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Morphine-Induced Tolerance Is Reduced By The Consumption Of A Ketogenic Diet, But Not A High Fat/high Carbohydrate Diet

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MORPHINE-INDUCED TOLERANCE IS REDUCED BY THE CONSUMPTION OF A
KETOGENIC DIET, BUT NOT A HIGH FAT/HIGH CARBOHYDRATE DIET

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Nina M. Beltran

2022

Dedication

To my better half, Jordan Aguon. Thank you for always believing in me.

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KETOGENIC DIET, BUT NOT A HIGH FAT/HIGH CARBOHYDRATE DIET

by

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Abstract

Opioid drugs like morphine are used medicinally for pain relief, but also have abuse liability that can lead to opioid use disorder (OUD), which contributes to nearly 70% of drug overdose deaths in the United States. Another rising health concern is the global obesity epidemic, and new evidence suggests that obesity and OUD might converge to impact individuals. For example, high body mass index (BMI) is associated with greater reports of pain among women. Further, opioid overdose risk is increased among individuals diagnosed with obesity, suggesting a potential relationship that might convey enhanced vulnerability among some populations. Given the high rates of obesity and pain-related conditions among women, in this study, 24 female rats ($n=8/\text{diet}$) eating either standard chow (17% kcal from fat), high fat chow (60% kcal from fat), or ketogenic chow (90.5% kcal from fat) were tested with cumulative doses morphine using a warm water tail withdrawal procedure to measure antinociception examined acutely (0.32 – 17.8 mg/kg; IP) and after 19-days of twice daily injections to assess for tolerance (3.2 – 56 mg/kg, IP). After chronic morphine administration, morphine was discontinued, and non-precipitated withdrawal signs were observed in rats for 5 days. Body weight, food consumption, and the adverse effects of morphine (e.g., fecal output, respiratory depression, body temperature) were recorded periodically throughout the study. It was hypothesized that rats eating high fat chow would be more sensitive to morphine-induced antinociception, tolerance following chronic administration, adverse effects of morphine (such as respiratory depression), and withdrawal following discontinuation of morphine, as compared to rats eating a ketogenic or standard chow. However, the antinociceptive effects of morphine tested during acute conditions did not differ among rats eating different diets. When tested during chronic administration, all rats developed tolerance to the antinociceptive effects of morphine; however, this effect was greater among rats

eating standard chow and high fat chow, as compared to rats eating ketogenic chow. The adverse effects of morphine (e.g., fecal output, respiration, and body temperature) were not different among rats eating different diets. Finally, observable withdrawal signs following morphine discontinuation was also not different between groups; however, rats eating ketogenic chow experience less withdrawal-related weight loss than other groups. These results demonstrate that while most of the effects of morphine are not altered by dietary intake, tolerance to the pain relieving effects of opioid drugs might not develop as severely for individuals eating a ketogenic diet. This might mean that patients taking opioids chronically for pain management should consider adopting a ketogenic diet to reduce the risk of tolerance, and therefore reduce the need to increase the dose of their pain medication over time.

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Introduction

The misuse of opioid pain-relieving drugs (e.g., morphine, oxycodone, and fentanyl) is a prevalent health concern, with nearly 3 million individuals in the U.S. currently diagnosed with opioid use disorder (OUD; Azafard et al., 2021). Opioid-related overdose deaths have increased nearly 6 times since 1999, contributing to 70% of drug overdose deaths in the U.S. in 2019 (Mattson et al., 2021; CDC, 2020). Opioid pain-relieving drugs bind to four G-protein coupled receptors, known as mu, kappa, delta, and nociception/orphanin opioid receptors and act as opioid receptor agonists, mimicking the actions of the endogenous opioid peptides (e.g., β -endorphin, enkephalins, and dynorphins; Al-Hasani & Bruchas, 2011; Gupta et al., 2021). Although these drugs bind to multiple receptors, their analgesic (or pain-relieving) effects are primarily due to their agonist activity at the mu opioid receptor which also mediates their rewarding effects (Al-Hasani & Bruchas, 2011). Agonist activity at the kappa opioid receptor induces pain-relieving effects but is also known to produce dysphoria and anxiety, while agonist activity at the delta opioid receptor induces anxiolytic effects (Valentino & Volkow, 2018).

While the misuse of opioids continues to rise, another prevalent health concern is obesity. It was reported that in 2018, nearly 43% of U.S. adults were diagnosed with obesity, and approximately 27% of patients diagnosed with obesity were prescribed opioids for long-term use (CDC, 2020; Stokes et al., 2020). Further, obesity diagnoses and opioid prescriptions are more prevalent among women as compared to men (CDC, 2020; Goetz et al., 2021). Pain-related conditions such as osteoarthritis, fibromyalgia, and lower back pain are also prevalent among patients diagnosed with obesity (Stokes et al., 2019). It has been reported that 77% of patients who used opioids chronically prior to bariatric surgery, increased opioid use following surgery (Raebel et al., 2013). Many genetic factors can contribute to the development of obesity, such as leptin

receptor, pro-opiomelanocortin receptor, and brain-derived neurotrophic factor mutations, along with overconsumption of high fat or high sugar foods (CDC, 2020; Thaker, 2017). There are a variety of current treatments for obesity, including pharmacotherapeutic approaches (e.g., phentermine), bariatric surgery, exercise, and dietary manipulations (CDC, 2020; Ruban et al., 2019). One example of a traditional dietary manipulation to treat obesity involves caloric restriction (e.g., restricting total calories consumed daily) or eating a high proportion of nutritionally balanced foods (e.g., diets high in fruits and vegetables, as well as rich in lean proteins; Aaseth et al., 2021). While food restriction has proven to be successful among patients, this kind of intervention can be quite challenging to implement and maintain. Additionally, this intervention might not be entirely accessible for all groups of individuals. For example, lower income households tend to buy less healthy foods as compared to high income households (French et al., 2019), which might be in part due to the high cost of produce and high-quality proteins as compared to more highly processed food items. One dietary manipulation that has been increasing in popularity for weight loss is a diet high in fat but very low in carbohydrates, known as a ketogenic diet (Batch et al., 2020; Moreno et al., 2014). A ketogenic diet has been shown to decrease medical costs for patients with diabetes and might improve metabolic markers (e.g., triglycerides) for patients diagnosed with obesity as compared to patients consuming low fat diets (Choi et al., 2020). However, it remains unknown if the consumption of distinct types of diets alters individual sensitivity to the therapeutic effects of opioids.

While opioid drugs like morphine remain a gold standard treatment option for pain management, these drugs have been over prescribed in recent decades, and nearly 30% of patients who take opioids for pain relief misuse use their prescription and are at risk for developing OUD (Al-Hasani & Bruchas, 2011; Upp & Waljee, 2020). Opioids are also used recreationally, as these

drugs can cause euphoria through their agonist activity at the mu opioid receptor, which impacts the mesolimbic pathway (WHO, 2021). While opioids are the most effective medications for pain relief, continuous or chronic use of these drugs can lead to the development of tolerance and withdrawal (Morgan & Christie, 2011). For example, with chronic exposure, tolerance to the pain-relieving effects of these drugs can develop and is defined by the need for increasing doses of drug to achieve pain relief (Morgan & Christie, 2011). Opioids can also induce physical dependence, evidenced by the presence of withdrawal following discontinuation of treatment, or the administration of an opioid receptor antagonist, which can contribute to relapse and overdose (Kosten & George, 2002). In humans, withdrawal from opioids includes symptoms such as diarrhea, nausea, vomiting, insomnia, and increased heart rate, according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5). In rats, opioid withdrawal is characterized by observable behavioral and physiological effects, including ptosis (upper eyelid droop), teeth chattering, wet dog shakes, paw tremor, and diarrhea (Gerak et al., 2019). To date, it is not known if the pain-relieving effects of drugs like morphine or the chronic effects of these drugs (e.g., the development of tolerance and withdrawal) are impacted by dietary manipulation.

Opioids can also induce other effects besides antinociception, tolerance and withdrawal, including adverse physiological effects like constipation, respiratory depression, and changes in body temperature (Benyamin et al., 2008; Rawls & Benamar, 2011). It has been reported that opioid-induced constipation occurs more in adult female patients as compared to adult male patients (Sizar et al., 2022), and this is a relatively uncomfortable side effect. To prevent constipation induced by opioids, physicians recommend that patients consume a diet high in fiber and low in fat (Sizar et al., 2022; Vakilli et al., 2015). It is recommended that adults consume 14 g of fiber per 1000 kcal daily for beneficial effects (Kranz et al., 2017), which is comparable to

the nutritional composition of a standard laboratory chow used in previous rodent experiments (13.7% fiber; [Teklad 7912]). In addition, opioid-induced respiratory depression is the hallmark symptom associated with overdose in opioid use (Baldo & Rose, 2022). Respiratory depression occurs due to the buildup of oxygen (O₂) and inadequate removal of carbon dioxide (CO₂; hypercapnia) from the lungs (van der Schier et al., 2014). Individuals diagnosed with obesity are at risk for developing breathing disorders (e.g., hypoventilation syndrome and sleep apnea [Speretta et al., 2018]); however, it remains unknown if dietary intake impacts sensitivity of individuals to opioid-induced respiratory depression. Endogenous opioids are known to increase or decrease body temperature based on specific receptor activation (Rawls & Benamar 2011). For example, hyperthermia and hypothermia are induced by activation of the mu opioid and kappa opioid receptor, respectively (Rawls & Benamar 2011). Small doses of morphine induce hyperthermia, and large doses produce hypothermia in rats (Geller et al., 1983) presumably via morphine's non-selective actions at both mu and kappa opioid receptors. It remains unknown if body temperature is differentially impacted in rats eating different diets after acute or chronic administration with morphine. When patients experience some or all these adverse effects, they often either discontinue their medication, or take an insufficient amount (e.g., reduced dose; Benyamin et al., 2008). In other words, patients sometimes choose to experience chronic pain rather than reduce their pain while simultaneously suffering the side effects of opioid medications. As such, understanding the impact of dietary intake on these adverse effects could help shed light on factors that might reduce their severity, improving overall acceptability and adherence for patients with chronic pain. In the present report, it was hypothesized that rats eating high fat chow would experience more adverse effects of morphine compared to rats eating standard and ketogenic chow.

There is substantial previous literature in rodents with psychomotor stimulants that demonstrates that drug sensitivity is modulated by dietary intake. For example, rats eating high fat chow are more sensitive than rats eating standard chow to the locomotor-stimulating effects of cocaine and methamphetamine (Baladi et al., 2012; Baladi et al., 2015; Ramos et al., 2019). Unpublished results from our laboratory further suggest that ketogenic diets might also increase sensitivity of rats to methamphetamine; however, this effect was more modest as compared to the effects of a traditional high fat diet (Elsley et al., in preparation). These data, taken together with limited previous reports exploring the impact of dietary manipulation on general nociception (pain sensation [Ziegler et al., 2005; Ruskin et al., 2013]) or the antinociceptive effects of drugs like morphine (Nealon et al., 2018), informed the hypothesis that female rats eating high fat chow would be more sensitive to the effects of morphine (including the antinociceptive and adverse effects, as well as withdrawal induced either by discontinuation of chronic treatment) as compared to rats eating a ketogenic or standard chow.

Chapter 2: Materials & Methods

2.1 Body Weight & Food Consumption: 24 female Sprague-Dawley rats (Envigo, Indianapolis, IN) arrived on post-natal day (PND) 20 and were housed individually in an environmentally controlled room under a 12:12 hour light/dark cycle with water available *ad libitum*. Starting on PND 23-25, rats were randomly assigned to dietary conditions ($n=8/\text{diet}$) and thereafter continued to eat standard chow (Envigo Teklad 7912; 17% kcal from fat) or were assigned to eat a high fat chow (Envigo Teklad 06414; 60% kcal from fat) or a ketogenic chow (Envigo Teklad 96355; 90.5% kcal from fat) for the duration of the experiment (see Table 2 for nutritional content).

2.2 Drugs: Morphine was purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and was dissolved in 0.9% saline and injected intraperitoneally (IP) at a volume of 1 mg/mL body weight.

2.3 Antinociception: To examine morphine's effects on thermal pain, 24 female rats ($n=8/\text{diet}$) were tested using a warm water tail withdrawal procedure, during which three water baths (EW-12105-84, Cole-Parmer, Vernon Hills, IL) were maintained at constant temperatures (40, 50, or 55 °C) throughout the experiment (see Table 1 for experimental timeline), and antinociception (i.e., the latency for rats to remove their tails from water maintained at each temperature) was measured using a stopwatch. Sessions comprised of 15-minute cycles, with an injection of saline given at the start of each cycle. After each injection, the rat was returned to its home cage for 13 minutes. Next, the rat was positioned on the palm of the experimenter's hand, and 5 cm of the tail was lowered into a water bath. Three water temperatures were tested in a randomized order and separated by 15 seconds. The maximum possible latency was 15 seconds per bath. The 15

second latency periods allowed for the minimization of tissue damage in situations when rats did not remove their tails (e.g., due to the analgesic effects of the drug). Vehicle injections were given in the first cycle, and cumulative doses of drug were given in the remaining cycles. Cumulative dosing for morphine increased by $\frac{1}{2}$ log doses (e.g., 0.32, 1, 3.2, 10, 17.8 mg/kg; IP) during the 15 min cycles. The largest dose of 17.8 mg/kg of morphine was only injected under acute conditions if 80% of antinociception was not reached at the 10 mg/kg observation, and this was determined for individual rats. Rats were habituated to the procedure for two weeks (with saline injections) before being tested with morphine. After morphine dose-response curves were generated under acute conditions, rats next received twice daily injections of morphine for 19 days at 0800 hours and 1800 hours increasing in $\frac{1}{4}$ log increments every 3 days up to 56 mg/kg. On the third day of scheduled treatment with 56 mg/kg; IP (day 19 of chronic administration) morphine-induced antinociception dose-response curves were generated to examine tolerance (e.g., 3.2, 10, 32, 56 mg/kg; IP). At 1800 hours that same day, rats received their final dose of 56 mg/kg morphine. The following day at 0800 and 1800 hours, and continuing for the next 5 days, rats received saline instead of morphine to examine the effects of morphine discontinuation (i.e., non-precipitated withdrawal).

2.4 Adverse Effects of Morphine:

2.4.1 Fecal Output: Cages (with fresh bedding) were changed the morning before antinociception testing (during 0800 and 1000 hours). Fecal output was weighed in g during 0800 and 1000 hours before and after saline and morphine injections.

2.4.2 Respiration: Respiration rate (% CO₂ output) was recorded using Field Metabolic System (FMS) and a Respirometry Flow Multiplexer (RM-8) from Sable Systems International

(Las Vegas, NV). Respiration rate was recorded 10-min after the last observation period with saline or morphine administered under acute conditions or 10-min after the final injection of morphine administered under chronic conditions, by placing rats individually in the respirometer chamber for 2 1.5-min intervals (staggered between 1 rat at a time). The slope was calculated for the CO₂ output in seconds and averaged based on the 2 intervals that were recorded.

2.4.3 Body Temperature: Body temperature (°C) was recorded at the end of each warm water tail withdrawal observation cycle following saline or morphine injections (under both acute and chronic conditions) with a rectal thermometer (as been done in previous studies [Gerak et al., 2019]; PhysiTemp Instruments, Clifton, NJ).

2.5 Withdrawal: During withdrawal observation periods, rats were kept in their home cage, but were moved into the same room where warm water tail withdrawal observations previously occurred. Food or water were not available during observation sessions. Observable withdrawal signs were recorded for each rat beginning 30, 60, and 90 minutes after the single injection of saline. Body weight as well as vocalization were recorded during handling and 13 observational signs of withdrawal (ptosis, teeth chattering, tongue protrusion, salivation, lacrimation, chromodacryorrhea, jumping, abdominal writhing, wet dog shake, rearing, paw biting, paw tremor, and diarrhea) were scored as present or absent during four 15-second intervals, separated by 15 seconds. If a particular effect was observed during at least one interval, it was recorded as present for the observation period. Thus, the maximum score for each observation period was 14, following methodology outlined in previous studies (Gerak et al., 2019). Signs of withdrawal from the 3 observation periods were added together and averaged based on dietary group.

2.6 Statistical Analyses: Body weight (g) and food consumption (g and kcal) were analyzed using a two-way mixed model ANOVA with diet and day as factors. Warm water tail withdrawal latencies were analyzed using a two-way mixed model ANOVA with diet and dose as factors. ED₅₀ values determined from individual antinociception dose-response curves were examined using the largest dose for which tail-withdrawal latency remained below 25%, the smallest dose for which tail-withdrawal exceeded 75%, and all doses in between, analyzed using a two-way mixed model ANOVA with diet and week as factors. Withdrawal signs were analyzed using a two-way mixed model ANOVA with day and diet as factors. Body weight during withdrawal was further analyzed as a % change in body weight (from non-withdrawal weights) using a two-way mixed model ANOVA. Fecal output (g) was analyzed using a two-way mixed model with drug (pre/post injection observations) and diet as factors. Fecal output was further analyzed as a % change from non-drug day values using a two-way mixed model ANOVA. Respiration (CO₂ output in seconds) was analyzed using an ordinary one-way ANOVA with diet as the factor. Body temperature (as a % change from saline) was analyzed using a two-way mixed model ANOVA with diet and dose as factors. Individual group comparisons were made using Tukey's multiple comparisons when appropriate, with statistical significance set at $p < 0.05$.

Chapter 3: Results

3.1 Body Weight & Food Consumption

Figure 1 displays average (\pm SEM) body weight for rats eating different diets collected starting on the last day of morphine administration and continuing for 5 days following morphine discontinuation. A two-way mixed model ANOVA revealed a significant main effect of day [F (25, 525) = 15.22; $p < 0.0001$], and a significant day by diet interaction effect [F (50, 525) = 5.548; $p < 0.0001$]; however, no main effect of diet was revealed ($p > 0.05$). Multiple comparisons tests revealed that on the second and third day following morphine discontinuation (day 21 and 22), rats lost a significant amount of body weight regardless of diet ($p < 0.0001$).

Figure 2 displays average (\pm SEM) food consumption in g collected starting on the last day of morphine administration and continuing for 5 days following morphine discontinuation. A two-way mixed model ANOVA revealed a significant main effect of day [F (25, 525) = 4.515; $p < 0.0001$], a significant main effect of diet [F (2, 21) = 29.23; $p < 0.0001$], and a significant day by diet interaction effect [F (50, 525) = 1.731; $p = 0.0020$]. Multiple comparisons tests revealed that rats eating standard chow consumed more g daily as compared to rats eating high fat chow throughout the study ($p < 0.0001$).

Figure 3 displays average (\pm SEM) food consumption in kcal collected starting on the last day of morphine administration and continuing for 5 days following morphine discontinuation. A two-way mixed model ANOVA revealed a significant main effect of day [F (25, 525) = 2.57; $p < 0.0001$], a significant main effect of diet [F (2, 21) = 42.62; $p < 0.0001$], and a significant day by diet interaction effect [F (50, 525) = 1.292; $p = 0.0928$]. Multiple comparisons tests revealed that rats eating ketogenic chow consumed more kcal on average than rats eating standard and high fat chow ($p < 0.0001$).

3.2 Antinociception

3.2.1 Acute Conditions

A two-way mixed model ANOVA revealed no significant main effect of dose, diet, nor any dose by diet interaction effect for the latency in seconds for rats to remove their tails from all water bath temperatures (data not shown) during the saline tests with morphine [data not shown; $p>0.05$] that preceded the acute morphine antinociception test. That is, the antinociception was comparable among groups following saline injections at all 3 temperatures.

Figure 4 displays the average (\pm SEM) latency in seconds for rats to remove their tails from the 50°C water bath temperature following morphine administration under acute conditions. A two-way mixed model ANOVA revealed a significant main effect of dose [$F(5, 98) = 81.60$; $p<0.0001$]; however, no main effect of diet nor any dose by diet interaction effect was revealed ($p>0.05$). Multiple comparisons tests revealed that the latency in seconds for rats to remove their tails was significantly increased following cumulative doses of 10 mg/kg and 17.8 mg/kg morphine as compared to saline for rats eating standard chow, high fat chow, and ketogenic chow ($p<0.0001$). That is, at larger doses of morphine, rats left their tails in the 50°C water bath longer than when they were administered saline, but this did not vary based on dietary group. A two-way mixed model ANOVA examining the average latency in seconds for rats to remove their tails from the 55°C water bath temperature following morphine administration under acute conditions revealed a significant main effect of dose [$F(5, 97) = 59.39$; $p<0.0001$]; however, no main effect of diet nor any dose by diet interaction effect was revealed ($p>0.05$). Multiple comparisons tests revealed that the latency in seconds for rats to remove their tails was significantly increased following a cumulative dose of 17.8 mg/kg morphine as compared to

saline for rats eating standard chow, high fat chow, and ketogenic chow ($p < 0.0001$). That is, following larger doses of morphine, rats left their tails in the 55°C water bath longer than when they were administered saline, but this did not vary based on dietary group. No main effects of diet or dose, nor any dose by diet interaction effects were revealed for the antinociception tests using the 40°C water bath temperature when morphine was administered acutely (data not shown; [$p > 0.05$]).

3.2.2 Chronic Conditions

Figure 5 displays the average (\pm SEM) latency in seconds for rats to remove their tails from the 50°C water bath temperature following chronic administration of morphine. A two-way mixed model ANOVA revealed a significant main effect of diet [$F(2, 21) = 5.341$; $p = 0.0133$], a significant main effect of dose [$F(4, 84) = 32.14$; $p < 0.0001$], and a significant dose by diet interaction effect [$F(8, 84) = 2.052$; $p = 0.0489$]. Multiple comparisons tests revealed that following the cumulative doses of 10 mg/kg ($p = 0.0039$) and 56 mg/kg ($p = 0.0001$) of morphine, rats eating ketogenic chow kept their tail in the 50°C water bath temperature significantly longer than rats eating standard chow. That is, while all rats left their tails in the 50°C water bath longer following morphine injections as compared to saline, rats eating ketogenic chow left their tail in the bath longer as compared to rats eating standard chow.

To examine differences between the morphine-induced antinociception tests that took place when morphine was administered acutely versus chronically (i.e., to examine the development of tolerance), a two-way mixed model ANOVA analyzing log ED_{50} values revealed a significant main effect of diet [$F(2, 21) = 4.986$; $p = 0.0169$]; and a significant main effect of week [$F(1, 21) = 185.6$; $p < 0.0001$]. However, no diet by week interaction effect was revealed ($p > 0.05$). Multiple comparisons tests revealed that rats eating ketogenic chow had significantly

smaller ED₅₀ values as compared to rats eating standard chow ($p=0.0008$) following chronic morphine administration. Further, multiple comparisons tests revealed significantly larger ED₅₀ values for rats following chronic morphine administration as compared to when morphine was administered acutely ($p<0.0001$). That is, after chronic morphine administration the morphine-induced antinociception dose-response curves shifted rightward 8.2-fold for rats eating standard or high fat chow, and 4.4-fold for rats eating ketogenic chow. Thus, while all groups developed tolerance to morphine, tolerance was reduced (or less developed) among rats eating ketogenic chow as compared to the other groups (Fig 5).

3.3 Adverse Effects of Morphine

3.3.1 Fecal Output

Figure 6 displays the average (\pm SEM) fecal output in g for female rats eating different diets based on fecal output weighed pre-drug and post-drug during the saline test and under acute and chronic conditions. During the saline test (two weeks prior to examination of morphine under acute conditions), a two-way mixed model ANOVA revealed a significant main effect of diet [$F(2, 14) = 45.67; p<0.0001$]; however, no main effects of drug (pre-saline/post-saline) nor any diet by drug interaction effects were revealed ($p>0.05$). Multiple comparisons tests revealed that both before and after the saline test, fecal output from rats eating standard chow weighed significantly more as compared to fecal output from rats in other groups ($p<0.0001$). When morphine was examined under acute conditions, a two-way mixed model ANOVA revealed a significant main effect of diet [$F(2, 14) = 47.04; p<0.0001$], and drug (comparing fecal output from days with or without morphine injections) [$F(1, 7) = 9.760; p=0.0167$]; however, no diet by drug interaction effect was revealed ($p>0.05$). Multiple comparisons tests revealed that fecal

output for rats eating standard chow weighed significantly more as compared to rats eating high fat or ketogenic chow ($p=0.0223$) both before and after acute morphine administration.

Following chronic morphine administration, a two-way mixed model ANOVA revealed a significant main effect of diet [$F(2, 14) = 21.23$; $p < 0.0001$]; however, no main effects of drug (comparing fecal output from days with or without cumulative morphine injections) nor any diet by drug interaction effects were revealed ($p > 0.05$). Multiple comparisons tests revealed that fecal output from rats eating standard chow weighed significantly more as compared to rats eating ketogenic chow ($p=0.0001$) and rats eating high fat ($p < 0.0001$) before and after morphine administration that followed chronic morphine exposure. That is, throughout the study, the weight of fecal output was always greater for rats eating standard chow as compared to the other groups, and this did not change with morphine when administered under acute or chronic conditions.

To examine the % change in fecal output in g normalized from the day prior (when saline was administered), a two-way mixed model ANOVA a significant main effect of drug (e.g., cumulative doses of morphine assessed under acute conditions or following chronic morphine administration) [$F(1, 21) = 40.55$; $p < 0.0001$]; however, no effects of diet, nor any drug by diet interaction effects were revealed ($p > 0.05$). Multiple comparisons tests revealed that fecal output from all groups following chronic morphine administration was significantly increased as compared to following acute morphine administration (data not shown; $p < 0.05$). Rats in all groups produced more feces by weight after chronic morphine administration, as compared to when morphine was administered acutely.

3.3.2 Respiration

Figure 7 displays the average (\pm SEM) CO₂ respiration rate collected following saline injections or morphine-induced antinociception tests under acute conditions or following chronic morphine administration. An ordinary one-way ANOVA for CO₂ output revealed no group differences in CO₂ output/seconds among rats tested after saline or cumulative doses of morphine (assessed under acute conditions or following chronic administration; $p>0.05$). A two-way mixed model ANOVA for the CO₂ respiration rate % change from saline revealed a significant main effect of week [F (1, 21) = 12.36; $p=0.021$]; however, no main effects of diet nor any week by diet interaction effects were revealed ($p>0.05$). Multiple comparisons tests revealed that there were no group differences in respiration rate when rats were tested with morphine, regardless of if this was assessed under acute conditions or following chronic administration (data not shown [$p>0.05$]). That is, respiration rate was not differentially impacted among rats eating different diets throughout the study.

3.3.3 Body Temperature

A two-way mixed model ANOVA revealed no significant main effects of dose, diet, nor any dose by diet interaction effects in body temperature (data not shown) when rats were tested with saline (2 weeks before acute administration with morphine [$p>0.05$]). That is, average body temperature values for rats eating different diets collected after saline injections were not different.

Figure 8 displays the average (\pm SEM) % change in body temperature for rats eating different diets normalized based on their body temperature values collected during the saline observation period that occurred prior to cumulative morphine doses assessed under acute conditions. A two-way mixed model ANOVA revealed a significant main effect of dose [F (4, 76) = 10.93; $p<0.0001$]; however, no main effects of diet nor any dose by diet interaction effects

were revealed ($p>0.05$). Multiple comparisons tests revealed that body temperature became significantly cooler after cumulative doses of 1-17.8 mg/kg morphine as compared to saline for all groups ($p<0.05$). That is, following these smaller doses of morphine under acute conditions, all rats developed hypothermia, regardless of dietary condition.

Figure 9 displays the average (\pm SEM) % in body temperature for rats eating different diets normalized based on body temperature examined at the saline observation period that occurred prior to cumulative morphine doses assessed following chronic morphine administration. A two-way mixed model ANOVA revealed a significant main effect of dose [F (3, 63) = 23.61; $p<0.0001$]; however, no main effects of diet nor any dose by diet interaction effects were revealed ($p>0.05$). Multiple comparisons tests revealed that body temperature became significantly warmer following injections of 32 mg/kg and 56 mg/kg morphine, as compared to saline, in all groups ($p<0.0001$). That is, following these larger doses of morphine and chronic administration, all rats developed hyperthermia, regardless of dietary condition.

3.4 Withdrawal

Figure 10 displays the average (\pm SEM) withdrawal signs collapsed across the 3 observation periods observed for 5 days beginning the day of the final examination of morphine-induced antinociception (which occurred following chronic morphine administration), after which morphine injections were replaced with saline (e.g., when the discontinuation of morphine began). A two-way mixed model ANOVA revealed a significant main effect of day [F (5, 105) = 7.074; $p<0.0001$]; however, no main effects of diet nor any day by diet interaction effects were revealed ($p>0.05$). Multiple comparisons tests revealed that, as compared to 1 day following

morphine discontinuation, observable withdrawal signs for all groups of rats were significantly increased 3 days after morphine discontinuation ($p<0.05$).

Figure 11 displays the average (\pm SEM) % change in body weight for rats eating different diets normalized based on body weight examined on the last day of chronic morphine administration. A two-way mixed model ANOVA revealed a significant main effect of day [F (4, 84) = 60.82; $p<0.0001$], diet [F (2, 21) = 4.943; $p=0.0174$], and a significant day by diet interaction effect [F (8, 84) = 4.054; $p=0.0004$]. Multiple comparisons tests revealed that, as compared to 1 day following morphine discontinuation, body weight for all groups of rats significantly decreased 2 days after morphine discontinuation ($p<0.0001$). Further, as compared to day 4 following morphine discontinuation, body weight for all groups significantly increased 5 days after morphine discontinuation ($p<0.0001$). Multiple comparisons tests also revealed that body weight (examined as a % change from the day before morphine discontinuation took place) was significantly greater for rats eating ketogenic chow as compared to rats eating standard chow on day 4 ($p=0.012$) and 5 ($p<0.0001$) and for rats eating high fat chow ($p=0.0123$) of withdrawal observations. That is, rats eating ketogenic chow gained back significantly more weight after 4 and 5 days following discontinuation from morphine, as compared to rats eating standard or high fat chow.

Chapter 4: Discussion

4.1 Summary

The goals of this thesis were to examine the effects of eating high fat or ketogenic chow on morphine-induced antinociception, adverse effects (e.g., respiration, fecal output and body temperature) and morphine-induced tolerance and withdrawal among female rats. Acute and chronic injections of morphine did not differentially impact body weight (Fig 1) food consumption (Fig 2-3), fecal output (Fig 6), or respiration (Fig 7) among rats eating different diets. Further, morphine-induced antinociception (Fig 4) under acute conditions was not different among rats eating different diets. A major finding from this study was that while all groups of rats developed morphine-induced tolerance, this tolerance occurred to a greater extent among rats eating a high fat or standard chow, as compared to rats eating the ketogenic chow (Fig 5). Following the discontinuation of chronic morphine, observable withdrawal signs among rats eating different diets were not significantly different (Fig 10); however, as rats recovered from withdrawal, those eating ketogenic chow gained more of their body weight back as compared to rats eating standard chow, 4 and 5 days after morphine discontinuation (Fig 11).

4.2 The effects of acute administration of morphine in rats eating different diets

To assess the antinociceptive effects of morphine under acute conditions, rats eating different diets were administered cumulative doses of morphine (0.32 – 17.8 mg/kg; IP) using a warm water tail withdrawal procedure. It was hypothesized that rats eating high fat chow would be more sensitive to the acute antinociceptive effects of morphine as compared to rats eating standard and ketogenic chow. However, in the present experiment, the antinociceptive effects of morphine (administered under acute conditions) were not significantly different for rats eating

different diets (Fig 4). This suggests that when given acutely, morphine might be equally effective at providing pain relief, regardless of the dietary habits of patients. This is consistent with literature describing the effectiveness of morphine for patients with and without an obesity diagnosis (Patanwala et al., 2014). This is also consistent with some previous work assessing acute morphine antinociception using the hot plate procedure in female mice fed either a high fat or standard chow (Nealon et al., 2018). However, these results are not consistent with other reports of dietary impact on pain processing assessed in rodent models (Nealon et al., 2018; Kanarek et al., 1997; Ruskin et al., 2013). For example, in contrast to our results (Fig 4), two separate reports demonstrated that rats eating a high fat diet (either a manufactured high fat chow, [Nealon et al., 2018] or a hydrogenated vegetable fat [Crisco ©] plus 32% sucrose solution [Kanarek et al., 1997]) were more sensitive to morphine-induced antinociception using the tail flick procedure as compared to mice eating standard chow. Further, in another previous study, rats eating ketogenic chow were hypoalgesic (i.e., had decreased sensitivity to thermal pain) as assessed using the hot plate procedure (Ruskin et al., 2013). Notably, in this latter report pain thresholds were assessed in the absence of an opioid (Ruskin et al., 2013); however, this could be compared to the saline values for our acute morphine-induced antinociception tests, during which no group differences were observed (Fig 4). One possible explanation for these discordant results could be the use of different types of antinociception assays in previous studies as compared to ours (e.g., hot plate or tail flick as compared to warm water tail withdrawal). While hot plate, tail flick and warm water tail withdrawal assays all examine thermal pain, the physical responses (dependent variable) are different for each assay (e.g., paw removal from a hot plate, tail flick from a light beam, and tail withdrawal from a warm bath) and the physiological mechanisms underlying these responses also differ. For example, the tail-flick and

warm water tail withdrawal responses are mediated spinally, while the removal of a rat's paw from a hot plate is mediated supraspinally (Nealon et al., 2018; Gardmark et al., 1998). While tail-flick and warm water tail withdrawal are similar in terms of the physiological mechanism underlying the animals' response, it has been reported that the location of the thermal stimulation on a rat's tail might contribute to differential changes in morphine-induced antinociception observed in one assay versus the other (Yoburn et al., 1984). These differences between assays might contribute to the differences observed in the present report as compared to previous studies.

4.3 The effects of chronic administration of morphine in rats eating different diets

To assess the antinociceptive effects of morphine in rats eating different diets after 19-days of twice-daily injections with morphine, rats were tested with cumulative doses of morphine using a warm water tail withdrawal procedure (3.2 – 56 mg/kg; IP). It was hypothesized that rats eating high fat chow would be more sensitive to the antinociceptive effects of morphine following chronic administration, as compared to rats eating standard or ketogenic chow. In the present experiment, the morphine-induced antinociception dose-response curves shifted 8.2-fold to the right among rats eating standard and high fat chow, but only shifted 4.4-fold to the right among rats eating ketogenic chow. That is, all groups developed tolerance to morphine after chronic administration; however, this effect was greater for rats eating standard or high fat chow as compared to rats eating ketogenic chow (Fig 5). These results are not consistent with some previous studies assessing the impact of dietary intake on sensitivity of rodents to tolerance induced by opioids. For example, female mice eating high fat chow were more tolerant to morphine as compared to rats eating standard chow using the tail flick procedure (Nealon et al.,

2018). However, in that same report, rats eating high fat chow were less tolerant to morphine as compared to rats eating standard chow using the hot plate procedure (Nealon et al., 2018).

In the present report, we used specific methodology to induce tolerance (Gerak et al., 2019); however, there are other schedules of drug administration that also induce tolerance and withdrawal (Mucha et al., 1979). It is possible that using a different schedule of chronic drug administration might lead to different results, perhaps by inducing tolerance to a different degree than observed here. Additionally, in this study we only assessed tolerance at one time point (e.g., at the end of chronic administration) due to delays related to drug availability from our supplier (Sigma-Aldrich, St. Louis, Missouri, USA). It is possible that group differences in tolerance might have been revealed had we examined tolerance at other time points during the chronic administration schedule. Finally, the use of different thermal pain assays might also have yielded group differences that are distinct from the results here, because as mentioned the different assays result in different types of responses which are differentially mediated by distinct physiology (Nealon et al., 2018; Gardmark et al., 1998). It is therefore possible that tolerance development might not follow a common timeline when examined across all assays. Future experiments could incorporate these variations of methodology to explore these possibilities.

The differences in tolerance observed between groups of rats eating different types of chow in the present report could also be due to distinct pharmacological mechanisms. For example, there are 3 types of tolerance that can occur with chronic administration of opioids: metabolic, pharmacodynamic, and behavioral tolerance (Collett, 1998). Metabolic (or enzymatic) tolerance is associated with changes in drug absorption, distribution, and metabolism, and can occur when repeated exposure to drug results in an increase in the number of metabolic enzymes available to break down the drug (Dumas & Pollack, 2008). Opioids are metabolized by a family

of enzymes called the cytochrome P450 enzymes, which are found primarily in liver cells and break down morphine into inactive and activate metabolites (McDonnell & Dang, 2013; Smith, 2009). For example, morphine is broken down to the inactive metabolite, normorphine, and the active metabolites morphine-3- and 6- glucuronide (Smith et al., 2009). Previous work suggests that these cytochrome P450 enzymes are differentially altered following the intake of different diets (Yang et al., 1992; Sadler et al. 2018). For example, cytochrome P450 enzymes increase during a state of ketosis, and decrease for mice eating a traditional high fat chow (Yang et al., 1992; Sadler et al., 2018). In the present study, we did not determine whether the animals eating the ketogenic diet developed ketosis; however, other studies have demonstrated that the consumption of this same ketogenic chow (Teklad 96355) results in ketosis within 48-hours of access (at least in male rats, see Ródenas-González et al., 2022). Importantly, if in the present study ketosis did develop among rats eating the ketogenic chow, previous work might suggest that due to an increase in cytochrome P450 enzymes (Yang et al., 1992), this group would have developed tolerance more rapidly than rats eating other diets. However, the opposite occurred (Fig 5); rats eating ketogenic chow developed tolerance to a lesser extent than rats in other groups. Therefore, it seems likely that the group differences seen in the present study are not driven entirely by enzymatic tolerance, though this type of tolerance might have occurred, and could be contributing to these effects in combination with other types of tolerance.

Pharmacodynamic tolerance can occur when binding availability of a receptor decreases over time (Dumas & Pollack, 2008). This can be caused by a variety of factors, but one potential possibility is a change in total number of available receptors on the cell surface for drugs like morphine to bind to (e.g., upregulation or downregulation of receptors). For example, chronic administration with morphine has been shown to lead to the downregulation of the mu opioid

receptor (Stafford et al., 2001). If there are fewer opioid receptors available for morphine to bind to, this would impact the dose-response curve for morphine-induced antinociception.

Specifically, in other studies demonstrating mu opioid receptor downregulation, the ability of morphine to relieve pain decreases, and corresponds to a rightward shift of the morphine-induced antinociception dose-response curve following chronic administration (Morgan & Christie, 2011). In our experiment, morphine dose-response curves shifted rightward 8.2-fold for rats eating standard or high fat chow and shifted 4.4-fold for rats eating ketogenic chow. While the impact of dietary intake on the effects of chronic administration of morphine on opioid receptor availability are not fully characterized, it is possible that binding availability of the mu opioid receptor is decreased overtime differently among rats eating different diets independently of downregulation that might be caused by chronic morphine administration. There is some evidence suggesting this might be the case; however, most studies have focused on mu opioid receptor mRNA changes (which are generally decreased following consumption of a high fat diet; see Pitman & Borgland, 2015 for a review), rather than cell-surface available (e.g., biotinylated) receptor expression. This distinction is important because while mRNA levels might be altered, this does not always correspond to a comparable change in cell-surface available receptor protein expression (Murgas et al., 2022). Indeed, our results suggest that even if receptor mRNA is decreased by eating a high fat diet, this did not correspond to differences in antinociception between rats eating standard chow and rats eating high fat chow, which might suggest that cell-surface available receptors are not altered by eating a high fat diet. To date, there is little known about how eating a ketogenic diet could alter opioid receptor availability; however, one report demonstrated no differences in mu opioid receptor mRNA expression between male mice eating ketogenic or standard chow (Blanco-Gandia et al., 2021). It remains

unclear how pharmacodynamic effects (e.g., changes to available mu opioid receptor, or changes in expression of other opioid receptors that also contribute to antinociception, such as kappa opioid receptors) alone or in combination with other types of tolerance might contribute to changes in antinociception after chronic morphine administration.

Finally, the last type of tolerance that can occur following chronic opioid exposure is behavioral tolerance. Behavioral tolerance can occur when environmental cues are paired with drug administration, resulting in associative learning (Collett, 1998; Vogel-Sprott, 1997). Changes in environmental cues (e.g., the room where drug is administered) impact the development of opioid-induced behavioral tolerance (Siegel, 1976). For example, when opioid administration occurs within the same environment, behavioral tolerance might develop. If the context changes (e.g., rats are administered the same opioid in a different or distinct environment), rats will overdose, suggesting that the tolerance in those studies was context/environment specific (Siegel et al., 1976). In the present experiment, rats were always administered morphine in the same environment; however, no assessment of the development of behavioral tolerance was specifically conducted. It is possible that behavioral tolerance developed, and it is also possible that this might have been different among rats eating different diets. The extent to which eating different diets impacts the development of opioid-induced behavioral tolerance is not yet known. However, some reports suggest that rats eating high fat chow do develop behavioral tolerance to the locomotor effects of a mu opioid receptor agonist (Lee et al., 2019; though importantly, this study did not include a group fed a control diet for comparison).

4.4 The adverse effects of morphine in rats eating different diets

The adverse effects of morphine (e.g., changes in fecal output, respiration, and body temperature) were assessed periodically throughout the study. It was hypothesized that rats eating high fat chow would be more sensitive to the adverse effects of morphine during acute and chronic administration, than rats eating standard or ketogenic chow. In the present experiment, rats eating high fat or ketogenic chow had less fecal output as compared to rats eating standard chow, throughout the duration of the study (Fig 6). The weight of fecal output in g was not changed by acute injections of saline or cumulative doses of morphine for rats eating different diets (Fig 6). In previous studies, there was greater fecal output from rats eating a diet high in fiber as compared to rats eating a diet low in fiber following acute injections of morphine (Niwa et al., 2002). In the present experiment, standard chow (Teklad 7912) contained more fiber per g as compared to the high fat (Teklad 06414) and ketogenic (Teklad 96355) chows, which might account for the increase in weight of fecal output from rats eating standard chow (Fig 6). One important methodological note is that in our study, fecal output was weighed just one time a day (in the morning the day before and the day after morphine administration). Previous studies have assessed fecal output hourly (up to 6 hours) following morphine injections (Gerak et al., 2019). It is possible that rats in our study might have experienced constipation in a time-dependent manner which was not observable using our specific methodological approach. Additionally, while constipation is commonly reported by patients taking opioids for pain relief (Benyamin et al., 2008; Swegle & Logemann, 2006), some individuals instead experience frequent bowel movements and diarrhea during chronic use (Bril et al., 2011) and withdrawal following discontinuation from opioids (Shah & Huecker et al., 2022; Camilleri et al., 2017). In the present study, rats eating high fat or ketogenic chow had reduced fecal output following acute morphine administration (i.e., these rats appear to have developed constipation) while rats eating standard

chow did not have reduced fecal output (i.e., appear to not have developed constipation). However, all rats had increased fecal output following chronic morphine administration, but for rats eating standard chow (which as mentioned contains more fiber than the other two diets) fecal output was significantly greater at the time of the tolerance test as compared to rats eating high fat or ketogenic chow (Fig 6). Therefore, it is possible that the different fiber content of the individual diets provided might have impacted overall fecal output weight following morphine administration, in ways that yielded group differences. However, besides macronutrient distinctions, the diets used in the present study also differ in terms of ingredient refinement (e.g., refinement and use of types of grains) which might also contribute to differences in fiber absorption (Envigo, Indianapolis, IN).

In the present study, we were especially interested in exploring any potential impact of dietary intake on respiratory depression, because this is the main adverse effect of opioids that can lead to overdose (Baldo & Rose, 2022). Individuals diagnosed with obesity are at a higher risk for developing breathing disorders and opioid-induced respiratory depression (Speretta et al., 2018; Freire et al., 2020). In contrast to this epidemiological data, in the present report, there were no differences in respiration among rats eating different diets throughout the study (Fig 7). There are different ways to measure respiration in rodents (Hsia et al., 2013). For example, some newer methods include the examination of changes in blood gases (as compared to the older invention of a respirometer), by collecting partial pressure of CO₂ from arterial blood in an anesthetized animal (Hsia et al., 2013). It is suggested that collection of CO₂ from arterial blood is more accurate than collection of CO₂ through a respirometer (Severinghaus & Astrup, 1986). In the present report, respiration was measured by examining respiratory rate for individual rats occurring in 2 1.5 min intervals (Sable Systems International [Las Vegas, NV]). While there

were no statistically significant differences in overall respiration rate for groups of rats eating different diets throughout morphine administration, during the first habituation test, rats eating high fat chow had significantly greater CO₂ output as compared to rats eating standard chow ($p=0.0184$ [data not shown]). However, this effect did not persist at additional time points or following morphine administration. It is possible that the way that CO₂ output was examined in the present report (e.g., as % respiratory rate) is less accurate than other methods and might have resulted in inconsistent results throughout the study. Future directions should consider the use of other respiratory tests (e.g., analysis of blood gases), or the calculation of volume of CO₂ expressed to include the factor of water vapor (Speretta et al., 2018) to examine potential differences in CO₂ output in rats eating different diets.

In the present experiment, there were no group differences in body temperature following acute morphine administration for rats eating different diets; however, morphine induced hypothermia in all rats regardless of dietary condition. (Fig 8). This is consistent with previous work demonstrating that female mice eating standard or high fat chow had significantly cooler body temperature following acute morphine administration (Nealon et al., 2018). However, previous studies have demonstrated that small doses of morphine induce hyperthermia, while large doses produce hypothermia in rats (Geller et al., 1983). In the present experiment, body temperature for rats in all groups was significantly warmer following 32 mg/kg and 56 mg/kg morphine injections following chronic morphine administration (Fig 9). This is consistent with previous work demonstrating that after the development of tolerance, morphine increases body temperature (Mucha et al., 1987). Opioid-induced hyperthermia is mediated by the mu opioid receptor, and opioid-induced hypothermia is mediated by the kappa opioid receptor (Handler et al., 1992; Rawls & Benamar, 2011). It is not clear if morphine's effects on body temperature in

the present study under acute versus chronic conditions are differentially regulated by distinct actions on mu versus kappa opioid receptors, but future work could explore this possibility. Additionally, other research suggests that while morphine's direct effects are on opioid receptors, indirect effects on other systems (e.g., glutamate) also contribute to the development of tolerance and hyperthermia (Rawls & Benamar, 2011). As such, it is possible that morphine's effects on non-opioid systems might also contribute to the distinct effects on body temperature observed in the present report, although this was not directly tested. Additionally, there is at least one previous report that has examined the impact of ketosis on non-opioid systems (e.g., glutamatergic systems; Gzielo et al., 2020) that might be implicated in the development of tolerance and hyperthermia for rats eating ketogenic chow.

4.5 Withdrawal observation signs in rats eating different diets

To assess observable withdrawal signs in rats eating different diets after chronic administration (see Table 3), morphine was discontinued and we observed 14 signs of withdrawal (vocalization, ptosis, teeth chattering, tongue protrusion, salivation, lacrimation, chromodacryorrhea, jumping, abdominal writhing, wet dog shake, rearing, paw biting, paw tremor, and diarrhea [Gerak et al., 2019]). That is, in the present study non-precipitated withdrawal (withdrawal following the discontinuation of chronic administration) rather than precipitated withdrawal (withdrawal induced by the co-administration of an opioid receptor antagonist) was assessed. It was hypothesized that rats eating high fat chow would display more withdrawal symptoms as compared to rats eating standard and ketogenic chow. In the present experiment, there were no differences in withdrawal signs in rats eating different diets (Fig 10); however, withdrawal signs were significantly greater among all groups on day 3 and 4 (i.e., 3 or

4 days after the start of morphine discontinuation) as compared to day 1 and 2 of withdrawal testing (Fig 10). That withdrawal signs were greatest after 3 and 4 days following morphine discontinuation is not consistent with previous studies examining non-precipitated withdrawal in male rats (Gerak et al., 2019). However, there are known sex-differences regarding the severity of non-precipitated withdrawal following chronic morphine administration (Bobzean et al., 2019). For example, female rats display less spontaneous withdrawal signs overall as compared to male rats, but the presence of withdrawal signs persist longer for female as compared to male rats (Bobzean et al., 2019). In the present experiment, as the effects of withdrawal dissipated, rats eating ketogenic chow gained back significantly more weight on day 4 and 5 following chronic morphine discontinuation as compared to rats eating standard chow (Fig 11). Previous studies have demonstrated that morphine can decrease body weight and food consumption; however, this did not occur in our study (Fig 1-3; Boghossian et al., 2001; Mucha & Kalant, 1979). There is limited research on the mechanism(s) of action underlying withdrawal signs and weight loss following chronic administration with morphine, and as such, it remains unclear why rats eating a ketogenic chow gained weight back sooner as compared to rats in other groups.

In the present study withdrawal was assessed after an injection of saline, corresponding to a discontinuation of chronic morphine administration (e.g., non-precipitated withdrawal). Our rationale for studying non-precipitated withdrawal, as opposed to precipitated withdrawal, was that the administration of an opioid receptor antagonist (e.g., naloxone) would yield more robust withdrawal signs in all groups, that might limit our ability to explore group differences. That is, we anticipated precipitated withdrawal would produce a ceiling effect, limiting the ability to detect small differences between groups (Gerak et al., 2019). However, studying precipitated withdrawal might have yielded different results and should be explored in future studies.

Although not much is known about how dietary intake might impact precipitated withdrawal, food-restricted female rats displayed more withdrawal signs as compared to rats with free access to standard chow (Kanarek et al., 2009). This suggests that while precipitated withdrawal does lead to more robust effects than non-precipitated withdrawal, the ability to detect group differences might remain intact (e.g., ceiling effect might not preclude the ability to detect group differences in precipitated withdrawal studies).

4.6 Conclusion

The present work investigated the effects of diet on sensitivity of female rats to the antinociceptive effects of morphine, morphine-induced tolerance and withdrawal, as well as the adverse effects of morphine on fecal output, respiration, and body temperature. Based on previous work, it was hypothesized that rats eating high fat chow would be more sensitive to the antinociceptive effects of morphine under acute and chronic conditions, and to tolerance and withdrawal following the discontinuation of chronic morphine as compared to rats eating standard or ketogenic chow. In the present experiment, dietary manipulation did not impact sensitivity of rats to the antinociceptive effects of morphine under acute conditions; however, rats eating ketogenic chow were less tolerant to morphine as compared to rats eating standard chow following chronic morphine administration. Further, there were no group differences in any other measure assessed (i.e., the adverse effects of morphine or withdrawal following chronic morphine administration), though rats eating ketogenic chow appeared to recover from withdrawal differently than rats eating one of the two other diets (i.e., body weight recovered differentially between groups). The results of this thesis suggest that morphine-induced tolerance is reduced (or perhaps not developed as completely) among rats eating a ketogenic diet, but not

rats eating a high fat/high carbohydrate diet. Individuals diagnosed with obesity taking opioids chronically for pain related conditions might therefore consider eating a high fat/low carbohydrate (i.e., ketogenic) diet to reduce the risk of developing robust tolerance to these drugs. Reducing the development of tolerance might provide more consistent, long-term pain-relief for individuals diagnosed with chronic pain conditions and might also reduce individual risk of the development of OUD (by eliminating or decreasing a need to escalate dose over time). This supports the growing literature that consuming a ketogenic diet has beneficial effects for several health conditions, including obesity and pain-related conditions (Masino & Ruskin, 2013; Ziegler et al., 2005; Ruskin et al., 2013).

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Appendix

Table 1. Experimental Timeline

The experiment began on postnatal day (PND) 20, when rats arrived at the facility. During PND 23-25 rats were assigned to groups and dietary manipulation began, such that some rats continued to eat standard chow, while rats in other groups began eating a high fat or a ketogenic chow thereafter. Starting on PND 97 rats were tested using the warm water tail withdrawal procedure under saline conditions to habituate to experimental conditions. Starting on PND 111, rats were tested using the warm water tail withdrawal procedure under acute conditions. Rats underwent 3 different acute conditions throughout the study due to disruptions occurring during observations, and delays related to drug availability from our supplier (Sigma-Aldrich, St. Louis, Missouri, USA). Starting PND 195, rats were tested a 3rd time under acute conditions (PND 194) to confirm antinociceptive effects did not differ based on age before starting chronic administration of morphine. Starting on PND 213, rats underwent discontinuation from morphine and were observed for withdrawal signs. * represents PND corresponding to data presented in the current thesis.

Arrival	Diet Change	Antinociception (Warm Water Tail Withdrawal)	Withdrawal
PND 20	PND 23-25	Saline: PND 97-99, 104-106, *181-183 Acute: PND 111-113, 118-120, *194-196 Chronic: *PND 195-215	*PND 213-219
		Fecal Output Body Temperature Respiration	Observe Withdrawal Signs

Table 2: Nutritional Content

Nutritional contents for the dietary conditions broken down by % by weight.

Feeding Conditions	Carbohydrates	Protein	Fat
Standard	44.3%	19.9%	5.7%
Keto	0.3%	9.2%	90.5%
High Fat	27.3%	23.5%	34.3%

Table 3: Withdrawal Timeline

The withdrawal phase of this experiment began on the final day of chronic morphine administration. On this day (PND 213), following morphine injections rats were observed though no observational signs of withdrawal or changes to body weight were anticipated. The following morning, instead of morphine injections at 0800 hrs, rats received an injection of saline to begin morphine discontinuation (i.e., non-precipitated withdrawal). On day 1-5 of morphine discontinuation, after the 0800 hrs injection of saline, rats were observed for four 15-second intervals and were scored as present or absent for signs such as ptosis, teeth chattering, tongue protrusion, salivation, lacrimation, chromodacryorrhea, jumping, abdominal writhing, wet dog shake, rearing, paw biting, paw tremor, and diarrhea. Additionally, body weight was measured daily to examine withdrawal-related weight loss and recovery following protracted abstinence.

Final Day of Morphine	Day 1	Day 2	Day 3	Day 4	Day 5
Warm water tail withdrawal (3.2-56 mg/kg; IP)	800 hrs Saline				
Withdrawal Signs (control)	Withdrawal signs				
1800 hrs 56 mg/kg morphine	1800 hrs Saline				

Figures

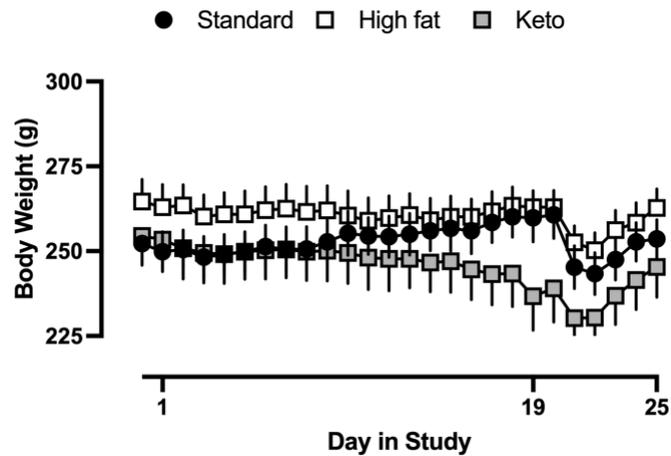


Figure 1. Mean (\pm SEM) body weight (g) collected during morphine administration and withdrawal (see Table 1 details) in female rats eating standard chow (closed symbols), high fat chow (open symbols), and ketogenic chow (shaded symbols).

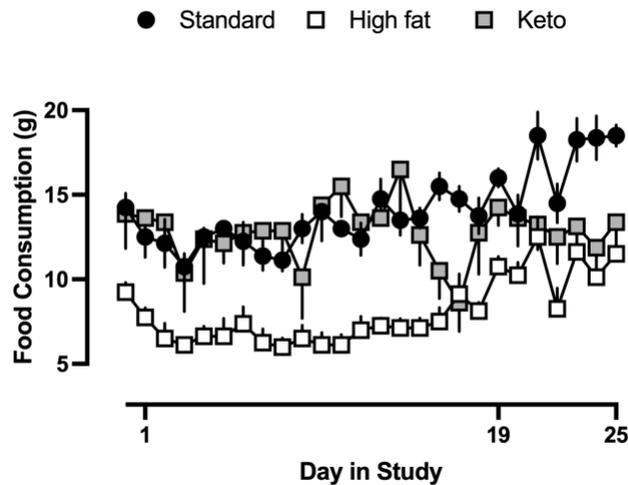


Figure 2. Mean (\pm SEM) food consumed (g) collected during morphine administration and withdrawal (see Table 1 details) in female rats eating standard chow (closed symbols), high fat chow (open symbols), and ketogenic chow (shaded symbols).

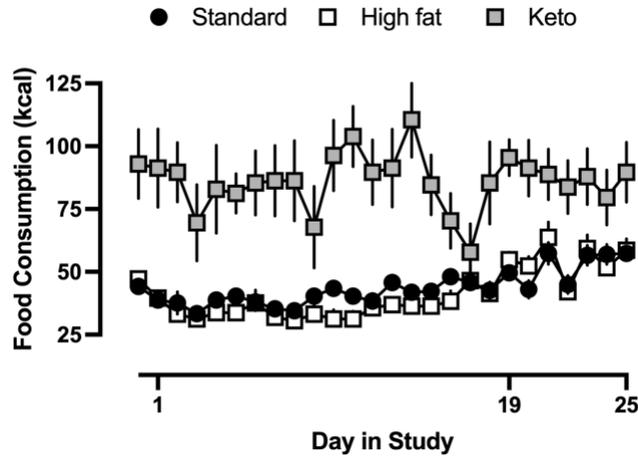


Figure 3. Mean (\pm SEM) food consumed (kcal) collected during morphine administration and withdrawal (see Table 1 details) in female rats eating standard chow (closed symbols), high fat chow (open symbols), and ketogenic chow (shaded symbols).

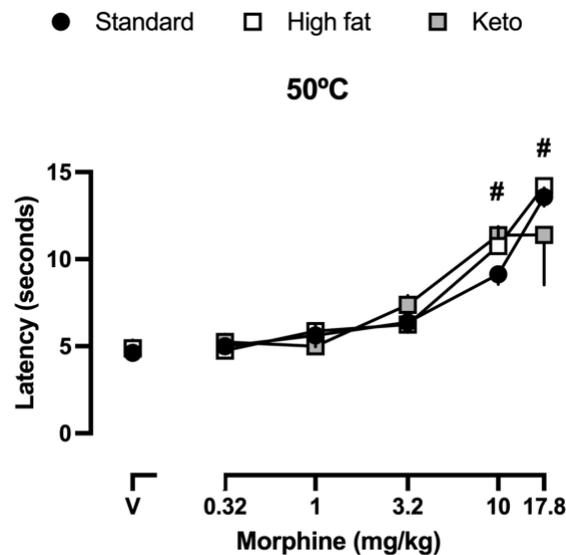


Figure 4. Mean (\pm SEM) withdrawal latency (seconds) for rats to remove their tails at 50°C during the warm water tail withdrawal procedure during acute conditions in female rats eating standard chow (closed symbols), high fat chow (open symbols), and ketogenic chow (shaded symbols). # represents a significant main effect of dose in rats eating standard, high fat, and ketogenic chow ($p < 0.05$). Diet did not impact sensitivity to the acute antinociceptive effects of morphine; however, latency did increase at the cumulative doses of morphine at 10 mg/kg and 17.8 mg/kg compared to the saline observation in all groups.

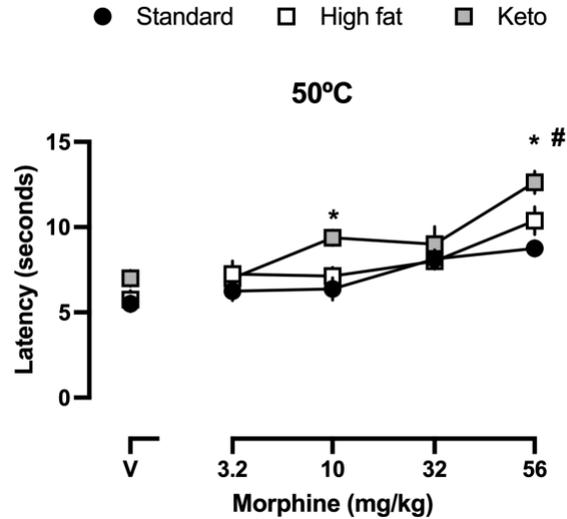


Figure 5. Mean (\pm SEM) withdrawal latency (seconds) for rats to remove their tails at 50°C during the warm water tail withdrawal procedure during chronic administration in female rats eating standard chow (closed symbols), high fat chow (open symbols), and ketogenic chow (shaded symbols). * represents a significant main effect of diet in rats eating ketogenic chow compared to rats eating standard chow. # represents a significant main effect of dose in rats eating standard, high fat, and ketogenic chow ($p < 0.05$). Rats eating ketogenic chow were more sensitive to the antinociceptive effects of morphine at the cumulative doses of 10 mg/kg and 56 mg/kg compared to rats eating standard chow. Latency increased at the cumulative doses of morphine at 56 mg/kg compared to the saline observation in all groups.

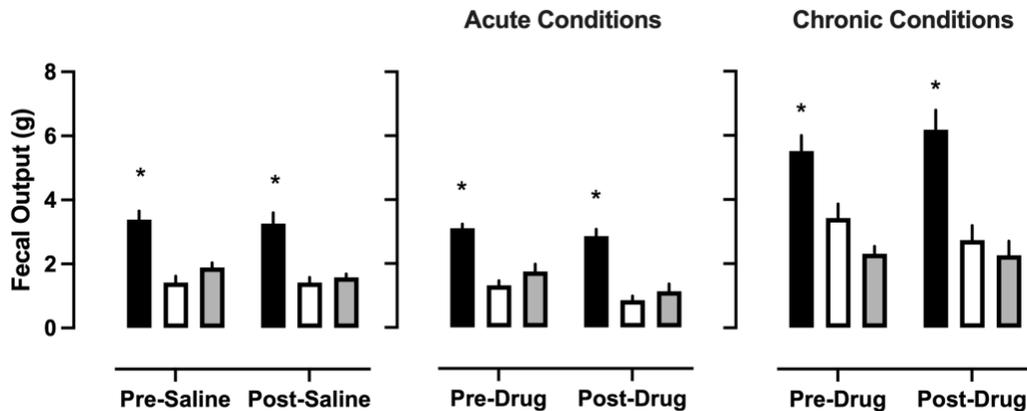


Figure 6. Mean (\pm SEM) fecal output in g for rats eating different diets based on fecal output weighed pre-drug and post-drug in rats eating standard chow (closed symbols), high fat chow (open symbols), and ketogenic chow (shaded symbols) during the saline test and under acute and chronic conditions. * represents a significant main effect of diet in rats eating standard chow compared to rats eating high fat and ketogenic chow ($p < 0.05$). Rats eating standard chow

produced more feces throughout the study as compared to rats eating high fat and ketogenic chow ($p < 0.05$).

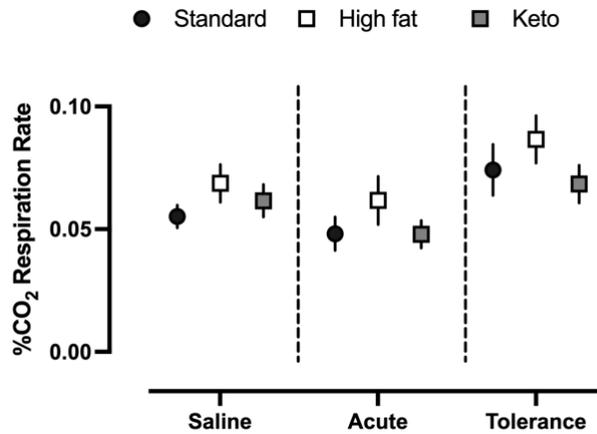


Figure 7. Mean (\pm SEM) CO₂ respiration rate collected during saline injections and acute and chronic conditions of morphine in female rats eating standard chow (closed symbols), high fat chow (open symbols), and ketogenic chow (shaded symbols). Respiration rate was not differentially impacted by rats eating different diets throughout the study.

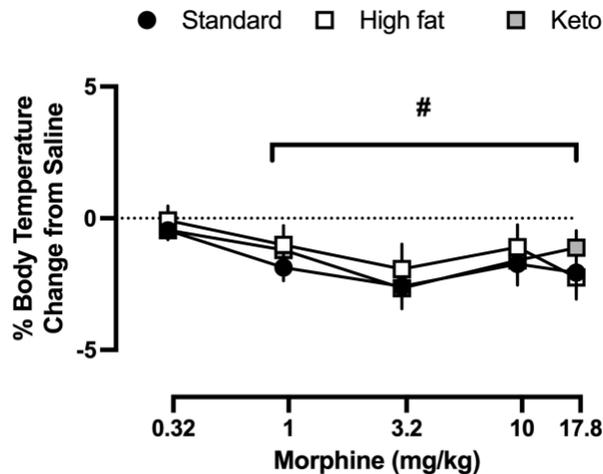


Figure 8. Mean (\pm SEM) % change in body temperature for rats eating different diets normalized based on body temperature examined at the saline observation period that occurred before assessing morphine-induced antinociception under acute conditions in female rats eating standard chow (closed symbols), high fat chow (open symbols), and ketogenic chow (shaded symbols). # represents a significant main effect of dose ($p < 0.05$). Body temperature was not different among rats eating different diets; however, body temperature was significantly cooler in all groups at the cumulative doses of morphine 1-17.8 mg/kg as compared to the saline body temperature ($p < 0.05$).

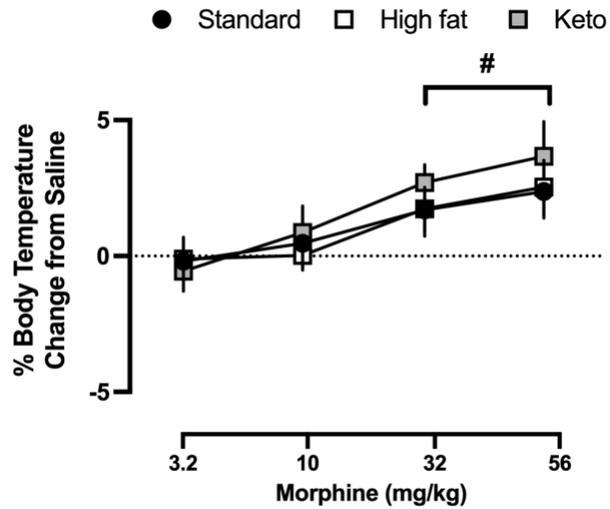


Figure 9. Mean (\pm SEM) % change in body temperature for rats eating different diets normalized based on body temperature examined at the saline observation period that occurred before assessing morphine-induced antinociception under chronic conditions in female rats eating standard chow (closed symbols), high fat chow (open symbols), and ketogenic chow (shaded symbols). # represents a significant main effect of dose in rats eating standard and ketogenic chow ($p < 0.05$). Body temperature was not different among rats eating different diets; however, body temperature was significantly warmer in all groups at the cumulative doses of morphine at 32 mg/kg and 56 mg/kg compared to the saline observation.

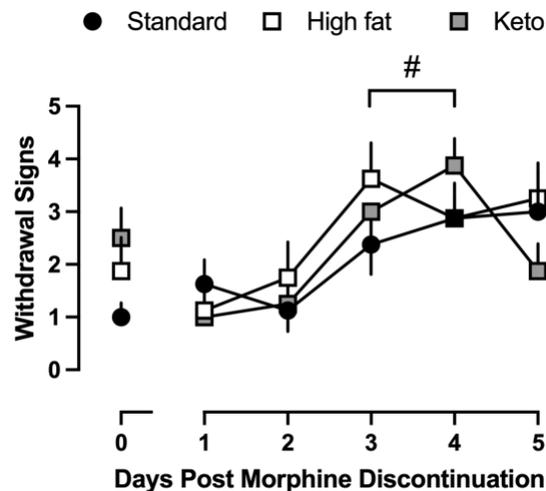


Figure 10. Mean (\pm SEM) withdrawal signs in female rats eating standard chow (closed symbols), high fat chow (open symbols), and ketogenic chow (shaded symbols). # represents a significant main effect of day in rats eating standard, high fat, and ketogenic chow ($p < 0.05$).

Withdrawal signs were not different among rats eating different diets; however, withdrawal signs did increase on day 3 and 4 compared to day 1 and 2.

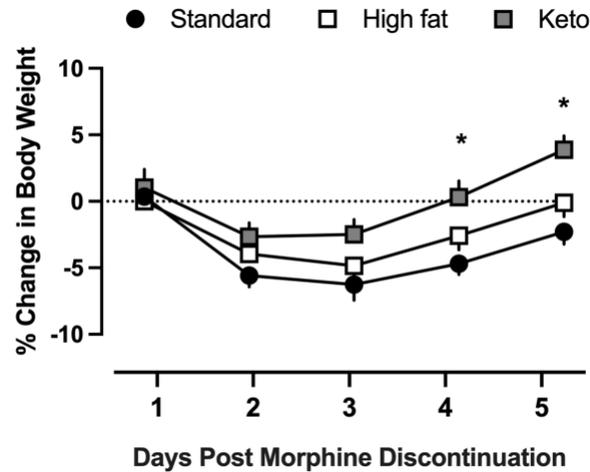


Figure 11. Mean (\pm SEM) % change in body weight for rats eating different diets normalized based on body weight examined on the day of the warm water tail withdrawal procedure during chronic morphine administration in female rats eating standard chow (closed symbols), high fat chow (open symbols), and ketogenic chow (shaded symbols). * represents a main effect of diet in rats eating ketogenic chow compared to rats eating standard chow ($p < 0.05$). Rats eating ketogenic chow gained significantly more weight on day 4 and 5 as compared to rats eating standard chow ($p < 0.05$).

Vita

Nina M. Beltran was born in Dyess Air Force Base in Abilene, Texas. As a daughter to an avionics technician in the military, Nina grew up in several states. Upon her father retiring from the Air Force in 2012 in Alamogordo, NM, Nina and her family moved to El Paso, TX, in which Nina graduated from Chapin High School in El Paso, in 2016. She thereafter attended the University of Texas at El Paso (UTEP) and became the first student at UTEP to graduate with a Bachelor of Science in Neuroscience in 2019. As an undergraduate at UTEP, Nina joined the laboratory of Dr. Katherine Serafine, who studies the behavioral factors that contribute to changes in drug sensitivity in rat models. Nina is now a PhD student in the Department of Psychology at UTEP. Throughout her time at UTEP, Nina has presented 13 poster abstracts as an undergraduate and 3 poster abstracts as a graduate student at several national conferences. Nina was also recently awarded a UTEP Dodson Research Grant to support her thesis work. She plans to continue her research and work towards her dissertation proposal and defense at UTEP under the mentorship of Dr. Katherine Serafine. Her long-term goals are to pursue a career in the pharmaceutical industry or a research-oriented career in government.