EXAMINING A NOVEL METHODOLOGY OF LONG-TERM LOW-DOSE EXOGENOUS CORTICOSTERONE SUPPLEMENTATION IN NEOPHILIC RATS

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ABSTRACT

Inhibited rats have baseline and reactivity levels of corticosterone (CORT) approximately 30 ng/mL higher than noninhibited ones (given basal CORT levels of approximately 100 ng/mL), although CORT levels during the recovery period do not mirror this difference. This research established an adapted method of CORT supplementation in drinking water with the purpose of artificially creating this 30 ng/mL CORT increase in noninhibited neophobic rats in order to determine if these rats would become inhibited (neophobic). Typical studies testing the effects of CORT on behavior use large CORT doses on the scale of mg/mL in order to establish the presence of an effect as opposed to the degree of one. We systematically determined that a CORT concentration on the scale of µg/mL was necessary to achieve this slight increase. The CORT supplementation method implemented showed an increase in circulating blood serum, but this increase was variably higher than 30 ng/mL in all three nonadrenalectomized rats assigned to the experimental group. Further refinement of the dosing is necessary for future studies. There were no apparent trends in pre-CORT and post-CORT behavioral tests, suggesting that the slightly higher CORT level seen in neophobic rats is not the causal mechanism of their inhibited behavior.
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INTRODUCTION

Glucocorticoids: cortisol and corticosterone

Glucocorticoids (GCs) are steroid hormones produced in the adrenal cortex. They are involved in a wide array of physiological processes such as glucose metabolism and immune functions. GCs play an integral role in stress and anxiety response, and there is a well-documented positive correlation between circulating GC levels and stress/anxiety level (Sapolsky et al., 2000). Circulating GCs at sustained abnormally high levels cause numerous pathologies including hypertension, immunodeficiency, impaired growth and development, and depression (Buckingham, 2006). The primary GC in humans is cortisol, while corticosterone (CORT) is the primary GC in rodent models. In humans, for example, circulating levels of CORT are 10-20-fold lower than circulating levels of cortisol (Raubenheimer et al., 2006).

Cortisol is commonly known as the “stress hormone” as a result of its direct involvement in the stress response. The physiological response to stress is moderated by a rather complex and not fully understood set of feedback interactions between the hypothalamus, pituitary gland, and the adrenal gland. This system together is termed the hypothalamic-pituitary-adrenal axis (HPA axis). The hypothalamus releases corticotropin-releasing hormone (CRH), which stimulates the anterior pituitary to release adrenocorticotropic hormone (ACTH), which in turn stimulates the adrenocortical cells of the adrenal glands to release GCs such as cortisol. Cortisol then suppresses CRH and ACTH through negative feedback mechanisms. Stress provokes an inflammatory response, and cortisol works to prevent these responses from dangerously overshooting by dampening immune system function (de Kloet et al., 2005). When the stressor
is chronic, and, as a result, cortisol levels are chronically elevated, the hippocampus can atrophy, resulting in memory problems among other neurological disorders (Lupien et al., 1998). This hippocampal decay has been similarly demonstrated in nonhuman primates given high-dose exogenous cortisol as well as aged rats with elevated basal CORT levels (Leverenz et al., 1999; Sapolsky, 1992).

GC circulation is pulsatile and follows both a circadian and ultradian rhythm (Hellman et al., 1970; Weitzman et al., 1971; Lightman et al., 2008; Follenius et al., 1989). The circadian rhythm of GCs is modeled by a peak right before waking and a minimum early in the sleep phase (Hellman et al., 1970; Weitzman et al., 1971). Furthermore, GC release in both rats and humans follows an ultradian rhythm where discrete peaks occur approximately every hour in rats, and every 95-180 minutes in humans (Lightman et al., 2008; Follenius et al., 1989). In humans, an aberrant circadian rhythm of cortisol has been linked to burnout and insomnia (Pruessner, 1999; Backhaus, 2004).

**Human behavioral inhibition**

Common personality descriptors like ‘shy’ or ‘outgoing’ are termed ‘inhibited’ or ‘uninhibited’ in the scientific literature, and such behavior profiles are associated with unique physiological predictors including variations in resting heart rate, hypothalamic activity, and basal cortisol levels (Kagan et al., 1984; Kagan et al., 1987). Salivary cortisol levels in inhibited toddlers become elevated when subjected to novel events (Nachmias et al., 1996). Furthermore, stable behavioral inhibition in children is known to predict a higher risk of development of anxiety disorders later in life (Hirshfeld et al., 1992). It has been speculated that this is a result of high
cortisol levels in inhibited children inducing changes in the amygdala, causing an even greater fear response (Schmidt et al., 1998).

**Neophobia in rats**

In animal models, the analogous trait of inhibition is ‘neophobia,’ which refers to a hesitation to explore novel environments (Cavigelli & McClintock, 2003). Neophobia, in contrast to ‘neophilia,’ which refers to a willingness to explore novel environments, is associated with a larger increase in circulating GC levels, an indication of a more active HPA axis (Cavigelli & McClintock, 2003). Furthermore, the neophobia trait makes male rats 60% more likely to die at any time point during their lifespan than male neophilic rats (Cavigelli & McClintock 2003). Similarly, female rats phenotyped during infancy as neophobic died approximately 6 months earlier than female rats phenotyped during infancy as neophilic (Cavigelli et al., 2006).

These mortality rates suggest abnormalities in immune function. Neophobic rats were shown to have slightly higher pro-inflammatory responses to lipopolysaccharide (LPS) and significantly diminished delayed-type hypersensitivity (DTH) responses to keyhole limpet hemocyanin (KLH) than neophilic rats, although a causal relationship with slight but chronic elevations in circulating GC levels remains to be determined (Michael et al., 2009). Research suggests that although linked, neophobia and chronic GC elevation have additive effects in determining longevity and are not just behavioral and physiological indicators of the same mechanism (Cavigelli et al., 2009).
Novel environments can be subdivided into novel physical environments, in which inanimate objects provide the novel stimulus, and novel social environments, in which a second rat provides the novel stimulus. The GC response is not necessarily similar for both types of novel environments, with social inhibition being a more accurate predictor of adrenal activity than non-social inhibition (Cavigelli et al., 2007).

Additionally, GC levels can be described in more specific terms as well. Baseline levels, stress reactivity levels, and levels during the recovery period from stress exposure back to baseline can all be independently examined. It has been shown that inhibited rats had baseline and reactivity levels of CORT approximately 30 ng/mL higher than noninhibited ones, although CORT levels during the recovery period did not mirror this difference (Cavigelli et al., 2007).

**Exogenous CORT supplementation**

This pilot study principally sought to develop and assess a methodology of increasing basal CORT levels in neophilic rats by 30 ng/mL in order to model the basal CORT levels seen in neophobic rats (Cavigelli et al., 2007). Drinking water provided the obvious vehicle to do so, although CORT, a steroid hormone, is a hydrophobic molecule. Several studies resolved this issue by first dissolving granular CORT in the amphipathic molecule 2-hydroxypropyl-β-cyclodextrin (HBC) (Oitzl et al., 2001; Mikics et al., 2003; Schridde & van Luijtelaar, 2004). Other studies used ethanol, a more economical alternative, to initially dissolve CORT, which was ultimately the method we adopted (Coppola et al., 2005; Snihur et al., 2007).
In order to determine the appropriate concentration, several factors such as CORT pulsatility, GC feedback inhibition, and rate and amount of water intake needed to be considered. CORT pulses in adult male Sprague-Dawley rats occur approximately every 90 minutes (Lightman et al., 2008). Additionally, adult male Sprague-Dawley rats have approximately 15-20 drinking bouts each day, taking in approximately 2 mL of water during each bout (Marwine & Collier, 1979). This suggests that adult male Sprague-Dawley rats drink approximately every 90 minutes, which roughly corresponds with their ultradian CORT pulsatility. Rats also drink more frequently during their active dark phase of the light cycle and less frequently during their light phase. This behavior is beneficial as CORT levels are naturally higher in rats during the dark/active period, allowing exogenous CORT intake to mimic endogenous CORT production.

Gavaging adult male Long-Evans rats with 20 mg/kg of CORT directly into their gut results in a spike of circulating CORT of 1000 ng/mL approximately 15 minutes post-gavage, with circulating CORT returning to pre-gavage levels 3-4 hours later (Pung et al., 2003). This method of CORT gavage is not useful for long-term exogenous CORT supplementation, but it does demonstrate that CORT received orally will end up in the blood stream and also gives an indication of how long it takes CORT to move from the gastrointestinal system to the circulatory system. Although 30-40 ng/mL was the target CORT increase for a rat during the active phase of the light cycle, an increase of 50-60 ng/mL provided a better target level when taking into account the CORT clearance rate between drinking bouts (Cavigelli et al., 2007; Marwine & Coller, 1979).
Objectives

Neophobic rats have stable circulating CORT levels approximately 30 ng/mL higher than the levels seen in neophilic rats (Cavigelli et al., 2007). We sought to replicate this 30 ng/mL increase in neophilic rats over a 2.5-month period in order to determine if neophilic rats would begin behaving neophobically. On a simpler more methodological level, we sought to develop a sound method to exogenously supplement circulating CORT in neophilic rats. There were two goals in developing this methodology: 1) the CORT increase needed to be slight (only 30 ng/mL) and pulsatile, which prevents the use of methods seen in the literature that typically imposed abnormally high spikes in circulating CORT, and 2) the method needed to be feasible for long-term implementation to mimic the long-term increased CORT production seen in neophobic rats.

MATERIALS AND METHODS

1. General methods

Six adult male Sprague-Dawley rats were individually housed in wire cages. The room in which they were kept was maintained at 21.1 °C and a 12L:12D lighting schedule, with lights turning on at 2200 h EST. Animal caretakers provided food ad libitum and changed the waste collection papers three times per week, but not during implementation of the fecal collection protocol, during which lab personnel temporarily assumed this task. Since exogenous CORT was delivered via drinking water (CORTerade), lab personnel provided drinking water weekly and monitored water levels every day, as required by facility authorities. Rats were acclimated to the housing conditions for 20 days, during which time they also became acclimated to handling by lab personnel through daily weighings between 1500 h and 1800 h EST. After this period, the
rats were weighed on average 3 times per week for the duration of the study. The following methods received approval by the Pennsylvania State University Institute for Animal Care and Use Committee (IACUC# 28290; Appendix A).

**Figure 1:** Study timeline (days). Behavioral tests appear on the upper half of the timeline; all other measures and methods appear on the lower half.

### 2. Subject selection

A total of 6 rats, 3 displaying neophilic traits and 3 displaying neophobic traits, were selected for this pilot study. The phenotypes of the rats were determined based on their behavior during a test in which their cages were opened. Rats that immediately explored the cage opening were categorized as neophilic, while rats that were hesitant to explore the cage opening were categorized as neophobic. It is important to note that these rats were not ordered from a biological supply company, but rather they were selected from an available stock facing termination after being subjected to a prior experiment. The prior experiment involved ad libitum feedings of the shortening Crisco® after controlled periods of fasting.
2.1. Behavioral testing and CORT radioimmunoassay for phenotype confirmation

The rats underwent one session of novel environment testing (Appendix C) and one session of novel social testing (Appendix C) in order to confirm the suspected phenotypes of the rats based on the rudimentary open cage test. Blood samples (Appendix F) and fecal samples were taken from each rat to obtain estimates for basal circulating CORT levels by using a commercial radioimmunoassay kit (Rat & Mice Corticosterone Kit, MP Biomedicals, Solon, OH). Rats E1, E2, and E3 had the lowest serum CORT levels (Table 1) and were subsequently assigned to the experimental group. Conversely, rats C1, C2, and C3 had the highest serum CORT levels and were subsequently assigned to the control group. The behavioral phenotypes, quantified as the addition of latency to approach novel object (novel environment test) and latency to approach caged rat (novel social test) were consistent with their serum CORT levels for 4 of the 6 rats: E1, E2, C1, and C2. E3 and C3 displayed converse phenotypes, with E3 having the second slowest time and C3 having the third fastest time. The individually ranked latencies from the novel environment and novel social tests matched exactly, except for the 5th and 6th place (slowest) rats, which switched rankings between the two tests, although it was only important that the rats be consistent in ranking in the top 3 or bottom 3. However, it was decided that serum CORT levels were the more important measure as our primary objective was to increase circulating CORT levels by 30 ng/mL; our secondary objective was to relate this increase to altered anxiety-like behavior.

3. Determination of CORT dose

A 500 g rat would need to get 500 µg of CORT (as determined from the gavage study; 20 mg/kg gavage = 1000 ng/mL CORT spike) in each 2 mL drinking bout to realize a 50 ng/mL spike in
CORT, which would presumably average an increase closer to 30 ng/mL if the CORT levels over the entire 90 minute period between drinking bouts were averaged (Pung et al., 2003). This yielded a target dose of 250 µg/mL (500 mg/2 mL). Given the small number of studies used in calculating this dose and the questionable reliability of the method of calculation, a wide range of doses were tested. The doses tested were 0, 30, 100, and 300 µg/mL, with the 0 µg/mL dose serving as the control. To determine the actual concentration needed, each rat received 4 different randomized doses of CORT solution over a 4-day period (1 dose per 24-hour time period). Blood samples and fecal samples were taken each day, and the serum and fecal CORT levels were determined by radioimmunoassay.

3.1. Administration of CORT in drinking water (CORTerade) (Appendix B)

30 mg corticosterone (Sigma; catalog no. C2505) was dissolved, through vigorous vortexing, in 2 mL ethanol (100%). The CORT solution was diluted with tap water to a concentration of 30 µg/mL (a starting dose of 30 mg CORT creates one liter of solution). A 0.2% ethanol solution served as the control. The CORTerade and control solution were given to the rats on Day 82 of the study (Figure 1) at approximately 22:00 h. The tap water was removed from their water bottles and rinsed before the bottles were filled with either the CORTerade or control solution. The CORTerade and control solution were replaced at precise weekly intervals (22:00 h). Any remaining solution was removed, and the water bottles were rinsed before being refilled with the appropriate solution. The water bottles were weighed at the beginning and end of each week as a measure of CORTerade intake. The water bottles were checked daily to ensure that each rat did not run out of solution before the scheduled refill as well as to monitor and correct any leaking bottles.
4. Behavioral tests

The rats were subjected to 6 different behavioral tests: novel environment, novel social, elevated plus maze (EPM), light/dark, free choice, and sucrose preference. The rats were tested prior to implementation of the CORTerade protocol and again approximately 2.5 months later. Apart from the sucrose preference test, which is a 24-hour experiment, rats were only tested between the hours of 15:00 and 18:00, the period of the day during which they were most active. The arenas where the rats were tested were illuminated indirectly by a red light source approximately 3 meters away.

4.1. Novel environment test (Appendix C)

The novel environment test was run in a square arena constructed of white polypropylene walls with a clear plastic lid (120 cm x 120 cm x 46 cm). The lid was divided into a 3 x 3 grid in order to quantify locomotion. A novel plastic object (igloo, tunnel, and disc; Figure 2) was placed into 3 of the 4 corners of the arena, with the final corner serving as the home base and starting location of the rat being tested. Clean gravel bedding covered the floor of the arena.

Rats were tested in a randomized order. The rat being tested as well as a small metal dish that was present in each rat’s cage at all times were transferred to the free corner of the arena using a plastic dish placed in a clear plastic rectangular container that was placed side-down in the arena so that the rat could easily leave it. Rats remained in the arena for a duration of five minutes, which was videotaped should data need to be recoded. Locomotion and latency to approach a novel object were the two measures of interest. Locomotion was quantified by tallying the number of lines of the 3 x 3 grid the rat crossed. Latency to approach a novel object was
measured using a stopwatch and was the time from initial placement of the rat into the arena to the time the rat first made snout contact with one of the three novel objects. Other behaviors tallied include number of digs, rears, times when nose to object contact was made, times when both hands touched an object, times a rat climbed on an object, times a rat entered an object, grooming bouts, and presence of fecal boli or urine. If a rat failed to make contact with a novel object, its latency was recorded as 310.0 seconds. After the five-minute testing period, the rat and its metal dish were removed from the arena and returned to the rat’s cage. Feces would have been removed and discarded, although this incidence never occurred. The plastic dish and plastic container were sprayed with ethanol and dried before being used for the next test subject.

![Figure 2: The novel environment arena.](image)

### 4.2. Novel social test (Appendix C)

The novel social test was run in the same arena as the novel environment test. However, instead of three novel inanimate objects placed in three corners of the arena, an empty cage and a cage
containing an unfamiliar male rat were placed in opposite corners of the arena so that they were equidistant from the starting corner of the test subject.

When the sentinel rat was present in the room, it served as the caged stimulus rat. When the sentinel rat was not present in the room, a random test subject rat was selected to serve as the stimulus for the first trial only. After the first trial, the rat tested in the first trial became the caged rat for the remainder of the trials, and the test subject that served as the caged rat was returned to its cage. This rat was then tested last in order to minimize any behavioral impact of having served as the initial stimulus rat.

Testing protocol was identical to the novel environment test except instead of recording latency to a novel object, latency to both the rat cage and the empty cage were recorded instead.

![Figure 3](image.png)

**Figure 3**: The novel social arena.
4.3. Elevated plus maze (Appendix D)

The rats were randomly subjected to 5-minute trials in the elevated plus maze (EPM). The trials were videotaped. The following behaviors were tallied: number of entries into closed arms, entries into open arms, head dips, stretch attends, freezes, sways, rears, moments of grooming, and presences of fecal boli or urine. The inside surfaces of the EPM were wiped down with 100% ethanol between trials.

Figure 4: The elevated plus maze (EPM).

4.4. Light/dark test (Appendix E)

The rats were randomly subjected to 5-minute trials in a light/dark box. The trials were videotaped. The rats were initially placed into the dark side of the box. Latency to emerge from the dark side of the box was recorded as were the number of light entries, the total time the rat spent on the light side, and presence of fecal boli or urine. The inside surfaces of the light/dark box were wiped down with 100% ethanol between trials.
Figure 5: The light/dark box.

4.5. Free choice test (Appendix C)

The rats were randomly subjected to 5-minute trials of the free choice test, which was conducted in the same arena as the novel environment and novel social tests. A wire cage was placed in each of the two corners closest to the ‘starting’ corner. One of the cages was the subject rat’s home cage; this cage contained a familiar object. The second cage was a novel cage, which contained a novel object. Latency to approach the home cage and latency to approach the novel cage were recorded. The following behaviors were recorded as well for both the home and novel cage: number of instances of nose-to-cage contact, number of times both hands were on a cage, and number of times the rat entered a cage.
4.6. Sucrose preference test

In the sucrose preference test, the water bottles containing the experimental and control solutions were removed, and a water bottle containing a 1% sucrose solution and a water bottle containing tap water were attached to each of the 6 rat cages. A 1% sucrose solution is frequently used in the literature for Sprague-Dawley rats (Bekris et al., 2005; Grippo et al., 2006; Koo & Duman, 2007). The bottles were weighed before being attached to the cages. The rats were allowed to drink from either bottle ad libitum over a period of 24 hours. After 12 hours, the bottles were briefly removed and weighed before being reattached to the cages in switched positions. After 24 hours, the bottles were weighed a final time, and the water bottles containing the CORTerade and control solutions were reattached to the cages. The mass of tap water consumed and mass of sucrose solution consumed were compared to the total mass of liquid consumed. Rats that
consumed sucrose solution 65% of the time or less (i.e. 65% or less of the total mass of liquid consumed was sucrose solution) were said to be anhedonic, a criterion established from rodent control groups that never exhibited less than 65% sucrose preference (Strekalova et al., 2004).

5. **Blood sampling and blood processing** (Appendix F; Appendix G)

Rats were bled by severing the tip of their tails (approximately 1 mm) with a disposable scalpel and draining approximately 100 µl of blood into a microcentrifuge vial. Rats were bled for four consecutive days when determining the appropriate CORT dose. Two baseline blood samples were taken, and the rats were periodically bled once the CORTerade protocol was implemented (Figure 1). The blood samples were immediately microcentrifuged and the serum extracted for use in the CORT radioimmunoassay.

6. **Fecal sampling and fecal extraction** (Appendix H)

Fecal samples were collected for each rat at baseline and then periodically once the CORTerade protocol was implemented (Figure 1). On days when fecal samples were taken, they were collected at the following time points: 8:00, 12:00, 16:00, 18:00, and 0:00. “Wet” and “dry” fecal samples were collected in separate tubes as urine leeches CORT from the fecal samples (Cavigelli et al., 2005). All samples were dried in a vacuum centrifuge. The total weight of each sample was recorded. Only the originally dry fecal samples were used in the CORT extraction protocol; the wet samples were only dried and weighed because their mass was necessary to calculate total fecal CORT (ng). 100% ethanol was used to extract corticosterone from
standardized amounts of the dry fecal samples (Appendix H). The CORT radioimmunoassay was then run on these samples.

7. CORT radioimmunoassay

Serum samples and fecal extract samples were appropriately diluted and prepared per instructions of the Rat & Mice Corticosterone Kit (MP Biomedicals, Solon, OH), a $^{125}$I radioimmunoassay. Serum samples were diluted 1:200 and fecal samples (reconstituted in 1 mL methanol) were diluted 1:50 (to ensure antibody binding to fecal CORT) with diluent from the RIA kit (Cavigelli et al., 2005). The diluent and 25 pg standard were mixed 1:1 to produce a 12.5 pg standard, which was used in addition to the 6 standards provided in the kit in order to produce a more accurate standard curve. All samples were assayed in duplicates except the Day 155 fecal CORT samples as a result of limited first antibody and tracer. First antibody, tracer, and second antibody were added to serum samples in amounts indicated in the kit instructions (100 µL, 100 µL, and 250 µL, respectively), and these amounts were appropriately scaled to accommodate the 1:50 fecal sample dilution. After centrifuging the samples and removing the supernatant, a gamma counter (LKB model 1272) was used to determine the CORT concentration of the samples.

8. Sacrificing procedure

After completion of the second round of behavioral testing and collection of a final round of blood and fecal samples, the rats were sacrificed by CO$_2$ suffocation followed by cervical dislocation. The brains were immediately extracted and preserved. Both the left and right
adrenal glands were weighed if extracted successfully, although only the left adrenal was preserved.

9. Method of data analysis

The study design lent itself to $t$-tests between baseline and post-experiment averages of relevant behavioral test data for the experimental and control groups. However, the very small sample size of the experimental and control groups ($n = 3$ for each group) invalidated $t$-tests as an appropriate statistical method of data analysis. Consequently, the results are presented in a “case study” format illustrating the baseline and post-experiment value change for each rat (where applicable).

RESULTS

As seen in Table 1, phenotype characterization based on a neophilic profile of low serum CORT/short latencies and a neophobic profile of high serum CORT/long latencies was possible for 4 of the 6 test rats (E1, E2, C1, C2). Rat E3 and C3 displayed “mismatched” serum CORT levels and latencies.

Table 1: Baseline serum CORT and baseline combined latency data

<table>
<thead>
<tr>
<th>Rat ID</th>
<th>Serum CORT (ng/mL)</th>
<th>Nov. Env. and Nov. Soc. Combined Latencies (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>2.208</td>
<td>25.5</td>
</tr>
<tr>
<td>E2</td>
<td>2.286</td>
<td>26.0</td>
</tr>
<tr>
<td>E3</td>
<td>1.402</td>
<td>41.0</td>
</tr>
<tr>
<td>C1</td>
<td>8.950</td>
<td>65</td>
</tr>
<tr>
<td>C2</td>
<td>4.492</td>
<td>37.5</td>
</tr>
<tr>
<td>C3</td>
<td>16.176</td>
<td>37</td>
</tr>
</tbody>
</table>

The green data represents the lowest serum CORT levels (ng/mL) and shortest latencies (s) and the red data represents the highest serum CORT levels and longest latencies.
Dose determination was established from serum CORT measurements from all 6 test rats regardless of phenotype characterization (Figure 7). The 30 µg/mL resulted in the target increase of 30 ng/mL over control levels. The 3 CORT doses tested appear to increase circulating CORT linearly (approximately 1 ng/mL serum increase for every 1 µg/mL dose increase). Regarding the circadian pattern of this increase, the difference in fecal CORT metabolites (ng/g) between the 30 µg/mL dose and a 0 µg/mL dose ranged from roughly 20 – 50 ng/g, with the 50 ng/g increase occurring during the rat’s active period (20:00 h; Figure 8).

**Figure 7:** Serum CORT increase (ng/mL) for three doses of CORTerade (30 µg/mL, 100 µg/mL, and 300 µg/mL).
Figure 8: Fecal CORT metabolites (ng/g) of the test rats on a 30 µg/mL CORTerade dose and a 0 µg/mL CORTerade dose over 4 time points: 12:00 h, 16:00 h, 20:00 h, and 0:00 h.

Given this 30 µg/mL CORTerade dose or a control solution, the test rats assigned to the experimental group (E1-E3) or control group (C1-C3) did not display consistent behaviors, whether characteristically neophilic or neophobic, as quantified by the following measurements: latency to approach a novel object (s), latency to approach a caged unfamiliar rat (s), total time spent on the light side of a light/dark box (s), latency to approach a novel rat cage (s), or preference for a 1% sucrose solution compared to tap water (%) (Figures 9-11, 13, 14). The only instance of consistent behavior was seen in the EPM as measured by latency to enter an open arm (s). While all rats in the experimental group had latencies within the same 81-second range for baseline and end values, no rat in the control group entered an open arm during the end test (5-minute time limit) despite having all entered one before the time limit during the baseline test (Figure 12).
Figure 9: Baseline and end latencies (s) to approach any object for the 3 experimental rats (E1-E3) and 3 control rats (C1-C3) in the novel environment test. Rat ID/percent difference (%): E1/25; E2/-10; E3/-62.5; C1/-12.5; C2/0; C3/264.5.

Figure 10: Baseline and end latencies (s) to approach the rat cage for the subject rats in the novel social test. Rat ID/percent difference (%): E1/310; E2/-78; E3/81; C1/81; C2/1282; C3/-61.5.
Figure 11: Baseline and end values for total time (s) spent on the light side of the light/dark box. Rat ID/percent difference (%): E1/45; E2/19.5; E3/51; C1/-61.5; C2/65; C3/55.5.

Figure 12: Baseline and end values for latency (s) to enter an open open of the elevated plus maze (EPM). Rat ID/percent difference (%): E1/5950; E2/-10.5; E3/-98.5; C1/15; C2/869; C3/675.
Figure 13: Baseline and end values for latency (s) to approach the novel cage in the free choice test. Rat ID/percent difference (%): E1/-38; E2/-98.5; E3/41.5; C1/23.5; C2/-89.5; C3/-88.

Figure 14: Baseline and end values for percent 1% sucrose solution preference compared to tap water. Rat ID/percent difference (%): E1/-24; E2/-7.5; E3/12.5; C1/-18; C2/202.5; C3/25.
Despite a general lack of discernible trends in behavioral test results, there was still a noticeable increase in serum CORT over time in the experimental group, with an average increase of 62.1 ng/mL from the Day 44 and Day 76 baseline average to Day 116 (Figure 15). The control group showed an average decrease over this same period of 6.9 ng/mL. Total fecal CORT (ng) measured over time (different time scale than serum CORT) did not mirror the trend demonstrated in serum CORT levels and is inconsistent (Figures 16 & 17).

Figure 15: Serum corticosterone concentrations (ng/mL) at 5 time points. Rat ID/percent difference from average of Day 44 and Day 76 baselines to day 116 (%): E1/817; E2/1329.5; E3/952; C1/136; C2/-87.5; C3/-63.
Figure 16: Total fecal CORT (ng) at 3 time points. The values from the 8:00 h and 12:00 h samples of each time point were averaged to represent the trough of the circadian CORT cycle. Rat ID/Percent difference from baseline to Day 155 (%): E1/-70.5; E2/549; E3/863.5; C1/-24.5; C2/32; C3/-53.

Figure 17: Total fecal CORT (ng) at 3 time points. The values from the 16:00 h, 20:00 h, and 0:00 h samples of each time point were averaged to represent the peak of the circadian CORT
cycle. Rat ID/Percent difference from baseline to Day 155 (%): E1/-81.5; E2/156; E3/-8.6; C1/46; C2/362.5; C3/-7.

Average weekly water consumption varied between the 6 test rats, from a low of 144.6 g (C2) to a high of 231.5 g (C3), representing a range of 86.9 g (Figure 18). If the value of rat C3 is treated as an outlier and excluded, the range was only 40 g. The average weekly water intake for all test rats was 171.1 g with a standard deviation of 15.2 g.

Figure 18: Average weekly water consumption (g) of the 6 rats, excluding time points during which a water bottle unintentionally reached empty or was topped off with an unmeasured amount of tap water. Rat ID/standard deviation: E1/10.9; E2/18.1; E3/34.4; C1/8.1; C2/7.5; C3/12.1; E1-E3/19.0; C1-C3/49.2.

The right adrenal gland of the 3 rats in the experimental group weighed less than the lightest adrenal gland of a rat in the control group (C3; Figure 19). The average weight of the right adrenal in the experimental group was .024 g compared to an average of .03 for the control
group. Two left adrenals were unsuccessfully extracted, and therefore left adrenal weights were not included.

![Figure 19](image)

**Figure 19**: The weight (g) of the right adrenal gland of each test rat taken immediately after extraction post-sacrifice.

**DISCUSSION**

We were able to determine a concentration of CORT that increased circulating CORT levels by the target 30 ng/mL, at least for a 24-hour period (Figure 7). This concentration may be too high for long-term implementation (Figure 15), suggesting a need for preliminary dose determination over a period longer than 24 hours. Although the 24-hour period was reasonable when considering the clearance rate of CORT, the absolute increase of 62.1 µg/mL is twice as high as the target increase of 30 µg/mL, suggesting that there may be some sort of compounding effect. It is important to note that the 30 µg/mL target increase was established from a group of inhibited and noninhibited rats that had average basal CORT levels of approximately 130 ng/mL and 100 ng/mL respectively (Cavigelli et al., 2007). The basal CORT levels for the rats in this
study were much lower, in single or low double digits (Table 1; Figure 15). This gross difference in basal CORT levels may have influenced results. Determining the circulating CORT increases for a large variety of concentrations of CORT over a longer period might provide a good starting point for future refinement of this project.

Given that the rats were not adrenalectomized, it is not possible to distinguish whether endogenous or exogenous CORT was the primary cause of the large increases in serum CORT. Spikes in CORT caused from environmental factors during the days when blood and fecal samples were obtained may have resulted in varying elevations of endogenous CORT. These environmental factors were not necessarily self-controlled for as rats were individually handled during blood collection and may have reacted to the blood collection process with different degrees of anxiety. Furthermore, there was not enough primary antibody or tracer in the Rat & Mice Corticosterone Kit to test serum samples beyond Day 116 of the experiment (Figure 1). Testing the Day 155 and Day 161 samples may provide a better indication of how the 30 µg/mL dose influences CORT levels over a longer term. However, there was a one-week period prior to the Day 155 blood collection where the rats unintentionally received tap water instead of CORT. This may invalidate a generalization of how CORTerade affected CORT levels over a longer term. The numerous inconsistencies seen in Figures 16 and 17, which include Day 155 fecal CORT values, show that this may be the case. It may be possible to determine if the exogenous CORT had any suppressive effects on endogenous CORT production (if serum CORT levels for the Day 155 samples are below baseline), which is known to occur with very large CORT doses, although fecal data suggest it did not (Pung et al., 2003). However, the weights of the right adrenals of all 3 rats in the experimental group were lower than those of all 3 rats in the control
group (Figure 19), which suggests the opposite — that exogenous CORT may have had a suppressive effect on adrenal CORT release, although this may just be the result of coincidental natural variation. Prolonged exposure to exogenous GCs is known to cause adrenal atrophy (Krasner, 1999).

Administering exogenous CORT via drinking water is a common method reported in the literature, but these studies typically seek spikes in circulating levels beyond endogenous levels as a means of demonstrating an association between the hormone in general and some behavioral or physiological effect. Achieving circulating CORT levels within the endogenous spectrum (especially in nonadrenalectomized rats), is more difficult as several factors like rate and amount of water intake need to be taken into account. The dose was calculated with a conservative average weekly water intake of 210 mL/week (2 mL/drinking bout x 15 drinking bouts/day x 7 days/week), but only one rat (C3) actually reached and surpassed this estimate. Despite this, CORT levels in the experimental group were still higher than anticipated. The relatively small standard deviations of weekly amounts of water consumed (Figure 22) for both an individual rat and when grouped into experimental and control populations suggest that variability in amount of water intake does not merit a lot of concern. Although rat C3 drank a significantly higher amount of water than rat E3 (the second highest water consumer), that rat was in the control group and therefore received no exogenous CORT. Specific weekly values that were noticeably aberrant for rats E1-E3 (i.e. weeks 4 and 5 for rat E3, which were responsible for its moderately higher standard deviation) did not correspond with a blood collection or fecal sampling date, so it is not able to be determined to what degree these higher or lower water intake amounts may have influenced circulating CORT levels. Another point to consider is the stability of CORT in
drinking water. CORT in solution is stable for at least two weeks at 4 °C; however, no studies involving chronic CORT supplementation in drinking water indicate the frequency at which fresh solution is provided (Pechinot & Cohen, 1983). How much a temperature of 21.1 °C shortens this two-week period is not known, and it is possible that CORT molecules could have begun decaying towards the end of a one-week period. However, given the higher than desired serum CORT increases (Figure 15), this is likely not relevant.

Slow-release implantable CORT pellets offer an alternative and perhaps more controlled method of administering endogenous CORT. In adrenalectomized rats, a 50 mg CORT pellet produces values comparable to resting basal levels, and a 75 mg pellet produces constant CORT levels slightly higher than resting basal levels (Spinedi & Gaillard, 1998; Wilcoxon & Redei, 2007). It stands to reason that a CORT pellet at a lower dose (25 mg or less) implanted into nonadrenalectomized rats could cause a stable 30 ng/mL increase in circulating CORT levels regardless of their drinking behaviors.

The problem with this method is that an implant would attenuate the circadian pattern of endogenous CORT. Even though there would still be circadian variation if the adrenals were left intact as suggested, the constant levels of CORT supplied by the pellet would upset the normal physiological mechanisms of the HPA axis when CORT is not being released in the bloodstream. CORT replacement in drinking water is more effective than these subcutaneous pellets in normalizing stress induced ACTH secretion (Lightman et al., 2008). This demonstrates that the pattern of CORT replacement is just as important of a consideration as the amount.
CORT replacement in drinking water is very effective at promoting the natural circadian and ultradian patterns of CORT secretion as rats drink more during their active dark phase and also drink at a frequency similar to the frequency of pulsatile CORT release. However, individual variations in drinking behavior among rats as well as a host of external factors that could alter drinking behavior allow for a certain margin of imprecision. Subcutaneous pellets, which provide exogenous CORT regardless of behavior, greatly reduce this imprecision, but does so at the expense of the important phasic response of the GC receptors. One way to at least recreate the diurnal circadian pattern of CORT secretion in rats implanted with the pellet is by giving them a subcutaneous injection of CORT at the onset of the dark phase (Huang & Herbert, 2006).

Currently, there is no physiologically realistic method of CORT replacement, particularly regarding the ultradian rhythm of CORT secretion (Lightman et al., 2008). One method that was explored, of which its feasibility for commonplace utility is suspect, is a computer-driven fusion pump that can inject cyclodextrin-dissolved CORT into an adrenalectomized rat’s femoral vein at selectable pulse rates and amplitudes (Lightman et al., 2008). This is overall a sound method, although infusion pumps would have to be momentarily detached from the rats to run behavioral tests. Additionally, this technology could not function as an exogenous CORT supplement to endogenous CORT production as the chronic stress of a permanent IV attachment mandates that rats be adrenalectomized.

There appears to be one fledgling technology that could in the future reconcile all of these aforementioned problems. Prototypes of a multi-pulse drug delivery resorbable polymeric microchip device have been designed with dozens of reservoirs that can be filled with desired
compounds and released in a pulsatile fashion based on varying polymer compositions (and hence varying biodegradability) of the reservoirs (Richards Grayson et al., 2003). A similar technology has been theorized that uses a threshold level of electric current to dissolve reservoir caps made of gold, titanium, or platinum, which, unlike the polymer caps, would allow precise and immediate release of a desired compound into the bloodstream (Maloney et al., 2005). Manufacture of the polymer microchips is highly customized and the overall technology still largely theoretical, but it hints at where the future of exogenous pulsatile hormone replacement might lay.

It is not possible to draw many conclusions from this experiment relating long-term exogenous CORT supplementation to behavioral changes in the subject rats. The sample size was too small to allow statistical analysis. However, latency data for the EPM suggest that long-term CORT administration keeps rats from scoring longer latencies when compared to a control group, although this trend was not seen in any other test measuring latency (Figure 12). This opposes the initial hypothesis that chronic CORT administration would cause neophilic rats to act more neophobically and measure significantly longer latencies. Also, when a test rat was used as the social stimulus in the novel social test when the sentinel rat was not available, it was possible that the social stimulus rat was related to some or all of the test rats since they could have come from the same litter at the biological supply company. Rats are capable of sibling recognition, even sometimes if those siblings have never previously encountered each other, and this likely could have affected results by making the rat being tested less apprehensive to approach the caged “novel” stimulus (Porter, 1988). What can be somewhat safely concluded is that the 30 µg/mL dose of CORT solution is not high enough to cause any extreme alterations in behavior
(extreme enough to show statistical significant with n = 3). Testing the rats on an array of immune responses as opposed to behavioral ones might be a more realistic starting point given this relatively small CORT dose.

Only 6 rats (3 displaying neophobic traits and 3 displaying neophilic traits) were selected for this study based on their behaviors during the open cage test. However, the initial phenotypes of these rats did not necessarily match up with the phenotypes determined from the more valid behavioral arena tests. Furthermore, the phenotypes determined from these behavioral arena tests did not necessarily match up with the target basal circulating CORT levels (Table 1). In future experiments, a number of rats greater than the desired sample size can be selected during the open cage test and subjected to behavioral arena tests and blood sampling tests. This will allow for a better chance of achieving the desired sample size with all of the test subjects having the target behavioral and hormonal profile (i.e. neophilic traits with lower basal CORT levels and neophobic traits with higher basal CORT levels). Additionally, Rat E1 had the most erratic serum and fecal CORT measurements (and those measurements were excluded from the dose determination analysis). Excluding these types of rats from the subject pool may provide more coherent data, especially when the sample size is small.

It is possible that the HPA axes of the subject rats were fundamentally altered in the prior experiment involving ad libitum feedings of Crisco®. More valid results likely would have been obtained had the rats been ordered directly from a biological supply company. High-fat feedings alter both basal and stress-induced HPA activity in rats, acting as a background form of chronic stress (Tannenbaum et al., 1997).
Neuronal tissue that is exposed to excessive levels of GCs is known to degenerate as a result of high intracellular calcium, but mammalian brains possess a defense mechanism in the form of the calcium buffer, calbindin, to protect against this (McMillan et al., 2004). In a study in which macaques were subjected to chronic cortisol exposure for a 1-year duration, an increase in hippocampal calbindin was seen, suggesting that the primates shifted towards a GABAergic phenotype as a compensatory inhibitive mechanism against the excitatory effects caused by hippocampal GCs (McMillan et al., 2004). This mechanism suggests that rats may physiologically adapt to exogenous CORT (at the level of the hippocampus, not necessarily the adrenals) before the supplemental glucocorticoids have a chance to provoke increased anxiety. This may explain why there were no consistent behavioral differences between baseline and end test behaviors of the rats in the experimental group.
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Tannenbaum, BM, Brindley, DN, Tannenbaum, GS, Dallman, MF, McArthur, MD, & Meaney,


APPENDICES

Appendix A:

IACUC Approval of Protocol IACUC #28290

Date: May 13, 2009
From: William G. Greer, IACUC Administrator
To: Sonia A. Cavigelli
Subject: Results of IACUC Protocol Review – Annual Review (IACUC# 28290)

Approval Expiration Date: May 12, 2010

“Pilot Immune Measures with Sprague-Dawley Rats”

The Annual Project Review form for your protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) for the continued use of animals in your research. This approval has been granted for a one-year period.

Approval for the use of animals in this research project is given for a period covering one year from the date of this memo. If your study extends beyond this approval period, you must contact this office to request an annual review of this research.

This Institution has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare. The Assurance number is A 3141-01. As of February 13, 2001, The Pennsylvania State University was awarded Full Accreditation by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

By accepting this decision, you agree to notify the Office for Research Protections of (1) any additions or procedural changes and (2) any unanticipated study results that impact the animals. Prior approval must be obtained for any planned changes to the approved protocol. Any unanticipated pain or distress, morbidity or mortality must be reported to the attending veterinarian and the IACUC.

On behalf of the IACUC and the University, I thank you for your efforts to conduct your research in compliance with the federal regulations that have been established for the protection of animals.

If you are interested in subscribing or being removed from ORP listserv, send an email to: L-ORP-Research-L-subscribe-request@lists.psu.edu to subscribe or L-ORP-Research-L-unsubscribe-request@lists.psu.edu to unsubscribe. There is no need to add any text in the subject line or in the message body of the email.

WGG/ci

Attachment

cc: Robert H. Bonneau
    Kerry C. Michael
To the Investigator:

Please forward the enclosed original approval letter to your funding agency, if applicable. This approval is effective for one year. During this time, you should notify this office of any changes in the protocol that will affect the care and use of the approved animals or that will result in the use of additional animals.

In a continuing effort to comply with federal regulations, this office reviews IACUC approvals on an annual basis. On the anniversary of this approval, you should expect to receive a letter soliciting your request for an "annual review" by the IACUC. It is my hope that this process aids researchers in maintaining active IACUC approvals and avoids the use of animals without the proper approval.

Also, in order for records of your animal usage at ARP and ORP to remain current, please review the information below. If you feel there is any discrepancy between this information and your request, please contact our office (ORP) immediately at 865-1775. Thank you.

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Appendix B:

Titrating CORT in drinking water to get a 30 ng/mL increase in circulating CORT levels

S.A. Cavigelli, J.T. Gillon, K.C. Michael, C.M. Ragan, PSU Behavioral Neuroendocrinology Laboratory, R.H. Bonneau, Penn State Milton S. Hershey Medical Center

1) Determine total volume of CORT solution needed, and weigh out the required amount of CORT into a weigh boat necessary for a concentration of 30 µg CORT/mL solution.

2) Transfer CORT into an appropriately sized centrifuge tube.

3) Pipet the necessary amount of 100% ethanol into the centrifuge tube so that the final concentration of ethanol will be 2 mL EtOH/L solution.

4) Cap centrifuge tube and vortex until the CORT is fully dissolved (this will take about 10 minutes).

5) Using a graduated cylinder, transfer the required amount of tap water (not distilled water) into an appropriate container.

6) Pour the CORT solution into the container. Close and shake thoroughly.
Appendix C:

Testing rats on exploration arena

*S.A. Cavigelli, PSU Behavioral Neuroendocrinology Laboratory*

Test during dark phase of the light cycle. Illuminate test room with red light bulb(s) only, deflecting them so they do not cast a glare on the arena cover.

Sprinkle the field with several handfuls of bedding collected from each rat cage in the test animal’s colony room.

Center test arena under camera.

For ‘Novel Physical’ testing, place 3 novel objects in each corner, far enough away from the walls so that the rat may pass easily around all sides of the novel object.

For ‘Novel Social’ testing, place two wire cages in the corners closest to the ‘start bowl’. There is nothing to place in the fourth corner – furthest from the start bowl. In one of the two cages, place another rat from the colony, being careful to control for estrous cycle if testing females (i.e. use a female in the same phase as test subjects), and control for body weight (use a subject that is close to the body weight of the test subjects. Be sure that the cage with the novel social partner is securely closed/latched!

For ‘Free Choice’ testing, place two wire cages in the corners closest to the ‘start bowl’. One of these wire cages in the test rat’s own cage, while the second wire cage is a clean, unfamiliar cage. There is nothing to place in the fourth corner – furthest from the start bowl. In the rat’s own cage, place a familiar object. In the unfamiliar cage, place an unfamiliar object.
Always double check that you have enough space on DVD before starting new day of recordings.

On data sheet, record amount of light in center and edge of arena (using light meter and recording lux), room temperature, date, time, cohort, and initialize.

Rats are tested one at a time for 5 minutes each.

Begin video recording before placing animal in arena. (Press ‘REC’ on DVD recorder.)

IMPORTANT: Make sure ‘DVD’ light is illuminated on DVD recorder.

Transport rat from colony room to testing arena using the familiar bowl in their home cage.
Make sure to cover rat if traveling through areas with white light.

Place rat & bowl into side-turned clear cage and place the rat, bowl and cage into the free corner in arena. Make sure the opening of the side-turned cage faces the arena corner. Do this as gently as possible. A rough landing for the rat will freak them out and really affect their behavior.

Set timer for 5 minutes.

Code 'lines crossed' during the testing session. Count one line crossed when all four of the rat’s paws cross the line. Record the latency (seconds) required to first investigate a novel object or
the latency to investigate the novel social partner. Finally, record each time the rat’s nose touches novel objects, places both paws on object, and climbs on top of objects. Code the number of times the animal rears as well.

After an animal has been in arena for 5 minutes, turn off video recording, remove animal from arena and return to home cage. Turn lights on in testing room and count and remove fecal samples and record number on data sheet. Check for urine in test bowl and record whether there was urine or not (Y or N) on data sheet. Rinse larger bowl with tap water and dry for next rat.

At the end of the day, burn data onto DVD and label DVD with date, test name, and animal IDs.
Appendix D:

Elevated plus maze

S.A. Cavigelli, PSU Behavioral Neuroendocrinology Laboratory

Two experimenters are ideal for performing an EPM testing

- one scribe

  o before testing begins record on data sheet:
    ▪ date (don’t forget to put the year!)
    ▪ time (military time: 8:00pm = 20:00; 9:00pm = 21:00; etc.)
    ▪ temperature of testing room
    ▪ experimenter initials

  o when testing begins record on data sheet:
    ▪ animal ID (also indicate animal sex next to ID using ♀ for female and ♂ for male)
    ▪ time start (time animal is retrieved)
    ▪ time in (time animal is placed on maze)

  o record behaviors and animal location for 5 minutes

  o when testing ends
    ▪ indicate if animal urinated (i.e. yes/no)
    ▪ indicate number of fecal boli animal left behind on maze

- one shout out behaviors/directionality

  o assist scribe with transitioning from animal to animal
    ▪ clean EPM with 70% ETOH, 30% H₂O
      ● always wipe down EPM before beginning testing
• wipe down EPM after each animal is tested
  • return animal tested back to home cage
  • if testing a cage of more than one animal, place tested animal
    in a clean new cage temporarily until whole cage has been
    tested
  • retrieve next animal on testing list
    o maintain attentive visual on animal on monitor and verbalize animals’
      behavior and location for scribe to record
    o sweep up testing room and hallway when finished testing – leave no bedding
      behind on floors
    o remember to shut off monitor and DVD recorder at end of testing day
Appendix E:

Light/dark box

S.A. Cavigelli, PSU Behavioral Neuroendocrinology Laboratory

1) Place the rat ID numbers in sight of the video camera. Begin recording. This procedure will last 5 minutes for each rat.

2) Place the rat by itself in the dark side of the box. (Do not use the rat bowls.) Start the first stopwatch. Measure the time it takes for the rat to first enter the light half of the box. ‘Entering’ the light side of the box means getting all four limbs into the light side. Mark this as “latency to enter light side.”

3) As soon as the rat enters the light side, start the second stopwatch. Pause it each time the rat leaves the light side, and restart it when it re-enters the light side. Mark this as “total time spent on light side.”

4) Use the clicker to count how many times the rat enters the light side. Mark this as “times entered light side.”

5) At the end of five minutes, stop the recording and retrieve the rat. Wipe the box down with 5% alcohol solution between each test.

Info taken from:


One protocol has no light on the dark side of the box, and red light on the light side. Another protocol has the dark chamber lit by a 60-watt red bulb and the light by a 60-watt white incandescent. Adamec put the rat down in the dark side. Chaouloff set the rat on both sides in different tests.... I think since we’re looking at novelty, setting the rat in the dark side would be more appropriate. Otherwise, the two studies measure the same things: latency to move to the light side, number of visits to light side, duration of time spent in light side.
Appendix F

Blood collection

S.A. Cavigelli, K.C. Michael, PSU Behavioral Neuroendocrinology Laboratory

1) Prepare working surface:
   - Cover with absorbent paper
   - Load a #10 scalpel blade onto a scalpel handle
   - Place microcentrifuge tubes in rack in an easy-to-reach/find location
   - Lay out the KLH and sterile syringes so they are easy to reach
   - Place ice bucket and sharps container in easy-to-reach location

2) Carry one rat in its cage into procedure room and record time cage removed from colony room.

3) Place rat on table, belly down. Identify the appropriate microcentrifuge tube for rat and time point.

4) In one rapid movement, sever the tip of the tail (approx. 1 mm) using a sterile #10 scalpel blade.

5) Collect 100 µL of blood into pre-labeled tube by gently massaging the tail and allowing blood to enter a capillary tube. Hold the tube down to let gravity work for you. To avoid blood clots, do NOT touch tail tip directly to tube, but rather just touch each blood drop to capillary tube. Gently tap the capillary tube in the blood collection tube to collect blood sample. Close tube.

6) Record the time blood sample started and the time sample completed.

7) If required, place gentle pressure on the tail nick with sterile gauze to aid in clot formation.
Appendix G

Blood processing

*S.A. Cavigelli, PSU Behavioral Neuroendocrinology Laboratory*

1) Label two 2.0 mL microcentrifuge tubes per blood sample, with one tube labeled ‘total blood serum’ and the other tube labeled appropriately for easy identification for the CORT radioimmunoassay.

2) Microcentrifuge blood samples for 5 minutes at 2,000 rpm.

3) Pipet the serum layer of each sample into pre-labeled microcentrifuge tube. Try to collect as much serum as possible without getting any RBCs in the sample.

4) From the total serum tubes, pipet 10 µL of each sample into the appropriate 2nd pre-labeled microcentrifuge tube.

5) Freeze at -80 °C until ready for use.
Appendix H

Fecal extraction


sample preparation:

1) thaw and weigh sample
2) dry in centrifugal/rotary evaporator
3) reweigh dry sample to determine % water in sample (optional)
4) in a clean container (plastic sample bag is ideal) crush the sample into a fine dust (a hammer works nicely here)

extraction - “short method”:

5) weigh 0.2g crushed feces into 15ml polypropylene centrifuge tube with 10ml of 100% EtOH
6) boil for 20min
7) centrifuge at 2000g for 15min at RT
8) decant supernatant into 16mm x 125mm glass tube
9) add 5ml of 100% ethanol to pellet
10) vortex for 1min and centrifuge at 2000g for 15min at RT and decant supernatant into same 16x125 tube
11) dry down supernatant and resuspend thoroughly in 1ml MeOH (an ultrasonic glass cleaner can assist in the resuspension process)
12) pour into clean storage vial and place in -80 freezer (can be dried down for long-term storage)

Acknowledgments:

I would like to thank Dr. Sonia A. Cavigelli, my thesis supervisor, for guiding me through every step of this project as well as Dr. David J. Vandenbergh for serving as my honors adviser. Additionally, I would like to thank Christina Ragan for her significant assistance throughout this study. Lastly, I would like to thank the following individuals for their assistance as well: Kerry Michael, Molly Woehling, Sammy Jane Leathers, Megan Seymour, Brian Roger Coleman, and Hashim Chaudhry.
Academic Vita of Jason T. Gillon

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EDUCATION

The Pennsylvania State University, Schreyer Honors College, University Park, PA
B.S. in Premedicine; B.S. in International Studies

University of Cape Town, Rondebosch, South Africa
CIEE Study Abroad Program, Fall 08 Semester

Braddock Scholar, The Eberly College of Science

MEDICAL-RELATED EXPERIENCE

Certified First Aid/CPR/AED Instructor, Centre Communities Chapter
Centre County, PA, November 2006 – present
• Teach classes to Centre County community members as well as Penn State students
certifying them in First Aid, CPR, and AED

Penn State Externship Program – Orthopedics and Sports Medicine
Upland, PA, May 16-17, 2007
• Shadowed orthopedist for two work days

Mount Nittany Medical Center Volunteer
State College, PA, June 2007 – May 2008
• Transported and delivered medical equipment, prescriptions, and medical files
• Escorted patients out of hospital after discharge

Penn State Externship Program – General Surgery
Wyomissing, PA, May 13-14, 2008
• Shadowed general surgeon for two work days

Hershey Summer Clinical Preceptorship Program
Hershey, PA, June 1 –27, 2008
• Shadowed physicians during a two-week surgery rotation and a two-week rotation in
family and community medicine

Free Medical Clinic Volunteer
Khayelitsha, South Africa, August – November 2008
• Took patient histories, filled pharmacy orders, and assisted volunteer physicians in any
capacity needed

RESEARCH EXPERIENCE

Research Assistant in Neuroscience Laboratory
University Park, PA, May 2007 – May 2008
• Worked towards lab goal of determining molecular basis of learning, memory, and drug
addiction
• Conducted behavioral conditioning experiments on Drosophila melanogaster
• Performed ethanol exposure experiments on *D. melanogaster*
• Ran immunostaining protocols to view molecule of interest in fly neuronal tissue after manual dissection of fly brain

**Research Assistant in Behavioral Neuroendocrinology Laboratory**
University Park, PA, January 2009 – present
• Run behavioral tests for stress and anxiety on rodent models
• Research the impact of the hormone CORT on anxiety-like behavior

**TEACHING EXPERIENCE**

**Assistant Undergraduate TA for Biology Courses**
University Park, PA, Fall Semester 2007, Spring Semester 2010
• Assist TA who speaks English as a second language and promote clear communication between TA and students

**Undergraduate TA for Biology Courses**
University Park, PA, Spring Semester 2008, Spring Semester 2009, Summer Session II 2009, Fall Semester 2009, Spring Semester 2010
• Teach biology laboratory course and maintain full responsibilities for running the class

**OTHER ACTIVITIES**

**THON 2007 Rules and Regulations Committee Member – Pass Team**
University Park, PA, October 2006 – February 2007

**Student Red Cross Club Blood Drive On-Site Coordinator**
University Park, PA, October 2006 – May 2007
Coordinated volunteer efforts at on-campus blood drives

**Active Member of an Independent THON Organization**
University Park, PA, September 2007– February 2009

**UNIVERSITY AWARDS AND HONORS**

• Braddock Scholarship
• Schreyer Honors College Merit Scholarship
• Academic Excellence Scholarship
• President’s Freshman Award
• Phi Beta Kappa
• Dean’s List (all semesters)