5-2018

The Effects of Adolescent Housing Condition and Voluntary Exercise on Alcohol Intake and Stress Response in Male Long-Evans Rats

Caroline Lynch
University of Dayton

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The Effects of Adolescent Housing Condition and Voluntary Exercise on Alcohol Intake and Stress Response in Male Long-Evans Rats

Honors Thesis
Caroline Lynch
Department: Psychology
Advisor: Tracy Butler, Ph.D.
May 2018
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Abstract
Can regular exercise during adolescence, combined with living in a social environment, decrease the negative effects of chronic stress and lower alcohol intake later in life? The aim of this research is to answer this question using a rat model that introduces a novel behavioral intervention in the form of regular voluntary exercise in order to counteract the negative effects of chronic stress caused by socially isolated housing during adolescence. Chronic stress has been linked to the development of alcohol use disorders (AUDs) in humans, and this study attempts to both model and hamper this phenomenon in rats using voluntary exercise. Gaining an understanding of how housing conditions and exercise can play a role in subsequent alcohol intake and stress hormone levels may be useful for the advent of new pharmacotherapies for individuals with an AUD.

Acknowledgements
I would like to thank my mentor, Dr. Tracy Butler, for her guidance and involvement in my thesis project. I would also like to thank the University of Dayton Honors Program, the Berry Family, and the Berry Family Foundation for their generosity in funding my project. Finally, I would like to thank my family and fellow lab members for their support and efforts in helping me successfully complete this project.
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Introduction

Due to the remarkable genetic similarity between rats and humans, experiments with rats as subjects have long served as corollaries to human research. Examples include research on the relationships between alcohol intake and exercise, stress levels and eating behavior, and exercise and adult neurogenesis (Leasure and Nixon, 2010). The research studies described herein follow in the footsteps of those previous studies by attempting to model a phenomenon in Long Evans rats that has been seen in humans: the development of an increased vulnerability to high anxiety levels and alcohol abuse after undergoing adolescent chronic stress (Sinha 2008; Enoch 2011). However, this project also introduces a behavioral intervention in the form of regular voluntary exercise in an attempt to counteract the negative effects of chronic stress such as increased anxiety and alcohol abuse. The chronic stressor that was used in these studies is socially isolated (SI) housing during adolescence (1 rat per cage), as opposed to group housed (GH) conditions (4 rats per cage). The studies described in this thesis took place over the course of one year and consisted of an original study performed in 2016 and a replicated study performed in 2017. They represent the first attempts at using a behavioral intervention for the specific purpose of reducing anxiety-like behavior and alcohol abuse in Long Evans rats. In the studies described in this thesis, the age of the rats is described in terms of post-natal day (PND). For example, PND 21 represents 21 days after a rat’s birth. In the twelve-week time span of each study in this project the rats were received on PND 21 and aged to PND 101, encompassing their period of adolescence and entry into adulthood (which begins around PND 63). In this project, eight rats in each cohort were put in SI conditions after the first week of each study while the other eight were in GH conditions for the entire twelve weeks, and four rats in each housing condition had access to exercise starting in the second week while the other four did not have access for the entire twelve weeks. Chronic exercise has been shown to be a means of decreasing stress and improving overall health in humans (Anderson and Shivakumar 2013; Broman-Fulks and Storey 2008; Cox et al., 2004), and previous studies has shown that it may have a similar effect on rodents by lessening anxiety-like behavior in rats (Fulk et al., 2004) and mice (Kim and Han 2016). To measure anxiety-like behavior in this project, we used an Elevated Plus Maze (EPM) and analyzed measures of open arm time and closed arm
frequency. Generally, the rats with less anxiety-like behavior will spend more time on the open arms compared to rats with more anxiety-like behavior. A swim stressor test was utilized in this project to examine the rats’ plasma corticosterone (CORT) levels in response to acute stress. CORT is a stress hormone found in rats that is analogous to the human stress hormone cortisol, and it was analyzed in blood samples taken at timepoints before and after the swim stressor to analyze and compare the rats’ stress responses. The stress response was measured because it has been observed that individuals with alcohol use disorders (AUDs) often show a blunted stress response, and SI rats have shown a failure to suppress plasma CORT levels when challenged with dexamethasone compared to GH rats (Butler et al., 2014b). Thus, the hypotheses of this study were principally that the rats with access to voluntary exercise (of both housing conditions) would exhibit less anxiety-like behavior, lower baseline corticosterone (CORT) levels, and less subsequent alcohol consumption than their counterparts without access to exercise and that for the rats without access to exercise, the GH rats would exhibit less anxiety-like behavior and alcohol intake than the SI rats, who were subject to the chronic stressor of an isolated housing condition during adolescence.

Materials and Methods

Animals and Housing—16 male Long Evans rats sourced from Envigo (Indianapolis, IN) were used in each study. Thus, the n for each study was 16 and the total N is 32. The rats were received on Postnatal Day (PND) 21 and were all group housed (GH; 4 rats/cage) in 55 cm x 38 cm polycarbonate cages until PND 27. In the 2017 study lids with filters were introduced and placed on top of each cage used in this study order to hinder the spread of allergens and dust. It is critical to note that PND 21 is immediately post-weaning, and the developmental period during which this experiment begins is equivalent to adolescence in humans. Beginning on PND 27, half of the cohort remained group housed while the other half became socially isolated (SI; 1 rat/cage) in 42 cm x 21 cm polycarbonate cages. This housing arrangement lasted for six weeks, during which time half of all GH and SI rats were allowed access to voluntary exercise (methods detailed below). The purpose for using this GH/SI model was to discern if socially isolated rats would display greater
anxiety-like behavior than group housed rats as has been repeatedly seen in previous studies (Chappell et al., 2013), or if voluntary exercise could prevent greater anxiety-like behavior in SI rats. Also of interest was how housing condition could affect exercise activity. This housing and exercise schematic is displayed in Table 1. On PND 69, all rats became SI in order to prepare for the behavioral tests and drinking procedure. Food and water were available ad libitum for the entirety of the experiment. The rats were housed in an environment with a 12 hour light/dark cycle (lights on 7:00-19:00), and all experiments were performed during the light portion of the cycle. All procedures were run in accord with guidelines provided by the National Institutes of Health (NIH) and were approved by the University of Dayton Institutional Animal Care and Use Committee (IACUC).

**Voluntary Exercise**- As previously described, from PND 28 to PND 69 in each study, a subset of GH (n = 4) and SI (n = 4) rats were given access to a running wheel for 30 minutes per day for five days per week between the hours of 8:00 and 11:00 am. Thus, there were n=8 runners in each study and a total of N=16 in the entire project. In each study, the access the rats received to the wheels on PNDs 28-31 and 34 was intended to serve as a chance for the rats to become acclimated to the equipment. Therefore, the distance the rats ran on these days was not included in the final totals and the official running protocol began on PND 35. The wheels were eight inches in diameter and made of wire mesh (Figure 2b). One BoGeer YT-813 Bicycle Odometer (Figure 2c) was attached to each wheel to record distance run (in km), in accordance with a similar technique from a previous study (Sasse et al., 2008). Each wheel was contained in a clear plastic tub (46 cm x 31.1 cm x 29.2 cm) with sawdust bedding so that each rat could exercise separately and had room to move around the wheel if he chose to not run. The running equipment was kept in the same room that the rats were held in for both the 2016 and 2017 studies to eliminate moving the animals an excessive amount. Also, the running protocol was performed in this same room each day. A researcher was present each running day for the entirety of the access period to monitor the rats and record any notable or unusual behaviors. This setup worked well during both the 2016 and 2017 studies even though it was a novel setup for this lab and had never been used before in
previous studies. During the 2017 study, a behavior chart was created and implemented to track rat behaviors such as hiding under the wheel, touching the wheel, walking on the wheel, and running on the wheel (Table 1). This behavior data was not statistically analyzed but provided useful information about how active each rat was during each access period.

<table>
<thead>
<tr>
<th>Date</th>
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<tr>
<td>RAT #</td>
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Table 1. Behavior chart.

**Elevated Plus Maze (EPM)**- On PND 70 of each study, all 16 rats were individually given five minutes of exposure time on an Elevated Plus Maze (EPM) to assess anxiety-like behavior beginning at 9:00 am. The rats were put on the maze in a random order that was kept consistent between the studies. The Elevated Plus Maze (Figure 3c) consists of two open arms and two closed arms (each 50 cm in length) and is elevated 75 cm above the ground. The measures we were interested in were open arm time (Figure 3a) and closed arm frequency (Figure 3b). Generally, the more time a rat spends on the open arms of the EPM, the less anxiety-like behavior it exhibits. This phenomenon can be explained by the observation that rats generally do not enjoy being in open, well-lit spaces, represented in the EPM by the open arms. Thus, if they spend time on the open arms, it shows that they are able to overcome their instinct of hiding and explore an open area. Closed arm frequency is a measure of general locomotor activity, giving researchers an estimate of how active a rat is on the maze (Holmes and Rodgers, 1999). Each rat was placed on the EPM for approximately five minutes, and its movements and behavior were tracked using EthoVision XT software.
Swim Stressor- The swim stressor protocol consisted of placing each rat individually in a cylindrical bucket (15” tall and 9.5” wide) that was filled with 9” of water that was kept at 25 degrees Celsius. Rats swam in the bucket for five minutes, after which time they were taken out and dried off. One pre-stressor blood sample was taken from each rat via a tail-snip technique approximately one hour before the swim stressor, and two post-stressor blood samples was taken via the same technique at timepoints approximately five and thirty minutes after the swim stressor. The swim stressor protocol was administered to rats 1-4 and 9-12 on PND 71 and rats 5-8 and 13-16 on PND 72 of each study. Preparation for each swim stressor day began at around 9:00 am and each day’s entire protocol took about three hours to complete. All 32 rats from both studies underwent the swim stressor procedure in order to observe their subsequent CORT levels compared to baseline levels.

Determination of Plasma Corticosterone Concentrations- Pre-stressor and post-stressor blood samples were kept on ice immediately after being acquired, and were spun in a centrifuge for 10 minutes to allow for separation of the plasma. The plasma from each sample was removed and kept frozen at -80° C until plasma CORT concentration was determined using a 96-well plate ELISA method. Plasma concentrations were diluted to a 1:20 ratio before being placed into the appropriate wells. The corticosterone enzymeimmunoassay ELISA kit used in this experiment utilized a polyclonal CORT antibody coated onto the inner surface of each of the 96 wells that attracted any CORT present in the diluted plasma samples to it. Then, the wells were washed and color was developed by using a chromogenic substrate. Finally, absorbance of the samples at 450 nm was read in a plate reader, and CORT concentration of each sample was determined using a standard curve and through a series of equations and interpolations (Immunodiagnostic Systems Inc, Gaithersburg, MD).

Drinking Procedure- Beginning on PND 76 of each study, following the completion of behavioral testing, ethanol consumption was assessed using an intermittent access two-bottle choice homecage drinking paradigm during which each rat received access to a
20% ethanol solution and water (in separate bottles) on Mondays, Wednesdays, and Fridays in 50ml conical bottles. On Tuesdays, Thursdays, Saturdays, and Sundays each rat was given two bottles of water in 50ml conical bottles. Due to the nature of the paradigm, all rats were singly housed for the entirety of the drinking period (PND 76-100). Ethanol consumption and preference was measured at 30 minute and 24 hour timepoints. Consumption was measured by subtracting the weight of each tube at the 30 minute and 24 hour timepoints from the original starting weight, and 30 minute and 24 hour g/kg consumption were calculated using the following equation: 1000/ body weight *(30 min. or 24 hour consumption/5*0.0397*20). 30 minute preference percentage was calculated using the following equation: (30 min. ethanol consumption)/(30 minute ethanol consumption + 30 minute water consumption). 24 hour preference percentage was calculated using the same equation while substituting the values for 24 hour consumption in place of the 30 minute consumption values. This model was borrowed from a previous study described in an article covering Alcohol Use Disorders (Butler et al., 2016).

**Data Analysis**—Body weight, plasma CORT concentration, and ethanol intake and preference data were all analyzed separately using repeated-measures three-way ANOVA (housing x exercise x timepoint of measurement). Running distance data were analyzed using repeated-measures two-way ANOVA (housing x timepoint of measurement). Anxiety-like behavior on the EPM was assessed using a two-way ANOVA (housing x exercise), which was followed up with one-tailed t-tests based on our a priori hypotheses. All analyses were done and graphs were created using GraphPad Prism 7.0 software. Data are expressed as mean +/- SEM. The data from the 2016 and 2017 studies were initially analyzed separately, and then the data were combined and analyzed as a whole.
Results for 2016 Cohort

Body Weight- Body weight data taken of all 16 rats once weekly from PND 21-69 (Figure 1) were analyzed using a repeated-measures two-way ANOVA (group x day of measurement), with Tukey’s post hoc test. The purpose of measuring body weights was to discern if housing condition or exercise were creating significant weight differences among the groups of rats, since chronically stressed rats sometimes show less weight gain than their non-stressed counterparts (Retana-Márquez et al., 2003). A statistically significant interaction was found between group and day of measurement, [F(21, 84)= 1.829, p< 0.05] such that all 16 rats consistently gained weight throughout the entirety of the experiment. Significant differences between GH runners and SI runners were found on PNDs 55 and 69 (p<0.05).

Exercise- Exercise data were analyzed using a repeated-measures two-way ANOVA (group x day of measurement), with Sidak’s post hoc test. A statistically significant interaction was found between group and day of measurement, [F(23, 138)= 2.407, p<0.05]. GH rats ran significantly more than SI rats on PNDs 35, 37, and 38 (Figure 2a). We also observed that running distance dropped considerably after PND 43 for GH and SI runners. The total distance run for each rat over the five week period of the protocol can be found in Table 2.
Figure 2. Distance Run (km). (A) Daily measurements of distance run were taken of all GH runners and SI runners from PND 35 to 66. Significant differences in running distance were found on PNDs 35, 37, and 38 between GH runners and SI runners (*p<0.05). (B) Distance run was measured using BoGeer YT 813 Bicycle Odometers. (C) Each rat was individually placed in a plastic bin with sawdust bedding and a wire mesh running wheel to run on.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>1  (GH)</th>
<th>2  (GH)</th>
<th>3  (GH)</th>
<th>4  (GH)</th>
<th>9  (SI)</th>
<th>10 (SI)</th>
<th>11 (SI)</th>
<th>12 (SI)</th>
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<tbody>
<tr>
<td>Total Distance Run (km)</td>
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<td>0.09</td>
<td>0.18</td>
<td>0.85</td>
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</table>

Table 2. Total Distance Run (km) by All GH Runners and SI Runners in 2016 cohort.
EPM- Measures of open arm time and closed arm frequency were analyzed using a one-way ANOVA with Tukey’s post hoc test. No significant differences in open arm time were found \[ F(3, 12)= 2.682, p= 0.09, \text{n.s.} \] (Figure 3a). However, a significant difference in closed arm frequency was found between GH runners and SI runners \[ F(3, 12)= 3.540, p<0.05 \] (Figure 3b) such that GH runners exhibited the highest number of closed arm entries on average compared to all other groups.

Figure 3. Open Arm Time and Closed Arm Frequency from the Elevated Plus Maze. (A) Open arm time in seconds was measured for all groups of rats. SI runners displayed the most open arm time of all groups. (B) Closed Arm Frequency in number of entries was measured for all groups of rats. GH runners displayed the highest number of closed arm entries of all groups and significantly more closed arm entries than SI runners \( ^*p<0.05 \). (C) The Elevated Plus Maze used in this experiment contains two open arms and two closed arms.
CORT Assay- Plasma corticosterone (CORT) concentration in ng/ml was determined by finding the B/Bo% of each plasma sample, and then interpolating the CORT concentration from a standard curve of CORT concentration vs. B/Bo% (Figure 4). Then, CORT concentrations were analyzed using a repeated-measures two-way ANOVA (group x timepoint) with Tukey’s post hoc test. A main effect of timepoint was found $[F(2,24)=93.71, p<0.0001]$ such that CORT levels for all rats were significantly higher at the 5 minute and 30 minute post-stressor timepoints than at the baseline timepoint. The purpose of analyzing CORT concentrations was to observe how the post-stressor samples related to the baseline samples to gain an understanding the hormonal response to the stressor. A rise in CORT levels after exposure to the stressor was expected, but it could have occurred that the chronically stressed SI rats would show a blunted CORT response as has been found in previous literature (Adinoff et al., 1998).

![Rat Plasma Corticosterone Concentrations (ng/ml)](image)

**Figure 4.** Plasma CORT Concentrations (ng/ml). CORT concentration was determined using blood samples taken via tail-snip technique at three different timepoints. Baseline samples were significantly lower than the 5 and 30 minute post-stressor samples (*p<0.05).
Ethanol Intake- Ethanol intake (g of ethanol/kg of body weight) and preference ratio (ethanol solution consumed out of total liquid consumed) data from both 30 minute and 24 hour timepoints were analyzed using repeated-measures two-way ANOVA (group x PND) with Tukey’s post hoc test interpreted when appropriate. A significant interaction was found between group and PND in 24 hour g/kg consumption \([F(33, 132)=1.777, p<0.05]\) (Figure 5b). Post hoc tests revealed a significant difference in 24 hour g/kg ethanol intake on PND 83 between GH runners and SI runners in that SI runners drank more ethanol \(p<0.05\). No significant interaction was found between group and PND in 30 minute g/kg consumption (Figure 5a), but there was a main effect of PND \([F(11, 132)=3.1, p<0.05]\). This means that ethanol intake changed over time (increasing in this case). No significant interaction was found between group and PND in 30 minute preference ratio (Figure 5c), but there was a main effect of PND \([F(11, 132)=2.795, p<0.05]\) such that 30 minute preference ratio increased over time. No significant interaction was found between group and PND in 24 hour preference ratio (Figure 5d). No significant differences in 24 hour preference ratio were found between groups, but there was a main effect of PND \([F(11, 132)=9.985, p<0.05]\) such that 24 hour preference ratio decreased over time. It is important to note that there were several occasions of bottle leakage that resulted in inaccurate data values. To correct for this, values from the day before and the day after of the same measure from the same rat were averaged. Errors occurred at the 30 minute timepoint for rats 3, 14, and 15 on PND 97 and for rat 15 on PND 87. Errors occurred at the 24 hour timepoint for rats 3, 14, and 15 on PND 97 and for rats 12 and 14 on PND 85.
Figure 5. Ethanol Consumption and Preference. (A) 30 minute ethanol consumption in g/kg. (B) 24 hour ethanol consumption in g/kg. SI runners drank significantly more than GH runners on PND 83 (*p<0.05). (C) 30 minute ethanol preference ratio. A significant difference was found in 30 minute preference ratio between GH non-runners and SI non-runners on PND 90. The solid line at 0.5 indicates the point of equal preference for water and the ethanol solution (*p<0.05). (D) 24 hour ethanol preference ratio. The solid line at 0.5 indicates the point of equal preference for water and the ethanol solution.

Results for 2017 Cohort

Body Weight- Keeping with the methods used for the 2016 cohort, body weight measurements were taken of all 16 rats once weekly from PND 21-69 (Figure 6). This data was analyzed using a repeated-measures three-way ANOVA (group x exercise x day of measurement), with Tukey’s post hoc test. The purpose behind this was the same as for the 2016 cohort. A main effect of time was discovered [F(7, 96)=311.3, p<0.0001] such that the rats consistently gained weight over time. Tukey’s multiple comparisons test revealed significant differences between the rats’ weights on certain PNDs (p<0.05, depicted in Table 3). Also, there were no significant differences in body weight found between the groups of rats from PND 21-69.
Figure 6. Body weight (g). Rats were weighed upon arrival to the lab (PND 21) and then weekly thereafter. No significant differences in body weight were found between the groups of rats.

<table>
<thead>
<tr>
<th>PND</th>
<th>Significantly Different From PND(s)</th>
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<tr>
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<td>62, 69</td>
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<tr>
<td>62</td>
<td>69</td>
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</tbody>
</table>

Table 3. Significant differences in the rats’ body weights between PNDs found using Tukey’s multiple comparisons test (p<0.05).
**Exercise**- Exercise data for the 2017 cohort were analyzed using a repeated-measures two-way ANOVA (group x day of measurement), with Sidak’s post hoc test. A statistically significant interaction was found between group and day of measurement, [F(23, 138)= 2.416, p<0.05]. GH rats ran significantly more than SI rats on PNDs 44, 48, and 50 (Figure 7). It can be noted that all four GH runner rats in this cohort displayed running behavior after only two days of exposure to the wheels, while the four SI runners appeared more timid and took between two and four days to show running behavior. SI runners maintained a level of little to no running activity throughout the entire protocol. The total distance run for each rat over the five week period of the protocol can be found in Table 4.

![Distance Run by GH and SI Rats from PND 35 to 66](image)

**Figure 7. Distance Run (km).** Daily measurements of distance run were taken of all GH runners and SI runners from PND 35 to 66. GH runners ran significantly more than SI runners on PNDs 44, 48, and 50 (*p<0.05).

<table>
<thead>
<tr>
<th>Rat #</th>
<th>1 (GH)</th>
<th>2 (GH)</th>
<th>3 (GH)</th>
<th>4 (GH)</th>
<th>9 (SI)</th>
<th>10 (SI)</th>
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<tr>
<td>Total Distance Run (km)</td>
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<td>0.11</td>
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**Table 4. Total Distance Run (km) by All GH Runners and SI Runners in 2017 cohort**
EPM- Measures of open arm time and closed arm frequency were analyzed using a two-way ANOVA (exercise x housing) with Tukey’s post hoc test. No significant interaction between exercise and housing was found for open arm time \([F(1, 12)= 0.1067, p= 0.75, \text{n.s.}]\) (Figure 8a), although it can be noted that GH runners displayed the highest average open arm time of all groups of rats in this cohort. Following the GH Runners, the group with the next highest average open arm time was SI runners, followed by GH non-runners and SI non-runners. Also, no significant interaction between exercise and housing was found for closed arm frequency were found \([F(1,12)= 3.097, p=0.10, \text{n.s.}]\) (Figure 8b). Closed arm frequency was fairly equal among all groups of rats in this cohort.

![Figure 8. Open Arm Time and Closed Arm Frequency from the Elevated Plus Maze.](image)

(A) Open arm time in seconds was measured for all groups of rats. GH runners displayed the most open arm time of all groups. No significant differences were found between groups. (B) Closed Arm Frequency in number of entries was measured for all groups of rats, no significant differences were found between groups.
**CORT Assay**- In the same process used for the 2016 cohort, plasma corticosterone (CORT) concentration in ng/ml was determined for the 2017 cohort by finding the B/Bo% of each plasma sample, and then interpolating the CORT concentration from a standard curve of CORT concentration vs. B/Bo% (Figure 9). Then, CORT concentrations were analyzed using a repeated-measures three-way ANOVA (group x exercise x timepoint) with Tukey’s post hoc test. A main effect of timepoint \[F(2,24)=3.657, p<0.05\] such that CORT levels for all rats were significantly higher at the 5 minute and 30 minute post-stressor timepoints than at the baseline timepoint. An SI runner had an extremely high level of CORT in its blood that was collected at the 5 minute post-stressor timepoint (1061.74 ng/ml) compared to the other rats’ blood at that timepoint (ranging from 142.34 to 236.94 ng/ml), which shifted the appearance of Figure 9.

![Figure 9. Plasma CORT Concentrations (ng/ml). CORT concentration was determined using blood samples taken via tail-snip technique at three different timepoints. Baseline samples were significantly lower than the 5 and 20 minute post-stressor samples (*p<0.05).](image-url)
**Ethanol Intake** - Ethanol intake (g of ethanol/kg of body weight) and preference data from both 30 minute and 24 hour timepoints for the 2017 cohort were analyzed using a repeated-measures three-way ANOVA (housing x exercise x PND) with Tukey’s post hoc test interpreted when appropriate. No significant interaction was found between housing, exercise, and PND in daily 24 hour g/kg consumption $[F(11,144)=0.8946, \ p=0.4743, \text{n.s.}]$ (Figure 10b), although there was a main effect of time $[F(11,144)=4.322, \ p<0.0001]$ such that this measure was significantly different between PNDs 76 vs. 85, 92, 94, 97, 99, and 101, 78 vs. 85, 90, 92, 94, 97, 99, and 101. Post hoc tests revealed a significant difference in 24 hour g/kg ethanol intake on PND 78 between SI runners and SI non-runners in that SI runners drank more ethanol ($p<0.05$). No significant interaction was found between housing, exercise, and PND in 30 minute g/kg consumption $[F(11,144)=1.007, \ p=0.4432, \text{n.s.}]$ (Figure 10a), but there was a main effect of PND $[F(11, 144)=2.304, \ p=0.125]$ such that this measure was significantly different between PNDs 76 vs. 78 and 78 vs. 85. Also, on PND 78 significant differences in 30 minute g/kg consumption were found between GH runners and GH non-runners, GH runners and SI non-runners, and SI runners and SI non-runners ($p<0.05$). No significant interaction was found between housing, exercise, and PND in 30 minute preference percentage $[F(11,144)=0.8509, \ p=0.5898, \text{n.s.}]$ (Figure 10c), but there was a main effect of PND $[F(11, 144)=2.529, \ p=0.0060]$ such that this measure was significantly different between PNDs 87 vs. 90 and 90 vs. 101. No significant differences between groups were found in 30 minute preference percentage in this cohort. No significant interaction was found between housing, exercise, and PND in 24 hour preference percentage $[F(11,144)=0.7471, \ p=0.6917, \text{n.s.}]$ (Figure 10d), although there was a main effect of PND $[F(11,144)=0.7.464, \ p<0.0001]$ such that this measure was significantly different between PNDs 76 vs. 80, 83, 85, 87, 90, 92, 94, 97, 99, and 101, and 78 vs. 85 and 94. No significant differences in 24 hour preference percentage were found between groups.
Figure 10. Ethanol Consumption and Preference. (A) 30 minute ethanol consumption in g/kg. On PND 78 there were significant differences between GH runners and GH non-runners, GH runners and SI non-runners, and SI runners and SI non-runners (*p<0.05) (B) 24 hour ethanol consumption in g/kg. SI runners drank significantly more than SI non-runners on PND 78 (*p<0.05). (C) 30 minute ethanol preference percentage. The solid line at 50% indicates the point of equal preference for water and the ethanol solution. (D) 24 hour ethanol preference percentage. The solid line at 50% indicates the point of equal preference for water and the ethanol solution.

Combined Results for 2016 and 2017 Cohorts

Body Weight- Upon analyzing the combined body weight data from both the 2016 and 2017 cohorts using a repeated-measures three-way ANOVA (housing x exercise x day of measurement) with Tukey’s post hoc test, and a main effect of time was found [F (8, 252)= 958.9, p< 0.0001], such that all 32 rats consistently gained weight throughout the entirety of each experiment (Figure 11). Tukey’s multiple comparisons test revealed significant differences between the rats’ weights on certain PNDs (p<0.05, depicted in Table 5). Also, there were no significant differences in body weight found between the groups of rats from PND 21-69.
Figure 11. Body weight (g). All 32 rats in this project were weighed upon arrival to the lab (PND 21) and then weekly thereafter. No significant differences in body weight were found between groups of rats, although all rats’ body weights increased significantly over time (p<0.05).

<table>
<thead>
<tr>
<th>PND</th>
<th>Significantly Different From PND(s)</th>
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<tbody>
<tr>
<td>21</td>
<td>27, 34, 41, 48, 55, 62, 69</td>
</tr>
<tr>
<td>27</td>
<td>34, 41, 48, 55, 62, 69</td>
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<td>41, 48, 55, 62, 69</td>
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<td>41</td>
<td>48, 55, 62, 69</td>
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<td>48</td>
<td>55, 62, 69</td>
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<td>55</td>
<td>62, 69</td>
</tr>
<tr>
<td>62</td>
<td>69</td>
</tr>
</tbody>
</table>

Table 5. Significant differences in the rats’ body weights between PNDs found using Tukey’s multiple comparisons test (p<0.05).
Exercise- Exercise data for the combined 2016 and 2017 cohorts were analyzed using a repeated-measures two-way ANOVA (housing x day of measurement), with Sidak’s post hoc test. A statistically significant interaction was found between group and day of measurement, [F (23, 336)= 2.172, p<0.05]. GH rats ran significantly more than SI rats on PNDs 35, 37, and 38 (Figure 12). It can be noted that GH runners overall showed more running behavior than SI runners for the first week of the exercise protocol, then all rats’ running activity appeared to decline after PND 38. The GH runners displayed a significantly higher average total running distance (1.218 ± 0.182 km) compared to the SI runners (0.327 ± 0.131 km) (t(14)=3.818, p=0.0019)(Figure 13).

Figure 12. Distance Run (km). Daily measurements of distance run were taken of all GH runners and SI runners from PND 35 to 66. GH runners ran significantly more than SI runners on PNDs 35, 37, and 38 (*p<0.05).
Figure 13. Average Total Distance Run (km). The average distance run by all 8 GH runners in both cohorts is 1.218 ± 0.182 km and the average distance run by all 8 SI runners in both cohorts is 0.327 ± 0.131 km. These averages are significantly different (t=3.818, df=14, p=0.0019). (*p<0.05)

EPM- Measures of open arm time and closed arm frequency were analyzed using a two-way ANOVA (housing x exercise) with Tukey’s post hoc test. One outlier (an SI non-runner) was removed from analysis based on outlier detection using Grubbs’ test. There was a main effect of exercise [F(1, 27)=6.515, p< 0.05] such that the runners spent more time on the open arms than the non-runners. As our a priori hypotheses predicted specific differences between GH and SI non-runners and between SI runners and SI non-runners, follow-up one-tailed t-tests were also conducted which showed that the SI non-runners spent significantly less time on the open arms compared to GH non-runners (as predicted; t(13)=2.701, p<0.01), and SI non-runners spent significantly less time on the open arms than SI runners (as predicted; t(13)=2.882, p<0.01). No main effects or significant interaction between exercise and housing were found for closed arm frequency [F(3, 28)=0.7162, p=0.55, n.s.] (Figure 14b). Closed arm frequency was not different among groups.
Figure 14. Open Arm Time and Closed Arm Frequency from the Elevated Plus Maze. (A) Open arm time in seconds was measured for all groups of rats. GH and SI runners displayed more open arm time than GH and SI non-runners. SI runners (*p<0.01) and GH non-runners (Ψp<0.01) spent significantly more time on the open arms compared to SI non-runners. (B) Closed Arm Frequency in number of entries was measured for all groups of rats, no significant differences were found between groups.

CORT Assay- CORT concentrations for both cohorts were analyzed using a repeated-measures three-way ANOVA (housing x exercise x timepoint) with Tukey’s post hoc test. No significant interaction was found between housing, exercise, and timepoint but there was a main effect of timepoint [F(2,84)=13.21, p<0.0001] such that the rats’ 5
minute and 30 minute post-stressor blood samples had significantly higher levels of CORT than their baseline blood samples (p<0.05) (Figure 15). A follow-up two-way ANOVA (exercise x time) was run that compared runners and non-runners across time, and a main effect of timepoint was found \[F(2,60)=19.95, p<0.0001\], post hocs revealed that the CORT concentrations at 5 and 30 minutes post stressor were significantly higher than the baseline concentration (p<0.001).

![Plasma Corticosterone Concentrations (ng/ml) for Combined Cohorts](image)

**Figure 15.** Plasma CORT Concentrations (ng/ml). CORT concentration was determined using blood samples taken via tail-snip technique at three different timepoints. Baseline CORT concentrations were significantly lower than 5 minute and 30 minute post stressor concentrations (*p<0.05).

**Ethanol Intake**- Ethanol intake (g of ethanol/kg of body weight) and preference data from both 30 minute and 24 hour timepoints from both cohorts were analyzed using a repeated-measures three-way ANOVA (housing x exercise x PND) with Tukey’s post hoc test interpreted when appropriate. No significant interaction was found between housing, exercise, and PND in weekly 24 hour g/kg consumption \[F(3, 112)=1.013, p=0.3899, n.s.\] (Figure 16b), but a main effect of time was discovered \[F(3, 112)=4.791, p=0.0035\]. No significant interaction was found between housing, exercise, and PND in weekly 30 minute g/kg consumption \[F(3,112)=1.124, p=0.3424, n.s.\] (Figure 16a) and no main effects were found. No significant interaction was found between housing, exercise, and PND in weekly 30 minute preference percentage \[F(3,112)=0.5189, p=0.6701, n.s.\] (Figure 16c) and no main effects were found. A significant interaction
was found between housing, exercise, and PND in weekly 24 hour preference percentage \[F(3,112)=1.038, p=0.3785\] (Figure 16d) and a main effect of time was found \[F(3,112)=14.09, p<0.0001\].

Figure 16. Ethanol Consumption and Preference. (A) 30 minute weekly ethanol consumption in g/kg. (B) 24 hour weekly ethanol consumption in g/kg. (C) 30 minute weekly ethanol preference percentage. The solid line at 50% indicates the point of equal preference for water and the ethanol solution. (D) 24 hour weekly ethanol preference percentage.
Average Ethanol Intake and Preference- The average 30 minute ethanol intake in grams of ethanol consumed/kg of body weight was calculated for all groups of rats in both cohorts and was found to be 0.519 ± 0.066 g/kg for GH runners, 0.387 ± 0.055 g/kg for GH non-runners, 0.468 ± 0.078 g/kg for SI runners, and 0.358 ± 0.076 g/kg for SI non-runners (Figure 17a). The average 24 hour g/kg ethanol intake was 3.251 ± 0.298 g/kg for GH runners, 3.335 ± 0.445 g/kg for GH non-runners, 3.486 ± 0.421 g/kg for SI runners, and 2.360 ± 0.409 g/kg for SI non-runners (Figure 17b). The average 30 minute preference percentage for ethanol was 43.566 ± 3.813% for GH runners, 42.873 ± 5.060% for GH non-runners, 42.379 ± 4.747% for SI runners, and 50.584 ± 6.860% for SI non-runners (Figure 17c). The average 24 hour preference percentage for ethanol was 40.351 ± 3.963% for GH runners, 41.166 ± 5.725% for GH non-runners, 41.386 ± 3.410% for SI runners, and 26.006 ± 4.409% for SI non-runners (Figure 17d). No significant differences were found between groups for 30 minute intake [F(1,28)=0.021, p=0.885, n.s.], 30 minute preference percentage [F(1, 28)=0.510, p=0.480, n.s.], 24 hour intake [F(1,28)=1.398, p=0.247, n.s.], or 24 hour preference percentage [F(1, 28)=1.936, p=0.175, n.s.]. However, due to our a priori hypotheses that SI non-runners would drink more than both GH non-runners and SI runners, we ran some one-tailed t tests that revealed significant differences between GH and SI non-runners in average 24 hour preference percentage and between SI runners and non-runners in average 24 hour intake and 24 hour preference percentage (as shown in tables 6 and 7).
<table>
<thead>
<tr>
<th></th>
<th>GH non-runners</th>
<th>SI non-runners</th>
<th>t(df), p</th>
</tr>
</thead>
<tbody>
<tr>
<td>30m intake</td>
<td>0.387 ± 0.055 g/kg</td>
<td>0.358 ± 0.076 g/kg</td>
<td>t(14)=0.309, p=0.380</td>
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<tr>
<td>30m pref %</td>
<td>42.873 ± 5.060%</td>
<td>50.584 ± 6.860%</td>
<td>t(14)=0.904, p=0.190</td>
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<tr>
<td>24hr intake</td>
<td>3.335 ± 0.445 g/kg</td>
<td>2.360 ± 0.409 g/kg</td>
<td>t(14)=1.612, p=0.064</td>
</tr>
<tr>
<td>24hr pref %</td>
<td>41.166 ± 5.725 %</td>
<td>26.006 ± 4.409 %</td>
<td>t(14)=2.098, p=0.027*</td>
</tr>
</tbody>
</table>

Table 6. Average 30 minute and 24 hour intake and preference for GH and SI non-runners. *indicates a significant difference between the groups in the measure indicated.

<table>
<thead>
<tr>
<th></th>
<th>SI runners</th>
<th>SI non-runners</th>
<th>t(df), p</th>
</tr>
</thead>
<tbody>
<tr>
<td>30m intake</td>
<td>0.468 ± 0.078 g/kg</td>
<td>0.358 ± 0.076 g/kg</td>
<td>t(14)=1.008, p=0.165</td>
</tr>
<tr>
<td>30m pref %</td>
<td>42.379 ± 4.747%</td>
<td>50.584 ± 6.860%</td>
<td>t(14)=0.983, p=0.171</td>
</tr>
<tr>
<td>24hr intake</td>
<td>3.486 ± 0.421 g/kg</td>
<td>2.360 ± 0.409 g/kg</td>
<td>t(14)=1.916, p=0.038*</td>
</tr>
<tr>
<td>24hr pref %</td>
<td>41.386 ± 3.410 %</td>
<td>26.006 ± 4.409 %</td>
<td>t(14)=2.759, p=0.007*</td>
</tr>
</tbody>
</table>

Table 7. Average 30 minute and 24 hour intake and preference for SI runners and non-runners. *indicates a significant difference between the groups in the measure indicated.
Figure 17. Average Ethanol Intake and Preference for both cohorts. (A) Average 30 minute g/kg ethanol consumption. (B) Average 24 hour g/kg ethanol consumption. (C) Average 30 minute ethanol preference percentage. The dotted line at 50% indicates an equal preference for ethanol and water. (D) Average 24 hour ethanol preference percentage.
Discussion

Study Design- This project was initially designed in 2016 and the first study was conducted in the summer of 2016 during my involvement in the Berry Summer Thesis Institute (BSTI). Then, because of the relatively small sample size in the initial study, that study was duplicated in the summer of 2017 in an attempt to confirm the preliminary results from the 2016 study. Thus, the 2016 study can be thought of as the initial study and the 2017 study can be thought of as the replicated study, though all data have been combined for analyses as experimental design was identical. Throughout this discussion I will explain the similarities and differences that were observed between the studies as well as why those similarities and differences may have occurred. Although the experimental design was identical between the two studies, there was a difference between the studies in terms of the location of the lab; the 2016 study took place in a temporary lab space on the second floor of the Science Center and the 2017 study took place in the newly renovated vivarium space in the basement of the Science Center (room 048). For the first week of both studies, all sixteen rats were group housed (GH; 4 rats/cage), and then at the beginning of the second week they were split into four groups of four. One group consisted of socially isolated (SI) rats that did not have access to voluntary exercise. It was this group of rats from which we expected the greatest anxiety-like behavior and alcohol consumption, because they were subjected to the adolescent chronic stressor of SI housing and did not have access to the possible anxiety-reducing effects of voluntary exercise. The other three groups included four SI rats that had access to voluntary exercise, four GH rats that did not have access to voluntary exercise, and four GH rats that had exercise access. It is the group of GH rats with access to exercise from which we expected the lowest amount of anxiety-like behavior and alcohol consumption, due to the absence of a chronic stressor and their access to available and regular exercise. During the second week of each study, the GH and SI rats with exercise access were given thirty minutes of exposure to their own individual running wheels on Monday through Friday for acclimation purposes, although the distances the rats ran during this week were not incorporated into the final data. The third week of the study marked the beginning of official running data collection and the exposure time to the running wheels was kept at 30 minutes per day, five days per week, for five consecutive
weeks. During these five weeks, the SI and GH non-runners received no stimulation except during standard weekly weighings and cage changes. After these five weeks, all sixteen rats became SI in order to prepare for behavioral tests such as the Elevated Plus Maze (EPM) (Figure 3c) and a swim stressor that occurred during the eighth week of each study, as well as the alcohol procedure. During the four weeks following the week of behavioral testing, alcohol intake/preference was measured using an intermittent access homecage drinking paradigm during which each rat received access to a 20% ethanol solution and water on Mondays, Wednesdays, and Fridays. Alcohol intake and preference (over water) was measured at 30 minute and 24 hour timepoints. After the final week of the alcohol protocol (the twelfth week of each study), the rats were sacrificed and the project reached completion. To summarize the previous information, this study attempted to create a model where exercise, a behavioral intervention, could counteract the negative effects of SI housing during adolescence (a chronic stressor) and lower anxiety-like behavior as well as alcohol consumption later in life.

Housing- The reasoning behind using socially isolated housing as our model of chronic stress is that it has been shown in other previous studies with rats to lead to the display of more anxiety-like behavior and alcohol consumption later on in the rats’ lives compared to rats that were group housed during adolescence (Hellemans et al., 2004; Chappell et al., 2013; Hall et al., 1998). In a study performed by A.M. Chappell and colleagues in 2013, it was found that SI rats spent significantly less time on the open arms of an elevated plus maze than GH rats, meaning that SI rats displayed more anxiety-like behavior than GH rats. Also, the study found that SI rats had significantly higher levels of daily ethanol intake than GH rats during an intermittent home-cage drinking procedure that is almost identical to the one used in this thesis project (Chappell et al., 2013). SI rats have reliably shown these trends due to the stressful nature of spending their adolescence in social isolation, when normally their social nature would lead to an adolescence surrounded by members of their own species. Also, many previous studies that have used the SI/GH housing paradigm have found behavioral and neurobiological deficits in SI rats compared to GH rats. For example, SI rats have exhibited deficits in neural baseline
dopamine levels (Karkhanis et al., 2015), cognitive function (Hedges and Woon, 2011),
deficits in sensorimotor gating (Ko and Liu, 2015), and impaired fear extinction (Skelly et al., 2015). This study represents a first attempt to lessen maladaptive behaviors in SI rats using voluntary exercise.

**Age of Rats-** Considering the life span of rats is on average 2-3.5 years while the average human lifespan worldwide is about 80 years, it has been stated that rats have a brief and accelerated childhood in respect of humans (Sengupta 2013). Rats enter adolescence when they become sexually mature at around six weeks of age (PND 42) while humans don’t reach this milestone until around 12 years of age, so by performing studies with rats the period of adolescence can be more quickly and easily observed as compared to studies with humans. Although the time frames of adolescence may be different between rats and humans, similar behaviors such as increased risk-taking and social play have been observed in each species (Doremus-Fitzwater et al., 2010). We used adolescent rats in these studies because adolescence is a critical period of development in both rats and humans where exposure to stress can cause physiological and behavioral deficits (Chappell et al., 2013). Thus, we wanted to observe whether the chronically stressed SI rats displayed any of these deficits and if the behavioral intervention of regularly voluntary exercise could prevent these deficits from occurring in both SI and GH rats. Also, adolescence represents a time where behavioral changes such as enhanced interactions with peers and increases in novelty seeking are common and play a large role in the maturation of an organism (Doremus-Fitzwater et al., 2010). In particular, rats demonstrate higher levels of social activity during adolescence than any other period of their life (Doremus-Fitzwater et al., 2010). Therefore, rats raised in social isolation during the juvenile adolescent period (~PND 28-70) are essentially missing out on these rewarding social interactions and may develop differently compared to rats maturing in a social environment. In addition, we thought that by introducing voluntary exercise during this period of adolescence that the rats would be more inclined to explore the novel running wheel apparatus and that it could make a greater impact on their physical and social development than if it were introduced during adulthood. Ultimately, we thought that by introducing a behavioral intervention during the critical developmental period of
adolescence that it could have a greater impact on reducing the rats’ subsequent anxiety-like behavior and ethanol intake than if introduced during adulthood.

**Sex of Rats**- To address the inclusion of only male rats in this project, it was due to the larger availability of similar studies performed with all male rats and the relative lack of similar studies performed with female rats. Also, a study was published with data showing that the socially isolated housing paradigm does not result in increased anxiety-like behavior or ethanol intake in female long-evans rats (Butler et al., 2014a). Therefore, in terms of designing this project it made the most sense to include the sex that would display the desired anxiety-like behavior and ethanol intake. However, we appreciate the need to include more females (both rats and humans) in relevant research since their body systems are notably different than males’ and should be accounted for. The reason for including only 16 male rats in each cohort was that their were only one or two people taking care of the rats and performing the experiments, thus we could only handle a maximum of 16 rats per cohort. We realize that this small number of rats may have limited the strength of our data, and a future suggestion would be to increase the number of rats in similar studies to gain a better understanding of the behavioral trends and increase statistical power. It can also be seen as a strength if the same trends are seen in each cohort.

**Voluntary Exercise**- The typical recommendation for humans is to get 2-2.5 hours of moderate exercise per week in order to reduce one’s risk for getting a chronic disease (Anderson and Shivakumar 2013). A study that particularly inspired the exercise protocols used in this project was performed by LJ Fulk and fellow researchers at the University of South Carolina and published in 2004. This study attempted to create an animal model to test the effects of chronic physical exercise on anxiety-related behaviors (Fulk et al., 2004). It involved 32 Sprague Dawley rats that were divided into two groups: runners (R), n=17 and non-runners (NR), n=15. The runners ran on a motorized treadmill for 45 minutes per day, five days per week, for ten weeks at a moderate intensity. Non-runners did not have access to the treadmills during the study but were handled by researchers daily. After these ten weeks of running, two behavioral tests were performed
including the elevated plus maze (EPM) and the open field test. The results from the EPM were that R rats showed more open arm time and more entries into the center of the open field, both of which indicate decreased anxiety-like behavior. Thus, the results from this study indicated that there is an anxiolytic effect of chronic exercise in rats. However, it should be noted that the form of chronic exercise used in this study was not voluntary, as the treadmill was motorized and thus encouraged the rats to continue running. Also, gentle air puffs were used to motivate running behavior when necessary. What we hoped for when designing this project was that voluntary wheel running would produce a similar anxiolytic effect to that seen in the Fulk (2004) study. In the protocol designed for this thesis, the rats were given 2.5 hours per week of access to voluntary exercise under the assumption that this would be enough time to encourage significant running activity. Therefore, although I and the other members of Dr. Tracy Butler’s lab had never previously incorporated voluntary exercise into any previous project, we figured that because of its proven positive benefits to humans (Anderson and Shivakumar 2013; Broman-Fulks and Storey 2008; Cox et al., 2004), and its capability to reduce anxiety-like behavior in rats (Fulk et al., 2004) that it could act as a viable behavioral intervention for this project. While designing the running protocol for this study, I looked into the methods used by researchers who had performed similar studies in the past and tried to duplicate those methods to the best of my ability. Several of the most informative studies I found used running wheels that were either attached to or inside of the rats’ cages, which allowed the rats to have 24-hour access to the running wheels (Greenwood et al., 2012; Sciolino et al., 2012). Although this could have been a more effective approach for this study to take, budgetary constraints prevented us from purchasing cages with attached running wheels and spatial constraints prevented us from fitting the running wheels inside of the rats’ original cages. Therefore, we were limited to constructing our own distance-monitoring system and running environment that the rats would be placed into during their periods of access. Upon reflecting on these methods, we realize that giving the rats only thirty minutes of exposure to the wheels per day may not have been enough time for significant running activity to occur. We came to this conclusion after seeing that rats in studies with 24-hour wheel access ran much longer average weekly distances (5-25 km) compared to our rats (0.002-0.077 km in 2016 and 0.019-0.06 km in
However, what we hoped for was that the wheels would provide a novel stimulus for the rats that would stimulate their curiosity and allow them to explore a new environment. The exercise protocol and the behavioral testing took place during the early part of the light phase (0900-1200 hours). The timing of the exercise protocol each day may not have been optimal, since it was performed during the light phase of the 12:12-hour light-dark cycle and previous literature has described how rats tend to do most of their running during the dark phase (Kregel et al., 2006).

**Elevated Plus Maze** - The EPM is a measure of anxiety-like behavior that has been previously used in numerous rodent studies (Sciolo et al., 2012; Butler et al., 2014a; Chappell et al., 2013) because it is based on rodents’ innate avoidance of heights and open spaces. The EPM was used in this study because of its widespread acceptance as a measure of anxiety-like behavior and our ability to use an animal-tracking software, Ethovision XT, to track the rats’ open arm time and closed arm frequency on the EPM. Open arm time is one of the most established parameters to assess anxiety-like behavior on the EPM, and has been used frequently in studies because generally the less time a rat spends on the open arms the higher its level of anxiety-like behavior. In an interesting and recent study published in 2017, a group of researchers attempted to translate the EPM from a measure of rodent anxiety-like behavior to a measure of human anxiety (Biedermann et al., 2017). The researchers built a human-size EPM out of wood for participants to walk on and also a representation of the EPM in virtual reality that the participants would experience. The results from this study include that the human participants reported having higher anxiety when on the open arms of the EPM and generally avoided them, which is comparable to rats’ responses on the EPM. Therefore, this behavioral test was included in this study because of its long history as a reliable indicator of anxiety-like behavior in rats, which we wanted to assess in order to determine how it was affected by voluntary exercise.

**Swim Stressor and CORT** - The swim stressor protocol was constructed by myself in collaboration with Dr. Butler after analyzing similar methods used in previous studies (Zareian et al., 2011; Wulsin et al., 2016; Rabasa et al., 2016; Solomon et al., 2014). The
goal of employing the swim stressor protocol was to assess the concentration of corticosterone (CORT), a stress hormone, in the rats’ blood both before and after exposure to a stressor. It can be expected that a rat with normal endocrine functioning would have a higher level of CORT in its blood after exposure to a stressor than before the stressor. In this project we did see significant increases in blood CORT concentration from baseline to the 5 minute and 30 minute post-stressor timepoints in both the 2016 and 2017 rat cohorts, confirming this expectation. It has also been demonstrated that rats undergoing chronic stress will produce more CORT and have higher levels of it circulating in their blood than non-stressed rats (Lowrance et al., 2016). Thus, we hypothesized in this project that the chronically stressed SI rats (both runners and non-runners) would have higher baseline CORT levels than their GH counterparts. However, we did not find this trend in the data from this study, and found no significant differences in baseline CORT levels between the groups of rats. There are other possible stressors that could have been used in place of the swim stressor such as acute footshock, forced restraint, or audiogenic stress, but the resources of our lab were best suited for the swim stressor. Also, forced swimming is regarded as a classical model of physical-activity-induced stress because it exposes rats to a novel and uncomfortable environment where enhanced stress can increase chances of survival (Paredes et al., 2005).

Drinking Procedure—The two-bottle choice home cage drinking procedure used in this study was adopted from several previous studies (Butler et al., 2014b; Butler et al., 2014a) and has been shown to lead to relatively high levels of ethanol intake in male Long-Evans rats (Chappell et al., 2013). The purpose for employing this drinking procedure was to assess whether or not the chronically stressed SI rats would display an increased level of ethanol consumption compared to their non-stressed GH counterparts. It has been mentioned in literature that individuals who are chronically stressed or have heightened anxiety are more likely to develop alcoholism than those who are not as stressed or anxious (June Ruan et al., 2008). Also, stress and anxiety pose as major risk factors for the development of an alcohol use disorder (AUD) in humans (June Ruan et al., 2008). Thus, in this study we were interested in modeling the development of increased ethanol intake in chronically stressed SI rats while also introducing voluntary
exercise in an attempt to lessen this ethanol intake in SI rats. However, the data we gathered from both cohorts show that the SI non-runners, who were hypothesized to have the highest stress levels and ethanol intake, showed the lowest (non-significant) average 24 hour ethanol intake compared to all other groups of rats (Figure 24b). Also, the GH runners, GH non-runners, and SI runners all showed practically equivalent average 24 hour ethanol intake levels. In terms of average 30 minute ethanol intake, the GH runners showed higher levels than the GH non-runners, and the SI runners showed higher levels than the SI non-runners (Figure 24c). This does not line up with our hypothesis, which proposed that the SI and GH runners would drink less alcohol than the SI and GH non-runners. Therefore, the rats that we expected to drink the most ethanol drank the least per day on average, and the rats that received the behavioral intervention drank more ethanol in the first 30 minutes of exposure than the rats that did not receive it. Compared to a study using a similar ethanol protocol where rats drank up to 8 g/kg of ethanol per day (Chappell et al., 2013), the rats in this study did not drink nearly as much ethanol (2.5-3.5 g/kg per day). This could be due to the inclusion of a period of forced ethanol consumption before the beginning of the two-bottle choice drinking protocol in the similar study and the lack of such a period in this study.

**Comparison Between Cohorts**

**Voluntary Exercise**- The results from the 2016 cohort suggested that the GH runners generally ran farther total distances than the SI runners and were more inclined to start running sooner after initial exposure than the SI runners. Running activity for this cohort declined around PND 43 for possible reasons including lack of novelty, limited access to the wheels, or lack of motivation to run. A pilot study was performed in early 2017 with six male Long-Evans rats that incorporated adding a small amount of peanut butter onto each rat’s wheel to promote running behavior, but the results were not encouraging. Thus, we decided not to incorporate peanut butter into the 2017 study. The results from the 2017 cohort were similar to those of the 2016 cohort, with GH runners displaying a higher inclination to begin running sooner than the SI runners and a greater average total distance run than the SI runners. Running activity began to decline in this cohort around PND 51. When the data from both cohorts were combined, it was discovered that the GH
runners had an average total running distance that was more than three times higher than
that of the SI runners. This data provides interesting insight into the effects of chronic
stress on voluntary exercise activity, and may suggest that chronically stressed rats are
less motivated or inclined to exercise than non-stressed rats. Although the drop in running
activity seen in both cohorts was discouraging, it can be suggested that it was a result of
either the lack of novelty of the running environment, poor timing of the running
protocol, or the limited amount of time the rats had to run each day.

EPM- No significant differences in open arm time were found between the groups of rats
in the 2016 cohort, although the SI runners displayed the longest open arm time of all the
groups. This data was encouraging to us, because it suggested at the time that voluntary
exercise could help reduce anxiety-like behavior in chronically-stressed rats. In terms of
closed arm frequency for this cohort, we saw that GH runners had significantly more
entries into the closed arms than the SI runners, which suggested that the GH runners
were more active on the maze than the SI runners. The results from the 2017 cohort were
different than those from the 2016 cohort, although still informative. No significant
differences were found between the groups on either measure of open arm time or closed
arm frequency, but there was a trend in the open arm data. The trend revealed that the GH
runners showed the longest open arm time of all groups and thus had longer open arm
time than the GH non-runners, and the SI runners showed longer open arm time than the
SI non-runners (which showed the least open arm time or most anxiety-like behavior).
This trend shows that the runners of both housing conditions displayed less anxiety-like
behavior than their non-runner counterparts, indicating the exercise may possibly help
reduce anxiety-like behavior. The open arm time data for the combined cohorts showed a
similar trend to that of the 2017 cohort, with GH runners displaying the most open arm
time and SI non-runners displaying the least (although there were no significant
differences between groups in this measure). This data agrees with our hypothesis that the
GH runners would show the least amount of anxiety-like behavior on the EPM and that
the SI non-runners would show the highest amount of anxiety-like behavior. The closed
arm frequency data for the combined cohorts showed no major trends and was fairly
consistent across all groups.
Swim Stressor and CORT- The data from the 2016 and 2017 cohorts are similar in that they both display a significant rise in plasma CORT concentration from the baseline timepoint to the five minute and thirty minute post-stressor timepoints. This increase in CORT concentration was also seen in the combined cohort data and was expected, since normally following exposure to a stressor (such as the swim stressor used in this project), an increased amount of CORT is released from the adrenal glands. To our disappointment, no significant differences in CORT concentration were found between the groups in either cohort at any of the three timepoints. Thus, we were not able to gain much insight into how voluntary exercise affects the CORT levels of chronically-stressed rats following acute stress besides that their CORT levels showed a normal upward trend. A study published in 2016 by Steven Lowrance and associates that used chronically stressed rats which were exposed to acute stress revealed that the rats exposed to chronic stress had significantly higher levels of CORT after five minutes of acute stress than the control rats (Lowrance et al., 2016). Therefore, it is possible that the chronically stressed SI rats that exercised did not have significantly higher CORT levels than the non-stressed GH rats after the swim stressor due to the stress-reducing effects of exercise.

Ethanol Intake and Preference- Besides a few significant differences between the groups in 24 hour g/kg ethanol intake and 30 minute g/kg ethanol intake, the data from the 2016 cohort did not reveal any major trends or stark contrasts worth noting. In other words, there was not a group of rats that showed greater alcohol intake or preference for alcohol at either timepoint. The data from the 2017 was very similar, with a few significant differences found between groups in 24 hour g/kg ethanol intake, 30 minute g/kg ethanol intake, and 24 hour preference percentage. Thus, this data was not very enlightening on the effects of voluntary exercise on the alcohol intake of chronically-stressed or non-stressed rats. The averages that were calculated for the combined cohorts showed little variation for each measure. The GH runners displayed the highest average 30 minute g/kg ethanol intake, contrary to our hypothesis that this group would display the lowest ethanol intake. The SI non-runners, from which we expected the highest g/kg ethanol intake, showed the lowest average 24 hour g/kg ethanol intake and 24 hour
preference percentage compared to all other groups. Thus, the data we collected opposed what we thought the expected results would be. Possible reasons for the lack of differences in ethanol intake and preference between the groups of rats could be the lack of a period of forced ethanol intake before the beginning of the protocol, the inclusion of a rat strain that was not bred for high ethanol intake in this project, or perhaps the use of an ethanol solution that was too potent for the rats to enjoy drinking (Besheer et al., 2013; Chappell et al., 2013).

**Conclusions**- Although this project gave us good support for our hypotheses that exercise could decrease anxiety-like behavior in SI rats and that the GH rats would exhibit less anxiety-like behavior than the SI rats, there were several limitations including the use of only male rats and the limited wheel exposure time of thirty minutes per day. As I explained earlier in the discussion, the reason that only male rats were used in this study is that they have reliably shown increased anxiety-like behavior and ethanol intake following an adolescence spent in socially isolated housing whereas female rats have not. Although, if this project were performed again I would want to include female rats as well as male rats in order to be able to observe the difference between them in each behavioral measure. On a more general note, male and female rats are different physiologically similar to how male and female humans are different, and this should not exclude one sex from being studied more in depth. Finally, the main behavioral intervention in this study, voluntary wheel running, may not have been as effective as we had hoped for several reasons. One being that the rats did not have 24 hour access to the wheels since they would not fit in the rats’ cages and we could not trust the rats to leave the odometer equipment alone. Upon reflecting on the results of this project, it can be said that it shed light on some behavioral trends of chronically-stressed rats and provided some solid support for our hypothesis that exercise could reduce anxiety-like behavior and alcohol intake in chronically-stressed rats. We discovered that GH rats tend to run more than SI rats, that GH and SI runners display slightly less anxiety-like behavior than their non-runner counterparts, and that all groups of rats show an intact stress response. It is our hope that the data from this project can contribute to the design of future studies,
and that adjustments will be made in those studies to correct for the limitations that this project faced. Finally, it is our hope that the findings from this study will be useful to those who are researching ways to prevent the onset of anxiety disorders or alcohol use disorders (AUDs) in humans. Since we have shown in this project that exposure to voluntary exercise during adolescence can lead to a reduction in anxiety-like behavior in both chronically-stressed and non-stressed rats, it can be suggested that voluntary exercise should be encouraged in humans from a young age in order to help prevent future anxiety disorders and possibly AUDs as well. Although there is much more research that needs to be done before any definitive conclusions can be made, the results from this project shed a hopeful light on the possibility of using voluntary exercise as a behavioral intervention during adolescence to reduce subsequent anxiety and alcohol abuse in humans.

**Acknowledgements**

I would like to thank everyone who was involved with this thesis project from its beginning in 2016 to its end in 2018, including my mentor, Dr. Tracy Butler, my lab colleagues Maddi Griff, Morgan Pair, and Hanna Peterson, and the University of Dayton Honors Program for choosing me to participate in the 2016 Berry Summer Thesis Institute. I have learned a great deal about the process of designing and conducting my own individual research project through this experience, and couldn’t have done it without the help of all of those involved.
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