotor data, unique identifier code, time, time interval, and cumulative lateral and cumulative rearing scores. However, the code only included the data gathered in interval 12 (at the end of the 60 minute testing since it included all cumulative data) for all animals. An issue arose when the time column was listed as a decimal in the cell array and not as standard military time. Therefore, another code was created called *Time Conversion* that was used to convert the decimal time into an hour and minute time (specifically military time). For statistical analysis, the hour, not the minute, was used when binning generations/animals together.

Using a “for loop”, we were able to tailor the code to recognize the “string” or name of the folder, as male or female and generation number and then inserted two new columns into the cell array, labeling the animal as male or female and providing its specific generation number. We did the same for the *Time Conversion* function by creating a “for loop” and a new column. The last “for loop” that was created combined the cumulative lateral and cumulative rearing

scores for all animals into a cumulative locomotor column. Not that only this column was used as the dependent variable, not the individual rearing and lateral score columns.

Labeling bLRs and bHRs

Once all the data were imported into a cell array, we had to confirm that the animals that were bred as bHRs and bLRs had locomotor scores that fit their phenotype. We used several strategies to do this. We first chose to use a function called *k-means*, which finds data clusters and uses that information to find the centroid, or center, of the clustered data. In this case, *k-means* took the cumulative locomotor scores for all animals and found that the data had two centroids, and therefore, two clusters. The two clusters were bHR and bLR locomotor scores, respectively. Using *k-means*, we divided all the data into a bHR cell array (bHR cluster) and bLR cell array (bLR cluster). In the earlier generations, from generation 12 – 20, there were a few outliers or animals bred to bHR or bLR lines that had abnormal locomotor scores. The outliers were removed by creating another code that analyzed the unique identifier, which either began with an H or L, as that was assigned to every animal. After the function *k-means* ran, a "for loop" was used to cross-reference the bHR and bLR groups with the unique identifiers. If there was an animal that was grouped in with the bHRs, but had an L listed in unique identifier, or vice-versa, it was removed entirely from the sample. Overall, there were approximately 40 outliers between generations 12 – 20 out of a total of 10,833 rats.

Generating Graphs

Several types of graphs were generated based on the need to analyze the data in multiple manners. All of the analyses/graphs that were generated were separated based on sex. The first type of graph created was a histogram, with an x-axis of "time of testing" and y-axis of "count" for each phenotype and sex. These graphs were used to initially determine whether the time of

testing differed by sex, phenotype, or generation. The graph binned all generations together and was created for male and female bLRs and male and female bHRs. The purpose of this graph was to find a correlation between time of day and phenotype or sex. Another histogram generated had the same x and y-axis as before, but generations were split and binned by fours, for example generations 12 – 15 were grouped together and graphed. The purpose was to see if there was a phenotype and time of day correlation across generations. A third type of graph that was created was a bar graph, which averaged locomotor scores across all generations. This graph was used to examine time of day against the average locomotor score for each phenotype and sex. In addition, a line graph to analyze locomotor activity in relation to sex and phenotype was also used to similarly analyze this data. Lastly, in order to accurately analyze the locomotor data for time of day effects and remove any large changes due to generation, median centered graphs were created in which the median for the cumulative locomotor score was calculated for each generation. Once the median was determined, it was subtracted from every score in the generation and then graphed against time of day. This method was used to see how much the locomotor scores, for each generation, differed from the median at different times of day. It also removed any large generational differences that may have skewed the results.

Statistical Comparisons

We statistically analyzed our data using a linear regression model. We looked at four independent variables: sex, time of day, generation, and phenotype. The dependent variable was locomotor score. The following equation was used to analyze individual variables, as well as interaction terms within the data set:

Locomotor Score ~ Intercept + Phenotype (HR vs. LR) + Gender (M vs. F) +

*Binned Generation + Time of Day + Phenotype*Gender +*

*Phenotype*Generation + Gender*Generation + (Time of Day)*Phenotype + (Time of Day)*Gender (M vs. F) + (Time of Day)*Generation + (Time of Day)*Phenotype*Gender + (Time of Day)*Phenotype*Generation + (Time of Day)*Gender*Generation + Phenotype*Gender*Generation + (Time of Day)*Phenotype*Gender*Generation.*

Although this model is complex, we felt confident that we had sufficient power to evaluate each term because the final data set included over 10,000 data points. Similarly, visual inspection of the data suggested that a linear model was appropriate for modeling time of day effects across this time period.

Results

In total 5,664 bLRs and 5,169 bHRs were analyzed for this experiment. When subdivided by sex, there were 3,211 male bLRs, 2,453 female bLRs, 2,683 male bHRs, and 2,486 female bHRs. Overall, our data fit the model very well: $R^2 = 0.4365$, $F(15, 10{,}817) = 558.6$, $p = 2.2 \times 10^{-16}$.

In our investigation, only two independent variables, phenotype and generation number, showed an effect on the dependent variable, locomotor score (Phenotype: $\beta = 1566.29$, $p = 2 \times 10^{-16}$, Generation: $\beta = -6.4741$, $p = 0.0065$; Figures 1 and 2). In terms of phenotype, bHRs showed a higher locomotor score than bLRs, where the differences in scores increased as generation number increased. Statistically there was a significant interaction between the effects of phenotype and generation ($\beta = 37.4375$, $p = 2 \times 10^{-16}$) on locomotor score. A possibility for this finding could be that as the generations increased, the variability within phenotypes decreased, causing bLRs to become more anxious and vice-versa for bHRs. As seen by the line graph (Figure 1), bHRs (males and females) increase in locomotor score as generation number

increased, whereas bLRs (males and females) decreased, at a very similar rate, as generation number increased. The bLRs seemed to bottom-out around generation 40, but bHRs continued to increase, with the female locomotor scores increasing quicker than the male locomotor scores. Indeed, there was a significant interaction between phenotype and sex ($\beta = 330.9929$, $p = 2.68 \times 10^{-13}$), which can be seen in most of the figures, but most clearly in the line graph (Figure 1). However, a significant interaction between sex x generation x phenotype was not seen ($\beta = 6.3865$, $p = 0.2643$). In addition, sex also did not show a significant effect on locomotor score ($\beta = -35.7058$, $p = 0.2649$).

Initially when examining the average locomotor score bar graphs for all generations, it appeared that there might have been an effect of time of day on locomotor score that differed by sex and phenotype (Figures 2 and 3a-d). However, after analyzing the average locomotor score bar graphs binned by generation, there was no consistent effect of time of day. Therefore, we decided to median-center the locomotor scores to remove any generational differences and any outliers (any major locomotor scores causing the average to severely shift) to see if a subtle relationship between time of day and locomotor score existed (Figures 4a-d). While there seems to be a decreasing trend, where bHRs are more active in the morning than the evening (most easily seen with males), time of day did not have a significant impact on locomotor score ($\beta = -1.5560$, $p = 0.8964$). There was also no relationship between time of day and locomotor score across all generations for each phenotype ($\beta = -1.9558$, $p = 0.3598$). The appearance of the time of day effect in the original bar graphs that averaged locomotor score across all generations is most probably due to differences in the distributions of testing times, as illustrated by the histograms. Histograms were created to reflect the frequency of testing times across the day (each histogram was divided by sex, phenotype, and generation; Figures 5a-d). It is clear from

examining these histograms that the rats were tested at different time points during different generations.

To conclude, our results showed that phenotype, generation, and sex tend to have the largest impact on locomotor score for rats that were bred for anxiety-like traits.

Discussion

Our hypothesis that as the generations of inbreeding progress, the difference between bLR locomotor scores and bHR locomotor scores would increase, no matter the time of day, was supported by the results of our study. The hypothesis that bHR locomotor scores would peak in the morning and then decrease in the afternoon, and that bLR locomotor scores would peak later in the day was not supported by our results. More generally, our hypothesis that time of day, would affect locomotor score was not supported by our results. In addition, our third hypothesis, that we expected to see a higher sex difference amongst the bHRs than the bLRs, was supported by our results.

Previous studies have shown an effect of time of day on both positive and negative affect in depressed patients (Golder et al., 2011, Murray, 2006, Peeters et al., 2006). Our results showed no correlation between locomotor response to a novel environment and time of day in rats bred for different emotional behaviors. However, other animal studies have shown a relationship between circadian rhythms and emotional behavior, such as Verma et al. (2009) and Kelliher et al. (2000). Verma et al. (2009) conducted behavioral tests, such as open field and elevated plus maze, in outbred rats during light and dark phases of the day. They found that both circadian phase and sex significantly influenced behavioral responses to stress. Kelliher et al. (2000) examined rat behaviors in the forced-swim test under both light (diurnal) and dark (nocturnal) conditions. They found that rats adapted to stress better during the day than at night.

Although, these studies had light as a confounding variable, their results show that time of day may be important for the study of mood disorders in animal models. If our study had tested rats throughout the day (both morning and evening) and controlled for light variables (light and dark cycles) we might have seen similar results. Studies analyzing differences in phenotypic behavior, such as Kerman et al. (2011), similarly found that bHRs were more active than bLRs. In addition, the Kerman et al. (2011) study also found that circadian functioning in rodents that are behaviorally less anxious (bHR) are innately different from those that are more anxious (bLR). Although we did not see differences in the timing of locomotor behavior due to phenotype, we did see that phenotype, itself, was significant.

Our results suggest several things about circadian rhythms and mood disorders. The first is that studying circadian rhythms and emotional behavior in rats may vastly differ from the study of circadian rhythms affect in humans. The concepts of positive and negative affect are not easily represented in rats since we do not have the ability to measure what they subjectively feel. Although several studies have been conducted in humans to understand the patterns that positive and negative affect presents in depressed patients, it is hard to reliably state if this type of understanding can be applied to rats. We are only able to assess emotionally driven responses based on behavior. Studies conducted by Verma et al. (2009), Kelliher et al. (2000), and Chaudhury et al. (2002) have all used other behavioral tests to measure emotional behavior and daily rhythms in rats. These tests have included the elevated plus maze, open field test, forced swim test, and sucrose preference test. By using different anxiety- or depression-like behavioral tests, such as in the open field and elevated plus maze, we may see a more significant relationship between locomotor scores and time of day, and possibly even sex. In addition, different emotional behavioral tests, such as forced swim test and sucrose preference, have also

been shown to differ in both male and female rats (Verma et al., 2009). If these tests were added to our experiment, we may be able to see a significant effect of both sex and time of day.

Our study does show that bHR/bLR model is a reliable means for modeling mood disorders. Both phenotypes can be used to model not only anxiety-behaviors, but depressive states and even addictive behaviors (Kerman et al., 2011). Our results also emphasize how extreme the genetic influence on mood can be. As the generations progressed, beginning at generation 12, bHRs became more responsive to novelty and bLRs became less responsive to novelty, demonstrating the heritability of emotional traits and mood disorders.

In terms of sex, our results varied from those found in Roenneberg et al. (2004). We found no significant effect of sex on the influence of time of day on locomotor score in our rats. However, Roenneberg et al. (2004) had found that in humans females typically reach their maximum eveningness peak, or tiredness, at an age earlier than males and are more prone to being morning-type overall. Animal research has shown that other species also have free-running rhythms that differ by sex. These sex differences are most likely due to steroid hormones being released from the gonads (Gorman & Lee, 2002). Our results may have not shown any significant sex differences in daily rhythms since rats were tested at different stages of their estrous cycle and no significant time of day effects were seen for locomotor score overall (Gorman et al., 2002 & Hagenauer et al., 2011).

In addition, there is an interaction between the effects of phenotype and sex. Another phenotypic difference that we have obscured in our lab is that past generation 40, many bLRs are struggling to reproduce. The exact reasons for this behavior remains unknown but it is potentially due to stress-suppressing hormone release that inhibits reproduction (Gorman et al., 2002). Therefore, there may be smaller hormonal sex differences in bLRs than in bHRs. It might

be interesting to address these questions by doing a comparison of hormone binding in the brain tissue of both bLRs and bHRs or testing peripheral circulating hormones. It also would be important to test animals in later generations that have reached adulthood, rather than focusing exclusively on those in late adolescence (Hagenauer et al., 2011).

One weakness of this study is that every generation of rats was only tested during the lighted hours and more specifically during morning hours (with the exception of a few generations that were tested at 1 p.m. and 2 p.m.). None of the rats were truly tested during the dark cycle or late afternoon. This impacts our results because we do not truly know if time of day affects locomotor response. Not seeing any overall significant effect of time of day in our results could potentially change if we compared light cycle novel locomotor responses to dark cycle novel locomotor responses. Another weakness is that the rats were also only tested for novel locomotor scores once during the day. Testing the rats at multiple times of day could give us more locomotor scores to analyze, which would truly help us to conclude if time of day is a factor in anxiety-like behaviors in rats. However, it is worth noting that it would be difficult to ensure that the locomotor responses represented a reaction to novelty if the rats were repeatedly tested. Another weakness of this experiment could be that the rats were tested during the adolescent phase (between 45-60 days old). Ronneneberg et al. (2004) showed that sex differences usually appear during adulthood when sex hormones have been more clearly developed. Therefore, testing the animals during late adolescence could have given different results than testing the animals during adulthood. We also did not run a second set of analyses that might be less susceptible to the influence of outliers, such as robust regression. Since we had several data points (over 10,000), manually sorting through the data to remove outliers within bLRs and bHRs was impractical. Therefore, our data could potentially contain very large

outliers, which could be skewing our results. However, had we ran a robust regression, we would have been able to statistically remove any significant outliers helping to reduce variability within our results.

In the future we could address these weaknesses by designing an experiment that included only adult rats (past day 60), as well as screening female animals to see if they were undergoing the estrous cycle during testing. In addition, we would test the animals for each generation at the same time intervals during the day. We would also test the animals twice a day, once during the light cycle and once during the dark cycle. We would run a robust regression analysis to exclude any outliers in our statistical data compilation.

However, overall we feel this experiment is strong because we tested 29 generations of animals, with a total of over 10,000 animals. Our sample size was quite large and included many animals from each phenotype. The data that was collected for each independent variable was analyzed in several methods and overall our model fit the data well. In order to take into account different potential confounds, approximately 50 graphs were generated when analyzing this data set. In terms of breeding colonies, our results support the approach that was used by the lab in breeding these rats. The breeding protocol does not take into consideration time of day effects when gathering behavioral data between the hours of 7 a.m. and 2 p.m. This protocol aided in our breeding project because it demonstrated consistency between generation 12 through generation 40, thereby illustrating that the data in generation 14 is comparable to the data in generation 36. In a long – term lab project, this consistency in the breeding strategy reduces the influence of confounding variables within the entire data set.

These results are important and necessary for the further progression of circadian rhythms and mood disorder research because it helps to identify factors that could potentially affect

emotional behaviors. By understanding how sex, phenotype, time of day, and age affect emotional behaviors, we might encounter solutions for psychiatric disorders, not just anxiety but also MDD and even addiction. Understanding the relationships between circadian rhythms and mood disorders could potentially help psychologists tailor programs for their patients to specific times of the day (Carpenter, Kupfer, & Frank, 1986).

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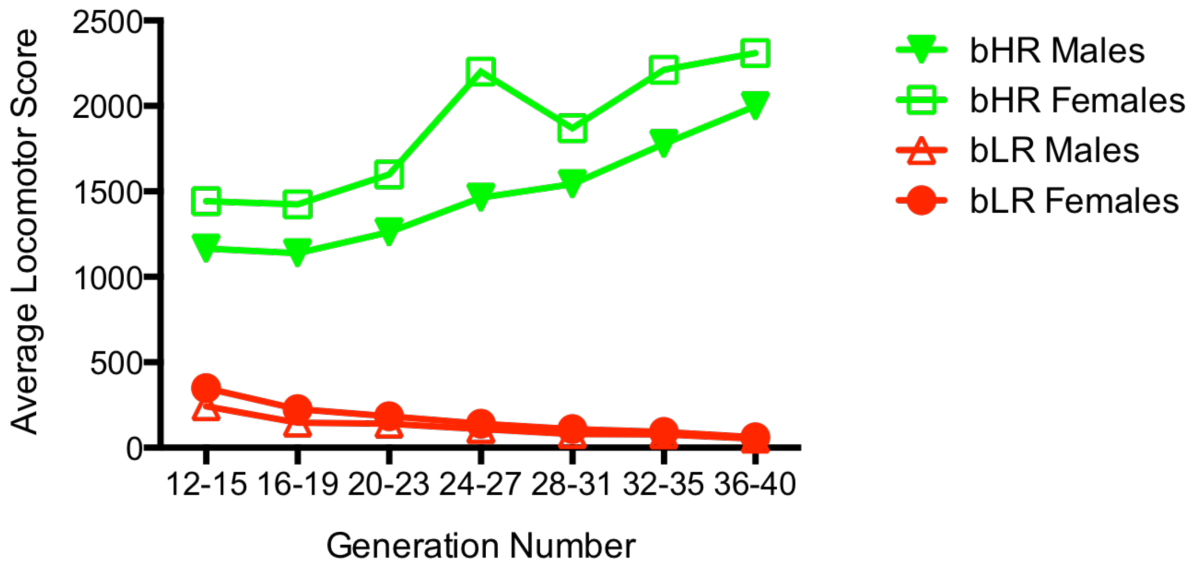

Figure 1: The graph demonstrates the increasing divergence between bLR and bHR phenotypes as generations progress.

This graph is a line graph that reflects average locomotor score on the y-axis and generations on the x-axis. The locomotor scores have been averaged together across all time intervals for each binned generation. The generations, reflected on the x-axis, are binned together in intervals of four. As the generations increased, average locomotor scores increased for both male and female bHRs, while significantly decreasing for both male and females bLRs. The two green lines are bHRs (high responders), where the upside down triangle refers to males and clear square are females. The two red lines refer to bLR (low-responders) rats, where the clear, facing up triangle are males and filled circle are females. Greater sex differences are present in the bHRs than the bLRs (phenotype*sex: $p = 2.68 \times 10^{-13}$).

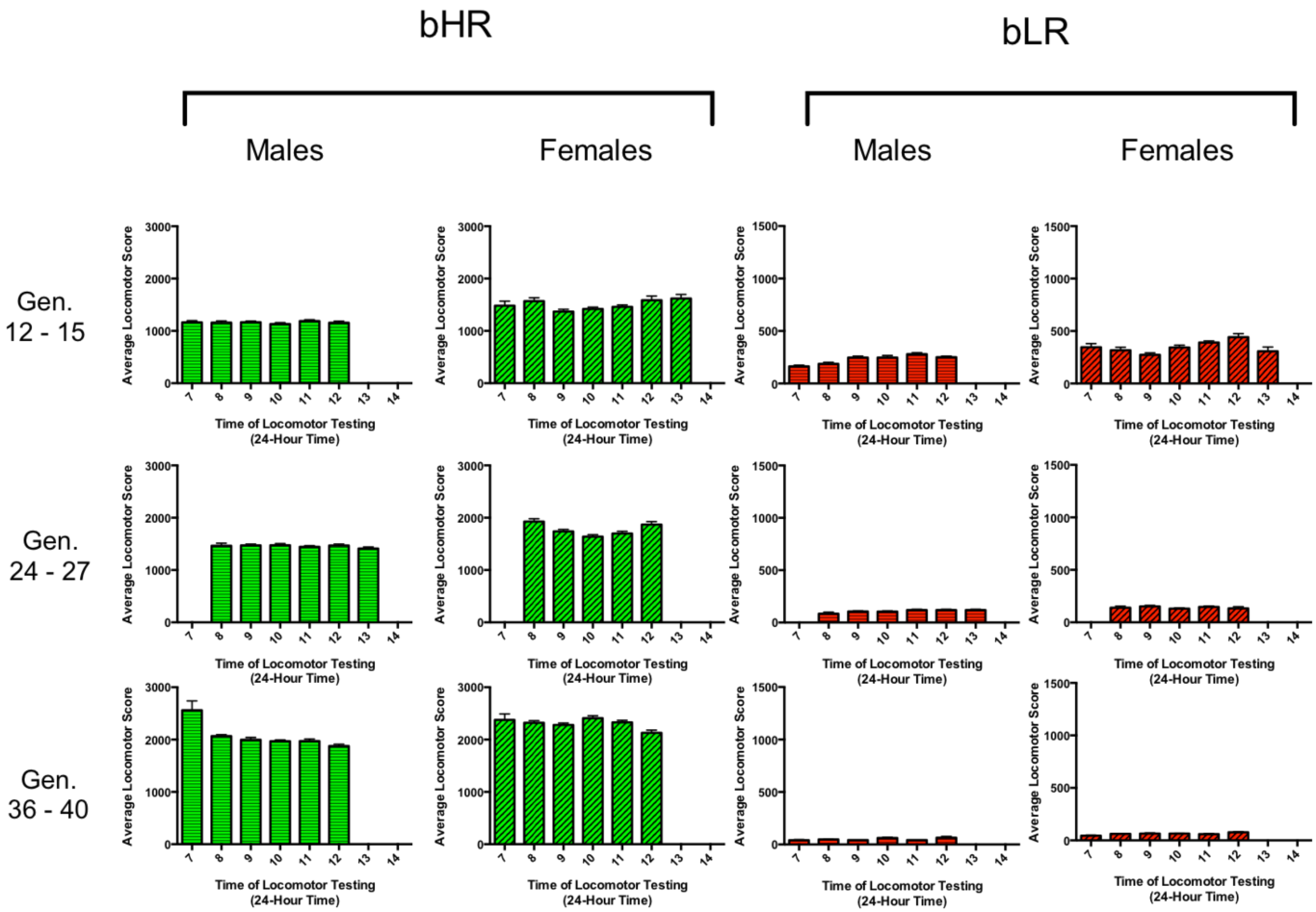

Figure 2: The amplification of phenotypic traits due to selective breeding with little evidence for time of day effects on locomotor score.

These bar graphs are sorted by phenotype, sex, and generation. On the x-axis for all graphs is the time of locomotor testing on a 24-hour scale. The generations shown are 12-15, 24-27, and 26-40 (earliest, middle, and latest generations). On the y-axis for bHRs, or high responders, (both males and females), is the average locomotor score on a scale from 0 – 3,000 beam breaks. On the y-axis for bLRs, or low-responders, (both males and females) is the average locomotor score on a scale from 0 – 1,500 beam breaks. Though the scale used for the bLR group is half the scale used for the bHR group, and the difference between average locomotor scores is still significantly visible. Overall, the figure is supposed to demonstrate and compare the extent to which phenotypic traits presented themselves as generations progressed for both males and females in relationship to time of day. The error bars on each bar represent the standard error of mean.

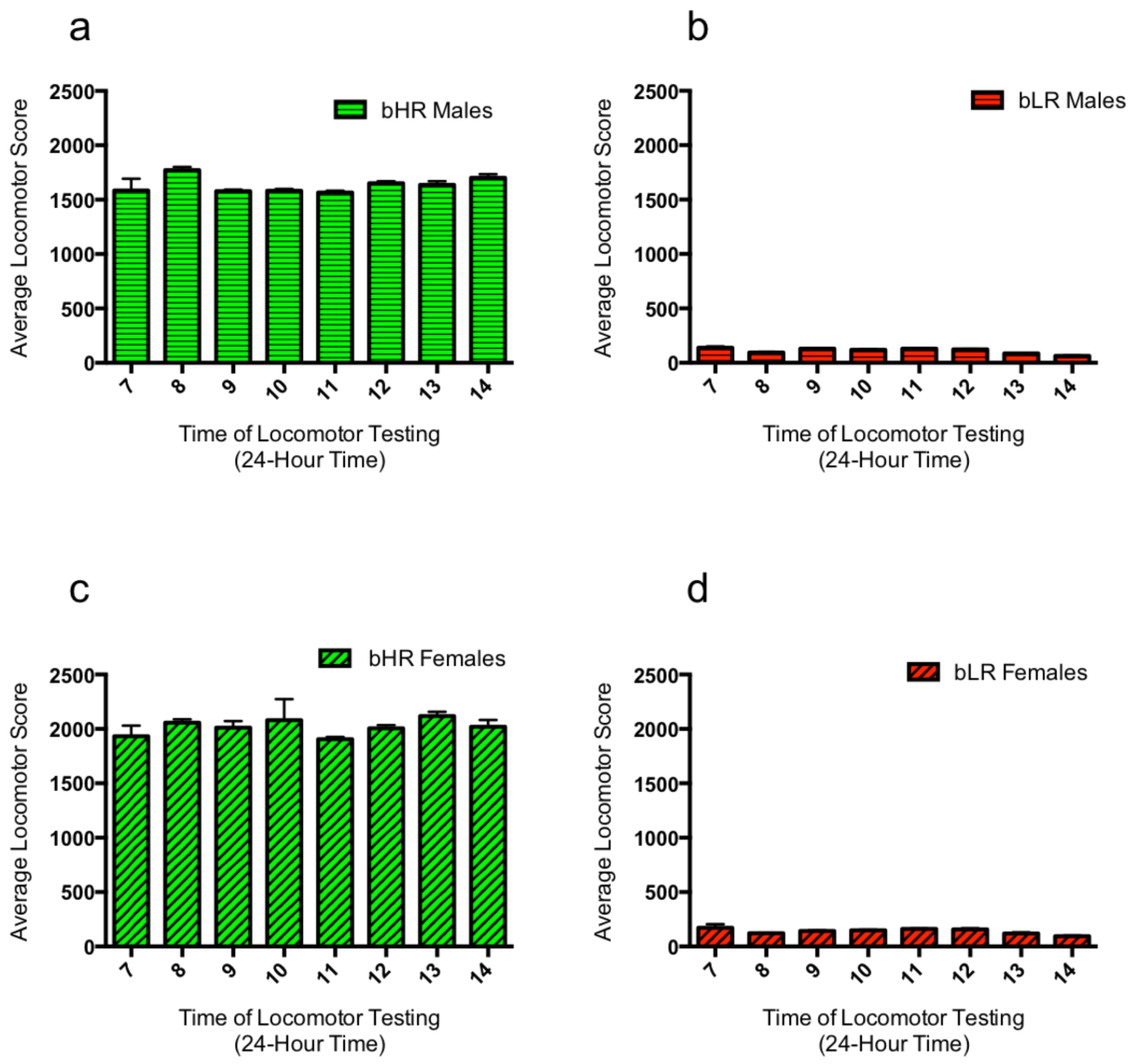

Figure 3(a – d): bHRs, across all generations and all times of day, have much greater average locomotor scores than bLRs.

All graphs bin data from all generations. The x-axis is the time of locomotor testing between the hours of 7 and 14, on a 24-hour clock. The y-axis is the average locomotor score for all animals for a specific sex and phenotype, which has been scaled to 2,500 beam breaks for all graphs. The error bars on each bar represent the standard error of mean. Overall, the graphs show how bHRs, both males and females, have a greater average locomotor score across the day than bLRs for both males and females, and little evidence of a change in locomotor score as the day progresses.

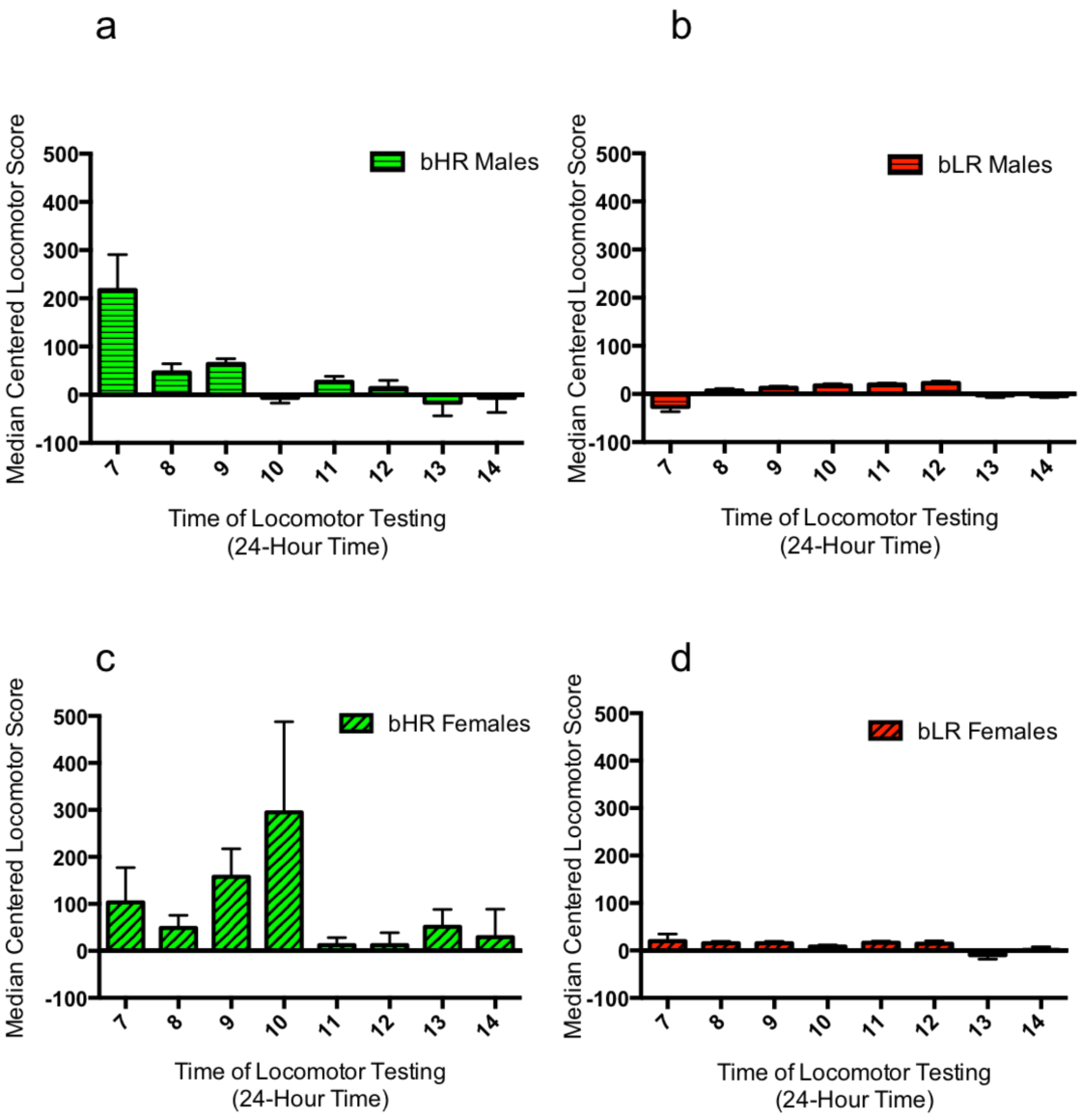

Figure 4(a – d): Median centered locomotor score graphs do not show a time of day effect on locomotor score for both phenotypes and sex.

This graph was created by subtracting the median score, for each hour of testing across all generations, from the average locomotor score. This was done to remove any generational effects and extreme outliers that may have existed when averaging the locomotor scores to test to see if time of day affects locomotor score. The y-axis has been made uniform by setting the maxium to 500 and minimum to -100 beam breaks relative to the median locomotor score for each generation. The x-axis is the time of day the locomotor score was collected between the hours of 7 and 14 on a 24-hour clock. The error bars on each bar represent the standard error of mean.

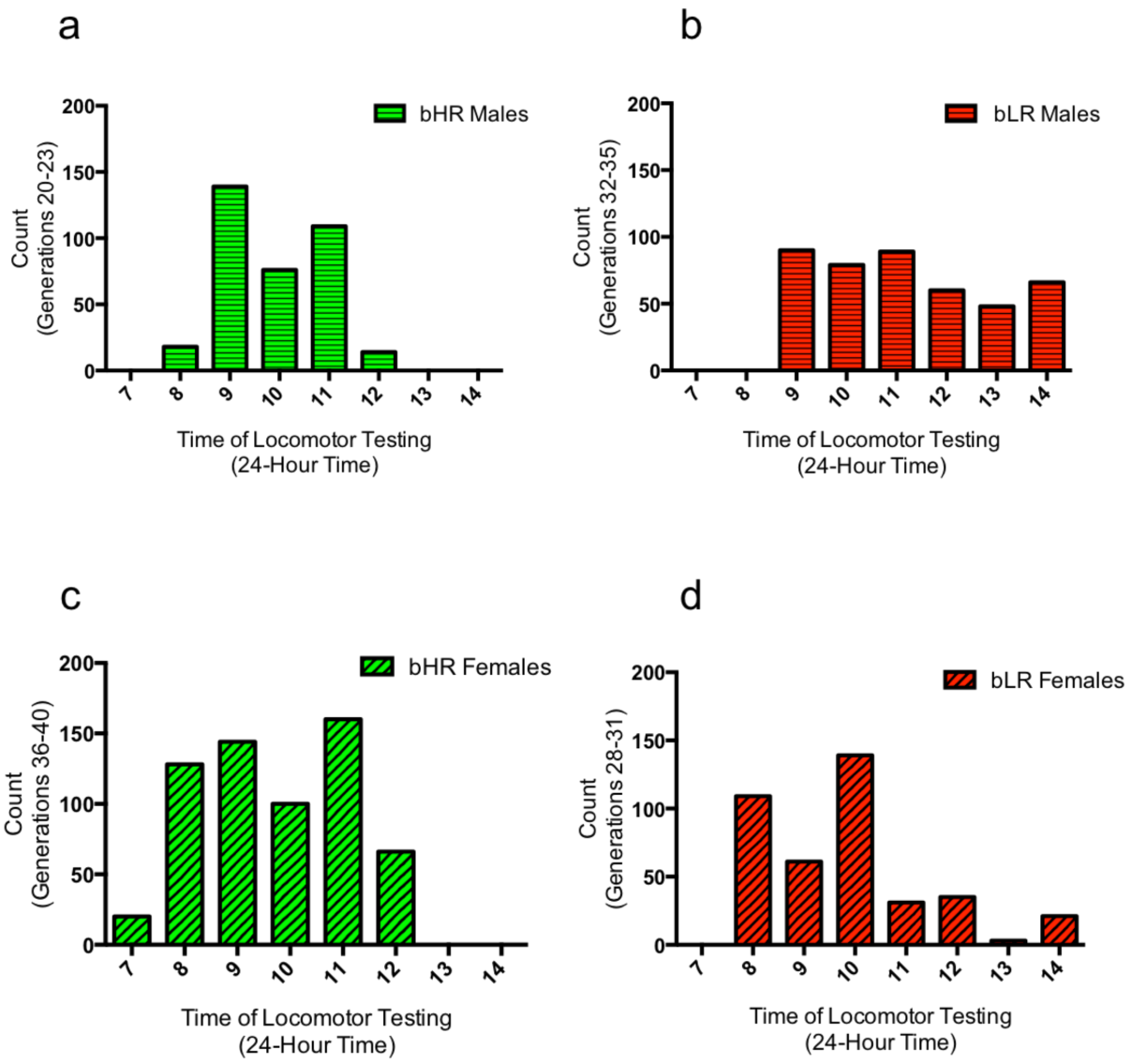

Figure 5(a – d): Representative generational histograms demonstrate the lack of consistency in times that the animals were tested.

These representative histograms are a sampling used to demonstrate the inconsistency in testing time across generations, phenotypes, and sex. The x-axis is the time of locomotor testing between from 7 to 14 on a 24-hour clock. The y-axis is the count or number of animals being tested for every hour for a specific binned generation. The y-axis has been scaled to 200 counts for all animals. bHR and bLR are abbreviations for high- and low-responders, respectively.