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# A meal pattern and time-course analysis of estrogen receptor agonists

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# A MEAL PATTERN AND TIME-COURSE ANALYSIS OF ESTROGEN RECEPTOR AGONISTS.

By

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## Abstract

Estradiol (E2) is an ovarian hormone that has a well-characterized anorexigenic effect in female animals that was originally believed to be mediated through the activation of nuclear estrogen receptors (ERs). However, recent studies from our lab and others have shown rapid anorexigenic effects after activation of membrane ERs (mERs), such as mERα and G-protein coupled estrogen receptor (GPER). The comparative action of the non-selective ER agonist, estradiol benzoate (EB), and the selective ERα and GPER agonists PPT and G-1, respectively, is poorly understood. In the current study, we analyzed meal patterns after acute administration of each of these agonists in OVX female Long-Evans rats. Both PPT and G-1 produced rapid decreases in food intake within 2 and 1 h, respectively, with associated decreases in the size of the first meal following drug treatment. It was also determined that EB produces a prolonged anorexigenic effect, suppressing food intake for three days beginning 12 h after drug treatment. Overall, these findings provide additional evidence that activation of mERs alone is sufficient to decrease food intake and that mER agonists produce more rapid but more transient effects than the non-selective ER agonist EB.

## Introduction

Estradiol (E2) is an ovarian hormone that exerts regulatory effects on homeostatic mechanisms in female animals, including those mediating body weight and food intake [3, 8, 10, 17, 19]. Bilateral ovariectomy promotes sustained hyperphagia resulting in weight gain and excessive adiposity in female rats [3], while administration of E2 to ovariectomized (OVX) rats decreases food intake and attenuates weight gain [11]. Despite the well-characterized anorexigenic effect of E2 administration, little is known about the impact of non-selective and selective agonist administration on meal patterns in OVX female animals. For instance, it is important to understand the onset and duration of the anorexigenic effect if E2 administration is to be used as a therapeutic treatment post-menopause. Furthermore, there are differing effects of nuclear and membrane estrogen receptor (ER) activation that could influence the targeted receptor or form of E2 used in these therapeutic treatments. This study seeks to address this gap in knowledge with a detailed meal-pattern analysis of the non-selective ER agonist, estradiol benzoate (EB), and the ERα agonist PPT and GPER agonist G-1.

The decrease in food intake following acute administration of non-selective ER agonists such as EB appears to occur after a long latency typically reported at 12-36 h after drug administration [11, 12]. The delayed effect of non-selective ER agonists suggests that EB's anorexigenic effect is mediated via nuclear ERs (ERα and/or ERβ), which function to regulate gene expression. Specifically, when E2 binds to these ERs, a conformational change in the receptor allows for the E2-ER complex to bind directly to the promoter regions of E2-responsive genes thus regulating their subsequent protein production [4, 8].

Available data suggest that E2's anorexigenic effect is mediated, at least in part, via ERα with limited or no involvement of ERβ. For example, ERα knockout (ERαKO) mice exhibit

increased body weight and body adiposity, as well as increased food intake while ERβKO mice do not [13]. This suggests that ERα has a more important role in modulating food intake and body weight regulation compared to ERβ. Pharmacological manipulation has also supported this claim as administration of the ERα agonist PPT has been shown to decrease food intake in OVX rats, whereas administration of the ERβ agonist DPN has not [12].

Despite the evidence of EB's anorexigenic effect, there is currently no published literature that observes a rapid (within 1 h) decrease in food intake after administration of EB. However, similar treatment with the ERα agonist PPT has been shown to decrease food intake within 3 h of injection [10], and additional unpublished data from our lab shows that PPT can decrease food intake within 1 h. Because ERα can be shuttled between the nucleus and the plasma membrane [13], it is hypothesized that this rapid action of PPT may be mediated by membrane-bound ERα (mERα). When E2 binds to mERα, it activates a series of intracellular signaling cascades that change the cytoplasmic environment of the cell. This change in the intracellular environment affects the activity of that cell and can result in rapid (within min) behavioral changes [8]. Nevertheless, decreases in food intake after administration of more selective E2 agonists, like PPT [1, 15], provide support for the anorexigenic effects of E2 being mediated, at least in part, through activation of mERs, including mERα and the more recently discovered G-protein-coupled estrogen receptor (GPER).

GPER, previously named GPR30, was not recognized as an ER until 2000, and since then it has been confirmed that it is genetically and structurally distinct from ERα and ERβ [17]. Available data suggest that GPER may be involved in mediating E2's inhibitory effects on feeding and weight gain. Activation of GPER using the selective GPER agonist G-1, results in modulation of behaviors that are also modulated by activation of ERα. For example,

administration of G-1 can reduce adipogenesis, and thus can reduce obesity [1]. Additionally, female GPER-KO mice exhibit decreased energy expenditure during dark onset [4], and unpublished data from our lab demonstrated that G-1 administration produced a rapid decrease in food intake within the first hour of the dark-phase.

Studies investigating the detailed feeding patterns of female rats have shown that E2 (specifically EB) decreases food intake by decreasing meal size, not meal number [11]. Thus, to better understand PPT's rapid anorexigenic effect, it will be important to investigate PPT's ability to decrease the size of the first meal consumed following drug treatment. The current study will thus employ a detailed meal-pattern analysis of the anorexigenic effects of the non-specific ER agonist, EB, as well as selective ER agonists that are thought to target mERs (i.e., PPT and G-1). This study seeks to replicate the previous findings regarding the rapid anorexigenic effects of PPT and G-1, elaborate on meal patterns after PPT and G-1 administration, and compare these effect to that of EB. Although the anorexigenic effect of EB is well-characterized, this analysis of meal-patterns in response to varying forms of E2 and E2 agonist administration could provide valuable insight into the behavioral mechanism underlying E2's anorexigenic effect.

## Methods

*Animals.*

Female Long-Evans rats (Charles River Breeding Laboratory, Raleigh, NC; weighing 300-330g at study onset) were housed individually in custom cages as described below. Rats were given *ad libitum* access to powdered chow (Purina 5001, St. Louis, MO) and tap water unless otherwise specified. Animal rooms were maintained at 20 ± 2°C with a 12:12 h reverse

light-dark cycle (dark onset = 1200 h). Animal usage and all procedures were approved by the Florida State University Institutional Animal Care and Use Committee.

*Surgery.*

Animals were anesthetized with 3% isoflurane (Butler Schein Animal Health, Dublin, Ohio) and then bilaterally OVX using an intra-abdominal approach. Immediately following surgery, animals received intraperitoneal (i.p.) injections of butorphanol (0.5 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) to reduce post-surgical pain, and subcutaneous (s.c.) injections of gentamicin (10 mg/kg; Pro Labs Ltd., St. Joseph, MO) to reduce the risk of infection. Animals were given one week of post-operative recovery prior to behavioral testing.

*Meal patterns.*

Following post-operative recovery, rats were transferred to custom cages designed to facilitate the analysis of spontaneous feeding behavior. The cages were equipped with feeding niches that provided access to spill-resistant food cups mounted on weight-sensitive load beams. Infrared beams, located on either side of the feeding niches and centered above the feeding cups, were also used to signal the occurrence of feeding behavior. Any food spillage was collected on a platform surrounding the food cup. Outputs from the load and photo beams were fed via an interface into a computer located in an adjacent room. Custom-designed software (ESP 500; T. Tang; Florida State University, Tallahassee, FL) recorded the weight of each load beam (± 0.001 g) and the activity of each photo beam at 30-s intervals. Additional software (Meal Weight Analysis V 2.0, T. Tang; Florida State University, Tallahassee, FL) was used to assess daily 22-h food intake, 12-h dark-phase food intake, dark-phase food intake at 1-h intervals, latency to

consume the first dark meal, and individual meals. A meal was defined as any feeding bout of at least 0.3 g that was separated by an intermeal interval of at least 15 min.

*Experiment 1: Anorexigenic effect of the non-selective ER agonist EB.*

OVX rats (n = 8) were adapted to the custom cages over a period of 10 days. Each day at 1000 h (2 h prior to dark onset), the software program collecting feeding data was halted and access to the feeding niches was prevented by a stainless steel blocker. Food intake during the preceding 22 h was recorded, body weights were measured, and food cups and water bottles were re-filled. The software program was restarted at 1100 h, access to food was restored at 1200 h (dark onset), and rats were left undisturbed until the following day at 1000 h. Rats were maintained on this maintenance schedule throughout the duration of the experiment.

Following adaptation to the cages, a within-subject design was used to investigate the time-course of EB's anorexigenic effect. On separate test days spaced 7 days apart, rats received subcutaneous (s.c.) injections of estradiol benzoate (EB, Sigma, 2 μg in 0.1 ml of sesame oil) or sesame oil vehicle alone. Injections were administered 30 min prior to dark onset. Custom software was used to assess 22-h food intake, 12-h dark-phase food intake and dark-phase food intake at 1-h intervals throughout the 7 test days.

*Experiment 2: Anorexigenic effect of specific ER agonists.*

A second group of OVX rats (n = 8) were adapted to the custom cages and the same daily maintenance procedures as described above. Following adaptation, a within-subject design was used to investigate the anorexigenic effects of specific ER agonists. On separate test days spaced 2-3 days apart to allow sufficient time for drug clearance, rats received s.c. injections (0.1 ml) of

50% DMSO vehicle (Sigma), the ERα agonist PPT (50 μg; Tocris, Minneapolis, MN), or the GPER agonist G-1 (0.5 μg; Cayman Chemical Company, Ann Arbor, MI). Custom software was used to assess 22-h food intake, 12-h dark-phase food intake, meal size and meal number, and dark-phase food intake at 1-h intervals throughout the 7 test days.

*Statistical Analysis:*

One- and two-factor repeated measures ANOVAs were used to assess the effects of the various ER agonists on food intake and meal patterns as appropriate. Significant ANOVA effects ($P < 0.05$) were further investigated via Tukey's posthoc tests.

## Results

*Experiment 1: Anorexigenic effect of the non-selective ER agonist EB.*

As, expected EB administration influenced daily 22-h food intake, $F(3,21) = 7.839$, $p < 0.001$. Post-hoc tests revealed that a single acute injection of EB decreased 22-h food intake for three consecutive days, relative to that consumed following vehicle treatment (ps < 0.05; data not shown). This action of EB was the result of a decrease in 12-h dark-phase food intake, $F(3,21) = 18.893$, $p < 0.001$, which persisted for three days (ps < 0.05; Fig. 1). Light-phase food intake over these same three days was not influenced by EB treatment (data not shown).

A more detailed analysis of dark-phase food intake at hourly intervals through the three days in which EB treatment suppressed feeding revealed a main effect of EB treatment on day 1, $F(1,7) = 7.839$, $p < 0.001$, and interactive effects of EB treatment and time on days 2 and 3, $F(11,77) = 2.804$ and 2.396, respectively, ps < 0.01 (Fig. 2). Post-hoc analyses revealed that EB first suppressed food intake during the last hour of the dark phase on day 1 ($p < 0.05$; Fig. 2A).

On day 2, EB suppressed food intake at all time points but one (ps < 0.05; Fig. 2B). On day 3, EB suppressed food intake at all time points except three (ps < 0.05; Fig. 2C).

*Experiment 2: Anorexigenic effect of specific ER agonists.*

Administration of selective ER agonists produced a reliable decrease in 22-h food intake, $F(2,14) = 36.116$, $p < 0.001$. This effect was due to a selective decrease in 12-h dark-phase intake, $F(2,14) = 83.135$, $p < 0.001$ (Fig. 3), as light-phase food intake was unaffected. Post-hoc analysis revealed that acute administration of PPT, but not G-1, decreased dark-phase feeding, compared to vehicle treatment ($p < 0.05$; Fig 3A). A closer examination of dark-phase feeding at hourly intervals revealed an interactive effect of ER agonist treatment and time, $F(22,154) = 9.871$, $p < 0.001$ (Fig. 3B). While G-1 suppressed food intake only during the first h of the dark phase ($p < 0.05$), PPT suppressed feeding at all but the first h of the dark phase (ps < 0.05).

Meal pattern analysis revealed an effect of ER agonist treatment on average dark meal size, $F(2,14) = 7.916$, $p < 0.005$ (Fig 5A), and dark meal number, $F(2,14) = 8.135$, $p < 0.005$ (Fig 5). While PPT significantly reduced average dark meal size compared to the vehicle (ps < 0.05), G-1 did not. Additionally, the number of meals consumed through the dark phase was not significantly reduced by PPT or G-1.

The size of the first dark meal was also reliably suppressed by ER agonist administration, $F(2,14) = 15.342$, $p < 0.001$. Both PPT and G-1 produced similar decreases in meal size compared to vehicle treatment (ps < 0.05 Fig. 5A). A trend for a reduction in the size of the second dark meal was observed, $F(2,14) = 3.278$, $p = 0.068$ (Fig. 5B). The size of the third meal was decreased by PPT, but not by G-1, $F(2,14) = 7.883$, $p < 0.001$ (Fig. 5C).

## Discussion

While E2's anorexigenic effect is well-characterized, the behavioral changes associated with decreased feeding are not as well understood. EB has been reported to decrease food intake at 12-36 h, but there has never been published data suggesting a more rapid decrease in food intake despite rapid effects of selective ER agonist administration. This may be due to preferential binding of EB to nuclear ERs, whereas other selective ER agonists, such as PPT and G-1 may preferentially bind to or specifically target mERs. EB is one of the most commonly employed forms of E2 in behavioral testing, thus a detailed time-course of its anorexigenic effects is valuable as researchers will better understand its prolonged effect on feeding. Futhermore, studies have provided evidence that E2 suppression in food intake is mediated by a decrease in meal size and not meal number [2]. However, a detailed meal-pattern analysis has not been done comparing the effects of EB administration to the administration of selective ER agonists, PPT and G-1. The goal of this study was to determine the effect of EB administration on meal patterns and time-course and compare that effect to those reported in the existing literature as well as to the effect of selective ER agonist administration.

We tested the effect of EB administration on daily 22-h food intake, and found the expected decrease compared to sesame-oil injections. Notably, food intake was significantly reduced for three consecutive days after a single acute injection of EB demonstrating the lasting impact of nuclear ER activation on behavior. It has been reported that E2 administration can influence the efficacy of other pharmacological treatments, such as antidepressants [15]. This raises questions regarding the duration of E2's behavioral effects as the timing and dosage of E2 could influence other pharmacological compounds, making a time-course analysis of common E2 compounds valuable. Considering this and the demonstration of a relationship between E2

and food intake, we performed a more detailed analysis of dark-phase food intake at hourly intervals through the three days in which there was a significant reduction in 22-h food intake. It was determined that EB exerted its first anorexigenic effect during the last hour of the dark phase on day 1, and this effect persisted for all the time points examined on day 2 excluding the 8-h time point. The anorexigenic effect also revealed a decrease in food intake at all but three time points on day 3. This further demonstrates that EB potentially binds to the nuclear ER's with a higher affinity than to mERs, exerting longer duration, but slower onset anorexigenic effects.

There has been no published literature demonstrating a rapid decrease in food intake after acute EB administration despite the growing amount of literature citing evidence of mER mediation of this anorexigenic effect. Our second experiment sought to compare the effects of selective ER agonist administration on meal patterns and food intake to the effects exhibited after acute EB administration. One candidate mER is mERα, a palmitoylated form of ERα that is shuttled from the nucleus to the membrane [9]. Previous data has already established that acute administration of PPT decreases meal size and exerts an anorexigenic effect within 3-6 h [12]. Furthermore, unpublished data from this lab shows a reduction in food intake within 1 h of administration. Similar to EB, PPT produced a reliable decrease in 22-h food intake, mostly due to a selective decrease in 12-h dark-phase food intake. However, PPT suppressed feeding during all time points excluding the first hour of the dark-phase. It is not clear why PPT administration did not replicate the currently unpublished finding that PPT can decrease food intake at 1-h post-injection, it did trend towards being significant and the anomaly may have been due to natural variance or a limitation of the current study. Altogether, these findings suggest that PPT, unlike EB, preferentially binds to mERα and thereby produces a more rapid anorexigenic effect.

The second candidate mER, GPER, has been shown to decrease food intake after GPER agonist administration. Unpublished findings from our lab indicates that G-1 administration can rapidly decrease food intake 1-h after injection, and other studies have also reported decreases in 24-h food intake after subcutaneous injection [20]. Here, we found that G-1's anorexigenic effect was limited to the first h of the dark-phase whereas PPT suppressed feeding at all but the first h. This supports our lab's currently unpublished literature citing the suppression in food intake after 1-h and demonstrates that the anorexigenic effect of mER activation may be more transient than that observed after nuclear ER activation. Importantly, G-1 produced a reliable and robust decrease in the size of the first dark meal following treatment. Because estrogens are known to suppress food intake by a selective decrease in meal size, our finding that G-1 exerts its effects via changes in meal size provides evidence of the behavioral specificity of G-1's inhibitory effect on feeding.

There has not been a detailed meal pattern analysis after administration of the selective ER agonists, PPT and G-1. This study sought to examine this by determining the effect of PPT and G-1 on average dark meal number, average dark meal size, and size of the first three meals of the dark-phase. Considering G-1 did not produce a significant reduction in overall dark-phase food intake, it should be expected that it did not significantly reduce average dark meal size or meal number. This was found to be the case, meanwhile, PPT administration significantly reduced average dark meal size. Additionally, G-1 significantly reduced the size of the first meal, which was taken within the first hour where G-1 was shown to significantly reduce food intake. Despite PPT not having significantly reduced food intake within the first hour, it did significantly reduce the size of the first meal. Neither PPT or G-1 reduced the size of the second meal, but PPT and not G-1 did reduce the size of the third meal. This is expected considering

PPT exerted an anorexigenic effect at later hourly intervals in the dark-phase compared to G-1, suggesting that GPER has a more transient role in mediating food intake compared to mERα. Because estrogens are known to suppress food intake by a selective decrease in meal size, our findings that both PPT and G-1 exerted their rapid effects via changes in meal size, rather than meal number, provides further evidence of the behavioral specificity of both compounds. That is, it is unlikely that the rapid anorexia induced by PPT or G-1 are secondary to adverse effects such as sedation or malaise, which would be more likely to reduce meal number.

In conclusion, the data presented here collectively supports previously published literature regarding the differential consequences of nuclear ER and mER activation. Furthermore, it has replicated previously found, but currently unpublished studies, from our lab demonstrating rapid anorexigenic effects after selective ER agonist administration. Overall, this behavioral analysis provides valuable insight into the onset and duration of the three ER agonists investigated, as well as their individual impact on meal patterns. This information is an important foundation in determining the physiological impact of mERs compared to the more classically studied nuclear ERs.

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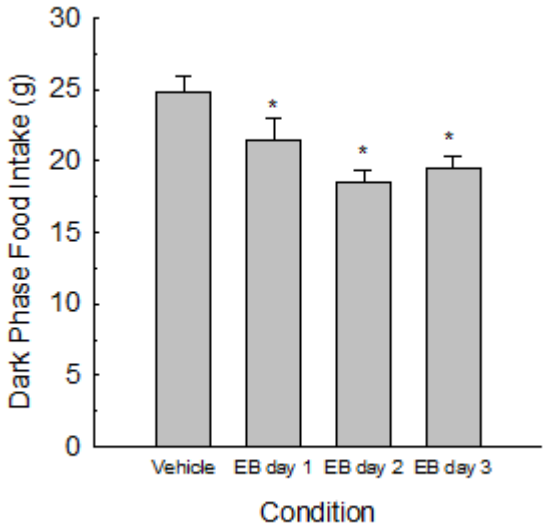


Fig. 1. Effect of EB treatment on dark-phase food intake up to three days after administration. On days 1-3, dark-phase food intake was significantly decreased by EB, relative to vehicle. *Less than vehicle, $P < 0.05$.

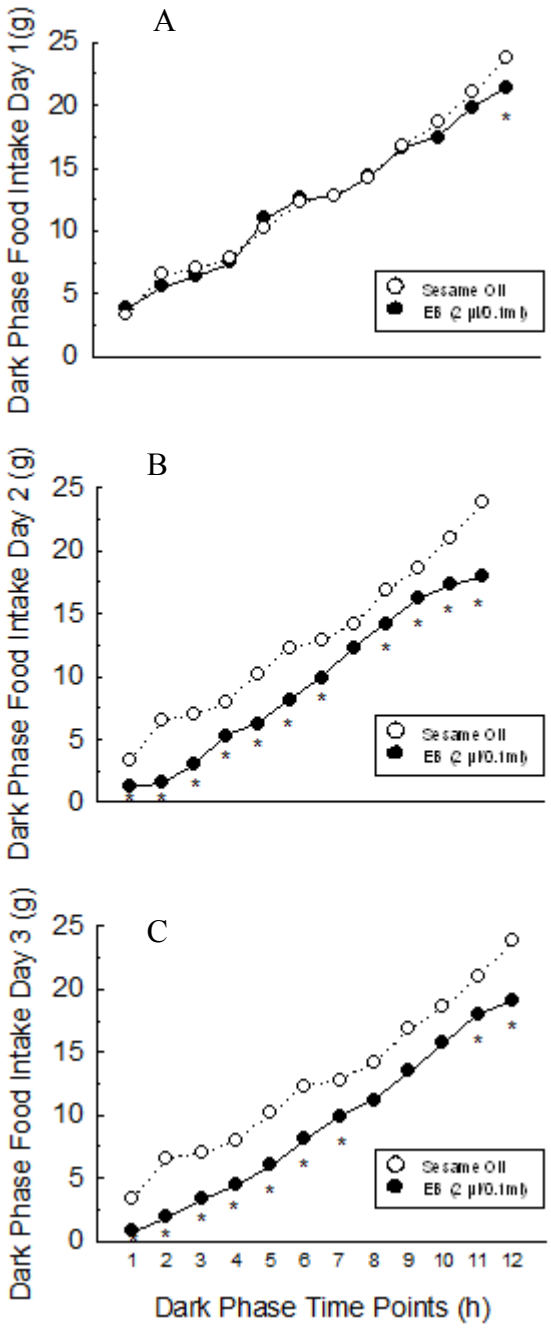


Fig. 2. Effect of EB treatment on hourly dark-phase food intake. (A) Food intake was significantly decreased at 12-h on day 1 by EB, relative to vehicle. (B) On day 2, all time points exhibited a significant reduction in hourly dark-phase food intake by EB, relative to vehicle. (C) On day 3, time points 2-7, and 11-12 showed significant reductions in dark-phase food intake by EB, relative to vehicle. *Less than vehicle, $p < 0.05$.

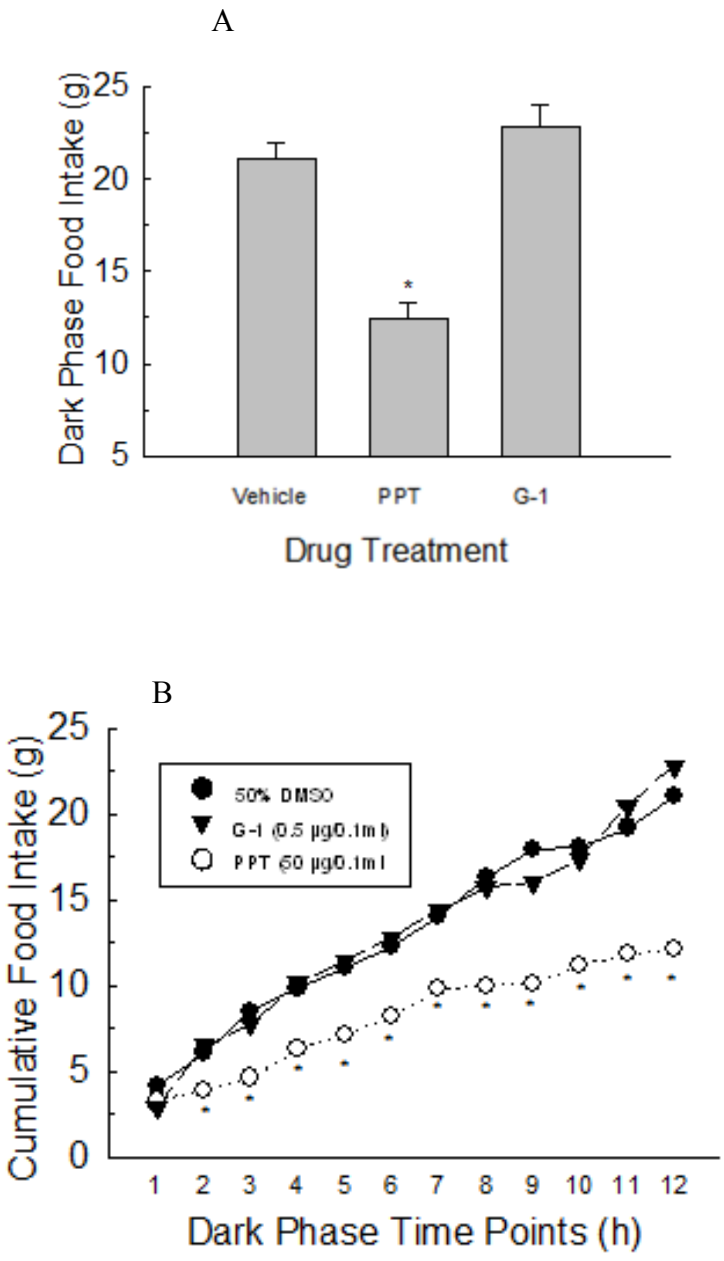


Fig. 3. Effect of selective ER agonist administration on dark-phase food intake, and cumulative hourly dark-phase food intake. (A) Food intake was significantly reduced by PPT administration, relative to vehicle. Food intake was not significantly reduced by G-1 administration, relative to vehicle. (B) PPT produced a reliable decrease in food intake at 2-12 hours post-administration, whereas G-1 only significantly reduced food intake in the first hour. *Less than vehicle, $P < 0.05$

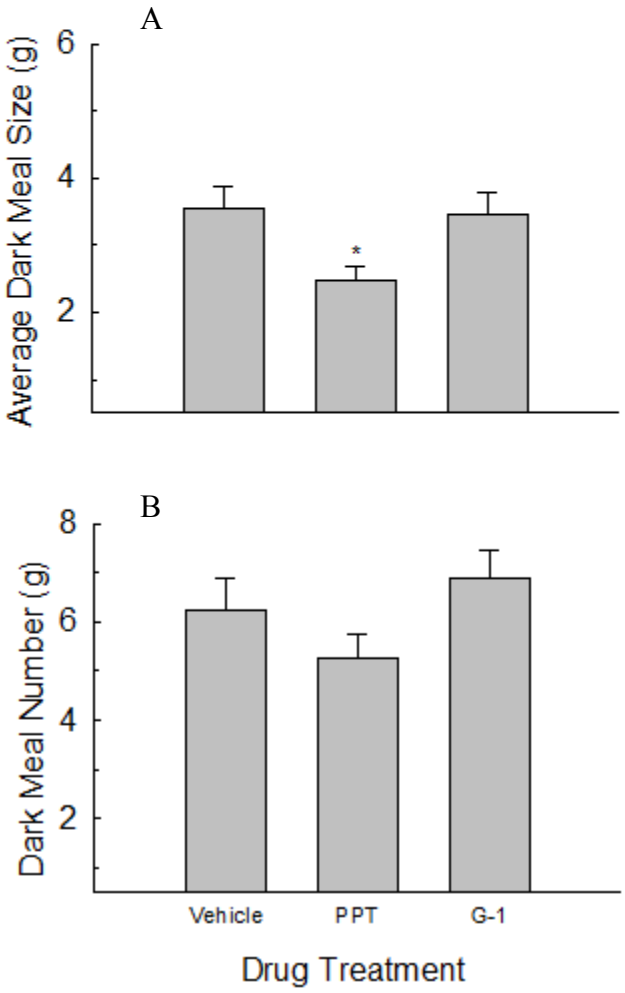


Fig. 4. Effect of selective ER agonist administration on average dark meal size and dark meal number. (A) Average dark meal size was unaffected by G-1, but showed a significant reduction after PPT, relative to vehicle. (B) Dark meal number was also unaffected by either G-1 or PPT, relative to vehicle. *Less than vehicle, $P < 0.05$.

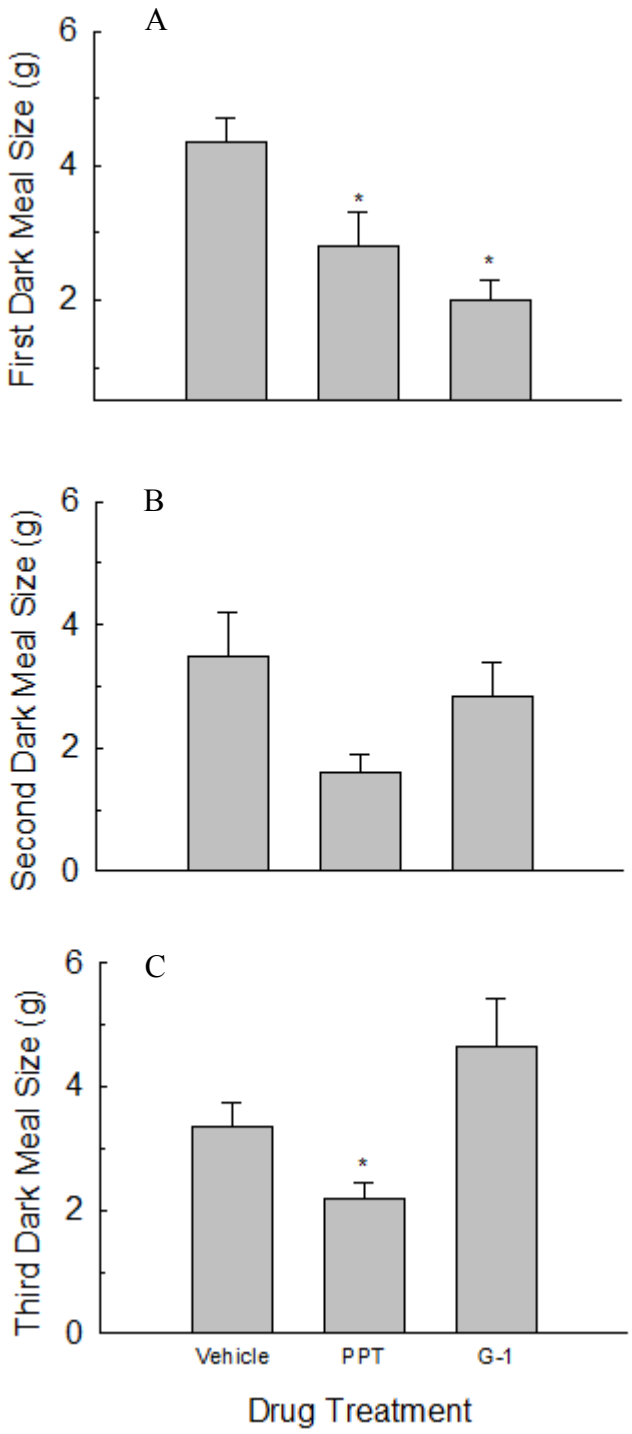


Fig. 5. Effect of selective ER agonist administration on first, second, and third dark meal size. (A) Both PPT and G-1 produced significant reductions in the size of the first meal. (B) The size of the second meal was trending towards being significantly reduced by PPT, but was unaffected by G-1, relative to vehicle. (C) The third dark meal was significantly reduced by PPT, and unaffected by G-1, relative to vehicle.