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Cellular activation in gonadotropin-inhibiting hormone-immunoreactive cells is associated with sexual motivation and food hoarding, but not sexual performance and food intake in female Syrian hamsters.

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Abstract

We hypothesized that putative anorectic and orexigenic peptides control the motivation to engage in conflicting appetitive behaviors, those behaviors that bring females in contact with either food or mating partners. Here, the putative orexigenic peptide, gonadotropin-inhibiting hormone (GnIH)(also known as RFamide-related peptide (RFRP)) and the putative anorectic hormones leptin, insulin and estradiol were examined during the course of food restriction. Female Syrian hamsters were restricted to 75% of their *ad libitum* food intake or fed *ad libitum* for 4, 8, or 12 days. Two other groups were food restricted for 12 days and then re-fed *ad libitum* for 4 or 8 days. After testing for sex and ingestive behavior, blood was sampled and plasma assayed for peripheral hormones. Brains were immunohistochemically double-labeled for GnIH and the protein product of the immediate early gene, *c-fos*. Cellular activation in GnIH cells, and appetitive (but not consummatory) ingestive behaviors were both significantly increased compared to baseline levels only at 8 and 12 days after the start of restriction and at 4 days after re-feeding, and returned to baseline levels at 8 days after the start of re-feeding. Food hoarding, but not food intake, was significantly positively correlated with cellular activation in GnIH cells, and vaginal scent marking, but not lordosis duration, was significantly negatively correlated with cellular activation in GnIH cells. There were no significant effects of food restriction on plasma insulin, leptin or estradiol concentrations. In the dorsomedial hypothalamus (DMH) of energetically-challenged females, strong projections from NPY cells were found in close apposition to GnIH cells. Together these results are consistent with the idea that metabolic signals influence sexual and ingestive motivation via NPY fibers that project to GnIH cells in the DMH.

Introduction

Metabolic control of the reproductive system has been demonstrated in every order of the class Mammalia, and we hypothesize that its primary function is to set behavioral priorities that optimize reproductive success in environments where food availability and energy demands fluctuate (Bronson, 1989;Wade and Schneider, 1992). The mechanisms that switch behavioral priorities from ingestive to reproductive behaviors might occur at multiple loci, including effects on behavioral motivation (the internal desire for food or sex), performance (mating and eating) and the hypothalamic-pituitary-gonadal (HPG) system, including the gonadotropin releasing hormone (GnRH) pulse generator, pituitary gonadotropin secretion and ovarian steroid secretion. Despite action at multiple loci, the majority of research has focused on metabolic factors, hormones, and neuropeptides that induce anestrus and stimulate food intake and vice versa (Kalra et al., 1988;I'Anson et al., 1991;McShane et al., 1992;Wade and Schneider, 1992;Foster et al., 1998;Henry et al., 1999;Cunningham, 2004;Schneider, 2004). Food deprivation and other metabolic challenges inhibit pulsatile GnRH secretion that, in turn, inhibits pituitary luteinizing hormone (LH) secretion, ovarian steroid synthesis and secretion, and ovarian-steroid-dependent copulatory behavior in a wide variety of species, including Syrian hamsters (McClure, 1962;Morin, 1975;Ronnekleiv et al., 1978;Bronson and Marsteller, 1985;Foster and Olster, 1985;Armstrong and Britt, 1987;Bronson, 1988;Sprangers and Piacsek, 1988;Schneider and Wade, 1989;Thomas et al., 1990;Cameron, 1996;Shahab et al., 1997;Temple et al., 2002;Terry et al., 2005;Shahab et al., 2006).

It is likely, however, that energy deficits influence behavioral motivation even before metabolic challenges become so severe that they induce anestrus. For example, in female Syrian hamsters, appetitive ingestive behaviors are stimulated and appetitive sex behaviors are inhibited after a period of food deprivation (Schneider et al., 2007). Appetitive behaviors bring animals in contact with the goal object (mating partners or food), and often occur separated in time from mating and eating (Sherrington, 1906;Craig, 1917;Lorenz, 1950;Johnston, 1974;Johnston, 1977;Lisk, 1983;Everitt, 1990). Syrian hamster appetitive sex behaviors include vaginal scent marking, an estradiol-dependent behavior that occurs with increasing frequency over days 1, 2 and 3 of the 4-day estrous cycle (with day 4 being proestrus) (Johnston, 1977). Consummatory sex behavior is commonly measured as the incidence of the lordosis reflex, a reflexive posture that allows male intromission on day 4 of the estrous cycle and requires physiological concentrations of plasma estradiol and progesterone, tactile flank stimulation, and male olfactory cues (Lisk, 1983). In Syrian hamsters, food hoarding is an example of appetitive ingestive behavior, whereas food intake is a consummatory behavior (Smith and Ross, 1950;Waddell, 1951). Consummatory sex and ingestive behavior can be simultaneously stimulated under special circumstances (Kaplan et al., 1992). Appetitive behaviors, however, are often in conflict, and females must choose between engaging in courtship or foraging for food. Thus, we have included appetitive behaviors and the choice between food and males in our experiments on energetic control of ingestive and reproductive behavior in female Syrian hamsters (*Mesocricetus auratus*).

In this experiment, appetitive and consummatory sex and ingestive behaviors were examined over the course of food restriction to test the following hypotheses: 1) Are appetitive

behaviors more sensitive than consummatory behaviors to the effects of mild food deprivation, and 2) Are changes in appetitive behavior correlated with increases in neural activation in cells expressing GnIH, a neuropeptide that inhibits GnRH secretion in response to environmental cues (Kriegsfeld, 2006)? GnIH was first identified as an inhibitor of gonadotropin secretion in cultured quail pituitary (Tsutsui et al., 2000) with orthologous neuropeptides found later across vertebrate species, including mammals (reviewed in (Bentley et al., 2010;Kriegsfeld et al., 2010;Tsutsui et al., 2010). We hypothesize that GnIH is a modulator of sex and ingestive motivation because intracerebroventricular treatment with GnIH rapidly inhibits LH secretion in hamsters, rats, mice and sheep (Kriegsfeld et al., 2006;Johnson et al., 2007;Clarke et al., 2008;Anderson et al., 2009), disrupts sex behavior of female white-crowned sparrows and male rats (Bentley et al., 2006;Johnson et al., 2007), and increases food intake in male rats (Johnson et al., 2007), sheep, mice and monkeys (I. J. Clarke, personal communication). If GnIH is important for the effects of mild food restriction on the observed changes in behavior motivation in female hamsters, it would be predicted that increases in ingestive behavior motivation (food hoarding) and decreases in sexual motivation (the preference for males vs. food) would be preceded by increases in neural activation in GnIH-immunoreactive (ir) cells in the dorsomedial nucleus of the hypothalamus (DMH). Our hypothesis would be refuted if there were no increase in cellular activation in GnIH-ir cells or if, for example, the activation occurred at 8 days of restriction even though behavior changed at 4 days of restriction. Thus, the present experiments examined cellular activation in GnIH-ir cells and appetitive sex and ingestive behavior after either 0, 4, 8, or 12 days of 25% food restriction or after 4 or 8 days of *ad libitum* feeding to females previously food-restricted for 12 days. In addition, we measured NPY--ir projections to the GnIH-ir cells, as well as plasma levels of progesterone, leptin, insulin, estradiol because they are putative orexigenic agents and anorectic hormones also implicated in control of reproduction (Clark et al., 1985;Stanley and Leibowitz, 1985;Kulkosky et al., 1988;Brady et al., 1990;Hardie et al., 1996;Ahren et al., 1997;Schneider et al., 2000;Corp et al., 2001;Buckley and Schneider, 2003;Jones et al., 2004).

Materials and Methods

All subjects were adult (60-90 days of age), female Syrian hamsters obtained from Charles River Breeding Laboratories (Wilmington, MA). Upon arrival, hamsters were housed singly in opaque, Nalgene cages (31 × 19 × 18-cm) in a room maintained at 23 ± 1°C with a 14:10 light-dark cycle (lights on at 2200 hours). Hamsters were fed Harlan Rodent Chow 2016 and water was available at all times. All procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the United States Department of Agriculture, and a protocol approved by the Lehigh University Institutional Animal Care and Use Committee.

Experiment 1: Effects of energy restriction on behavior, GnIH, and circulating hormones

This experiment was designed to examine cellular activation in GnIH cells and circulating hormones after testing for appetitive and consummatory sex and ingestive behaviors in animals subject to mild food restriction for varying durations.

Preference apparatus

Hamsters were acclimated, trained and tested in a preference apparatus designed to duplicate aspects of their native habitat, and to allow quantification of behaviors associated with the motivation to engage in either sex or ingestive behavior (Schneider et al., 2007). Hamsters in the wild live in isolation in underground burrows from which they emerge for only 90 min per day at dawn and dusk, and spend virtually every minute of this time foraging for and hoarding food (Gattermann et al., 2008). Matings have been observed only at the entrance to the female burrow. Together, these considerations suggest that decisions about whether to engage in ingestive or sex behaviors that occur near the burrow entrance during the 90 min above-ground foraging period are relevant to their reproductive success. Thus, each preference apparatus consisted of a home cage for the subject female connected via a vertical tube to two boxes: One with an adult male hamster (male box) and another box containing a food source (food box). Home cages were made from opaque, Nalgene cages (31 × 19 × 18-cm) lined with fine wood shavings with a specialized door that was kept closed when the animals were not being trained or tested. The door to the home cage led to an upward vertical tube (134 cm in length) connected to two more tubes in a T-configuration (both tubes 40-50 cm in length), one leading to food and the other to the male hamster. The food box contained a weighed amount (150 ± 5 g) of hoardable pellets made from standard laboratory chow (Harlan Rodent Chow 2016) that was broken into 2 cm pieces, a size that permits pouching and enables hamsters with full cheek pouches to fit readily through the tubes. The male boxes for the stimulus hamsters were made from clear, Plexiglas cages (27 × 20 × 15 cm) with wire barriers that allowed hamsters to interact, but prevented mating or fighting. The stimulus male boxes did not contain food or water.

Females were acclimated to the home cage for 24 h/day for at least 1 week prior to testing, which reduced any tendencies to sleep, move bedding, or hoard food into any other compartments during later preference testing. After acclimation to the home, females were trained to expect food in the food box and a male in the male box at the onset of the dark period. Hamsters experienced training sessions with the food source box once a day for 2 days on days 1 and 2 of the estrous cycle, and training sessions with the male box once a day for 2 days on days 3 and 4 of the estrous cycle. For 90 min at the onset of the dark period on days 1 and 2 of the estrous cycle, females were allowed access via the tubes to the food box and allowed to keep all food they carried from the food source box to their home cage. For 90 min at the same time on day 3 of the estrous cycle, females were allowed to enter the male box with an unrestrained male (females cannot become pregnant on this day) for 5 min or until fighting occurred, after which the male was placed behind a wire barrier to prevent injury to either animal. Females were placed back in their home cage and allowed to find the male box two more times before being placed in the home cage without access to the food source or male cage during the night. On day 4 at the same time of day, females were again allowed to directly interact with a male in the male box for 5 min and receive ectopic mounts. The experimenter prevented male hamster intromissions or ejaculations so that the females would not become either pregnant or pseudopregnant.

Females that showed at least two consecutive estrous cycles and had been acclimated and trained in the preference apparatus were first tested for baseline behaviors including food hoarding, vaginal scent marking, flank marking, and male preference calculated as (time spent with the male – time spent with food) / total time. During baseline testing, 24 h food intake was

measured for at least 4 days prior to the start of the experiment. After baseline testing, 48 hamsters were randomly placed into 1 of 6 groups that did not differ significantly in body weight (115-175 g). The groups included hamsters that were food-restricted by 25% (fed 75% of *ad libitum* food intake determined during baseline) for 4 days (n = 6), food-restricted for 8 days (n = 6), food-restricted for 12 days (n = 12), food-restricted for 12 days and re-fed *ad libitum* for 4 days (n = 6), food-restricted for 12 days and re-fed *ad libitum* for 8 days (n = 6), or fed *ad libitum* (n = 12).

Testing began at the onset of the dark phase of the photoperiod (1200 h) on day 3 and was conducted under dim, red light illumination. The door to the home cage was opened and females were allowed access to both the male and food boxes for a total of 90 min. During the first 15 min, vaginal marking, flank marking, food hoarding and eating as well as location (male, food, or home cage) were recorded. After 15 min of observation, the experimenter stopped recording and the test continued for an additional 75 min (90 min total); i.e., the females continued to have access to both the male and food boxes. After the 90 min test was complete, the hamsters were returned to their respective cages and the doors to the home cages were closed. Weight of food in the home cage and food box was measured and recorded to determine the amount of food hoarded and eaten during the 90 min test.

Blood collection and perfusion

Female hamsters were tested in the preference apparatus on day 3 of the estrous cycle, and at the same time the next day, they were euthanized and a terminal blood sample was taken. Plasma was assayed for estradiol and progesterone concentrations to determine effects of food restriction, and to determine whether levels were below those that would induce lordosis. Plasma insulin and leptin concentrations were assayed to determine the effects of chronic restriction. In order to avoid the confounding effects of meals and cephalic phase hormone release, both food-restricted and *ad libitum*-fed animals were given access to the amount of food normally fed to the food restricted females for 15 min 4 hours before blood collection. This schedule was chosen because previous results showed that Syrian hamsters do not show post-fast hyperphagia, and plasma insulin and leptin concentrations are not significantly increased in Syrian hamsters until more than 4 hours after a meal (Schneider et al., 2000). Thus, plasma hormone concentrations in our different groups of females would be expected to reflect length of food restriction (4, 8, 12 days of restriction) rather than effects of meals. All hamsters were sacrificed before the onset of the dark phase of the photoperiod (1200 hours) by an overdose of sodium pentobarbital (Ovation Pharmaceuticals, Inc., Deerfield, IL). Blood was centrifuged at 3000 rpm and 5°C for 20 min. Plasma was collected and frozen at -20°C until analysis.

Animals were perfused intracardially with phosphate buffered saline (PBS, pH 7.4 at 4^{o}C) followed by 4% paraformaldehyde in PBS at the same temperature. Brains were removed, post-fixed for 24 hours at 4°C in 4% paraformaldehyde, and stored at 4°C in 20% sucrose and 0.001% thimerosol until sectioning. All brains were sectioned within 30 days using a freezing microtome set at 40 µm. Hypothalamic brain sections were placed into polyvinyl pyrollidone (PVP) and stored at -20°C until immunohistochemical staining.

Immunohistochemistry

Cellular activation in GnIH-containing cells was measured by double-labeling for intranuclear FOS, the product of the immediate-early-gene, *c-fos*, a well established marker of changes in cellular activity in response to stimuli in rodents (Hoffman et al., 1993). Tissue was collected and every 4th 40 µm section was double-labeled using fluorescence immunohistochemistry. FOS (1:50,000; Jackson ImmunoResearch Laboratories, West Grove, PA) was amplified with biotinylated tyramine (0.6%) for 30 min at room temperature prior to incubation in CY-2 conjugated streptavidin (1:200; Jackson ImmunoResearch Laboratories) for 1 hour. Following labeling for FOS, sections were labeled using an antibody directed against GnIH specifically for Syrian hamsters (1:10,000; PAC 1365), with CY-3 donkey anti-rabbit (1:200) as the secondary antibody/fluorophore.

Leptin and insulin radioimmunoassay

Blood plasma was analyzed for leptin using the Multi-Species Leptin Radioimmunoassay (RIA) kit (Millipore, St. Charles, MO). Samples were run in duplicate in the same assay with assay limits between 1.0 ng/ml and 50 ng/ml. Similarly, plasma insulin was measured in duplicate using a Rat Insulin RIA kit (Millipore, St. Charles, MO) adjusted to use 50 µl of plasma with assay limits between 0.01 ng/ml and 10.0 ng/ml. Insulin and leptin assays were performed by Millipore Biomarker Services (St. Charles, MO).

Estradiol and progesterone radioimmunoassay

Blood plasma was analyzed for estradiol and progesterone using RIAs (TKE21 and TKPG2, Siemens Medical Solutions Diagnostics, Los Angeles, CA). Assay limits were between 10.0 pg/ml and 1035.4 pg/ml for the estradiol assay and 0.09 ng/ml and 13.0 ng/ml for the progesterone assay. For progesterone values to fall within the acceptable range, blood plasma was diluted 1:10 prior to analysis. Estradiol and progesterone assays were conducted by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA).

Statistical analysis

Behavioral, hormone, and immunohistochemical data were analyzed using one-way analysis of variance (ANOVA) to look for significant effects of different durations of food restriction. When main effects were significant, *post hoc* comparisons were made using Duncan's Multiple Range test. Correlation coefficients were calculated to determine whether there was a significant association between cellular activation in GnIH cells and each behavior variable, or between plasma hormone concentrations and each behavioral variable. Differences were considered statistically significant if $P < 0.05$.

Experiment 2: Effects of Food Deprivation and Body Fat Content on Cellular Activation in GnIH Cells and NPY projections to the DMH

These two experiments examined cellular activation in GnIH cells in the DMH that were either susceptible to or buffered from severe metabolic challenges (food deprivation). Previous

work determined that adult estrous-cycling hamsters below 120 g in body weight were highly likely to show anestrus after 48 hours or more of food deprivation, whereas those above 125 g were buffered from the effects of food deprivation due to their higher body fat content and the ability to oxidize free fatty acids from lipids stored in adipose tissue (Schneider and Wade, 1989).

Hamsters that were the same age, with the same diet composition, were created by feeding diets that differed in the energy required to ingest them. The low body weight group was fed 4 pellets (approximately 20 g) of standard rodent chow in the wire hopper that hangs into the ceiling of the cage. The high body weight group was fed powdered rodent chow *ad libitum* on the floor of the cage. The former group showed a high level of activity as they stood upright and gnawed at the pellets. The latter group, those fed the powdered chow, expended comparably less energy and gained body weight faster because they were not required to chew their food in order to consume it, and they slept in close proximity, if not right in the food.

In the first experiment, Experiment 2a, hamsters were either high (n = 5, 133.13 $\pm$ 2.9) or low body weight (n = 6, 113.6 $\pm$ 3.5) and half of each group was fed *ad libitum* or food-deprived for 96 hours ending on day 4 of the estrous cycle, the day of the LH *surge* and ovulation. This experiment was designed to determine whether cellular activation in GnIH cells on the day of the LH surge would be affected by the severe energetic challenge known to induce anestrous, and whether having a high body fat content prior to deprivation would buffer this effect. Two-way ANOVA, with food availability and prior body weight as the two main factors, was used to analyze the data for behavior, hormone concentrations and the immunostaining.

The second experiment, Experiment 2b, was designed to examine cellular activation in GnIH cells earlier in the cycle, during the time when severe food deprivation would be beginning to have its effects on the GnRH *pulse generator*. Thus, 18 hamsters of a high (n = 9, 121.2 $\pm$ 2) or low body weight (n = 9, 104.2 $\pm$ 3.1) were food deprived for either 36 hours (euthanized on day 2 of the estrous cycle) or 50 hours (euthanized on day 3 of the cycle). An additional group (n = 6, 131.4 + 2.5) served as *ad libitum*-fed controls and data were analyzed with a one-way ANOVA.

In both experiments, the blood was sampled, and hamsters perfused as described for Experiment 1. Animals were perfused intracardially with phosphate buffered saline (PBS, pH 7.4 at 4^{o}C) followed by 4% paraformaldehyde in PBS at the same temperature. Brains were removed, post-fixed for 24 hour at 4°C in 4% paraformaldehyde, and stored at 4°C in 20% sucrose and 0.001% thimerosol until sectioning. All brains were sectioned within 30 days using a freezing microtome set at 40 µm. Hypothalamic brain sections were placed into PVP and stored at -20°C until staining.

Percent FOS/GnIH and GnIH double-labeling with NPY was carried out as described in Experiment 1 on every fourth section. NPY fibers were immunostained using an antibody that stains fibers (1:10,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and was amplified with biotinylated tyramine (0.6%) for 30 min at room temperature prior to incubation in CY-2 conjugated streptavidin (1:200; Jackson ImmunoResearch Laboratories) for 1 h. Following labeling for NPY, sections were labeled using an antibody directed against GnIH specifically for

Syrian hamsters (1:10,000; PAC 1365), with CY-3 donkey anti-rabbit (1:200) as the secondary antibody/fluorophore.

Light microscopy

Sections were investigated using a Zeiss Z1 microscope. Sections were examined using the standard wavelengths for CY-2 (488 nm) and CY-3 (568 nm). Every 4th section through the dorsomedial hypothalamus (DMH) was assessed, and those areas expressing GnIH-ir were investigated for coexpression with FOS protein using confocal microscopy (see below). For light microscopy, areas identified as having double-labeled cells were digitally captured at 200x in 8 bit greyscale using a cooled CCD camera (Zeiss). Each label was captured as a single image without moving the position of the stage or plane of focus between captures. Images were superimposed digitally. Brain areas were examined for double-labeling using Photoshop software in which CY-2 and CY-3 channels could be turned on and off independently. Only those GnIH cells with a visible nucleus in which FOS expression was localized were counted as double-labeled cells. The total number of GnIH cells and the percentage of cells expressing FOS were recorded by two independent observers blind to the experimental conditions.

Confocal microscopy

To examine NPY contacts, GnIH-ir cells with putative NPY contacts were scanned though the extent of each cell in 0.5 μm increments. Only those cells in which the NPY- labeled fiber contacted a GnIH-ir cell in the same 0.5 scan were counted as close contacts. Cells characterized as double-labeled with FOS/GnIH or at the conventional microscopy level were confirmed in the same manner to ensure that FOS was expressed within the cells rather than in overlapping cells in the same field of view. Likewise, cells classified as single-labeled were assessed to ensure that the conventional microscopy strategy did not result in false negatives. At least 10% of those cells quantified using conventional microscopy were assessed in confocal scans for FOS colabeling. Regions of the brain with putative double-label identified at the light level were scanned at 400 × using confocal microscopy. Cells were observed under a Zeiss Axiovert 100TV fluorescence microscope (Carl Zeiss, Thornwood, NY) with a Zeiss LSM 510 laser scanning confocal attachment. The sections were excited with an Argon-Krypton laser using the standard excitation wavelengths for CY-2 and CY-3. Stacked images were collected as 1.0 μm multitract optical sections. Using the LSM 3.95 software (Zeiss), red and green images of the sections were superimposed. GnIH cells in the DMH were examined through their entirety in 1.0 μm steps.

Results

Experiment 1: Different Durations of Food Restriction, GnIH and Behavior
Ingestive Behaviors

One-way ANOVA showed a significant main effect of food availability on the amount of food hoarded ($F(5,42) = 2.64$, $P < 0.04$) (Fig. 1). Similarly, when the amount of food hoarded was subtracted from baseline, there was a significant effect of food restriction on the change in food hoarding ($F(5,42) = 2.75$, $P < 0.03$). Food hoarding was significantly higher in the 8-day and 12-day food restricted groups compared to the *ad libitum* fed group and the 4-day food-

restricted group ($P < 0.05$). This pattern matched that of the percent of GnIH-ir cells that were positive for FOS, but did not match that of the cells labeled for GnIH alone (discussed later). There was a significant positive correlation between the amount of food hoarded and percent of GnIH-ir cells that were positive for FOS-ir (r = 0.585; $P < 0.0001$), and a significant negative correlation between food hoarded and GnIH-ir cell count (r = 0.436; $P < 0.003$). The amount of food hoarded was significantly correlated with body weight loss (r = 0.368; $P < 0.01$), but not with raw body weight. The correlations between food hoarding and other variables were not statistically significant (body weight, leptin, insulin, estradiol, or progesterone concentrations).

There was a significant main effect of food availability on the amount of time spent eating during the preference test ($F(5,42) = 7.56$, $P < 0.0001$) (Table 1). Hamsters spent significantly more time eating after 4, 8, and 12 days of food restriction compared to hamsters fed *ad libitum* ($P < 0.05$) (Table 1). There was a significant negative correlation between time spent eating and body weight (r = 0.437; $P < 0.002$). The correlations between time spent eating and other variables were not statistically significant (change in body weight, number of GnIH-ir cells, and percent of GnIH-ir cells that were positive for FOS-ir, plasma insulin, leptin, estradiol, and progesterone concentrations).

The amount of food eaten during the 90-min test (Fig. 3) did not differ significantly among groups fed *ad libitum* or food-restricted for varying durations, and the correlations among 90-min food intake and the other variables were not statistically significant (body weight, change in body weight, number of GnIH-ir cells, or percent of GnIH-ir cells that were positive for FOS-ir, plasma insulin, estradiol, progesterone concentrations).

Reproductive behaviors

The effect of food availability on the number of vaginal scent marks per 15 min was significant ($F(5,42) = 4.66$, $P < 0.002$) (Fig. 1). Hamsters food restricted for 8 and 12 days showed significantly fewer vaginal scent marks than those fed *ad libitum* and those food restricted for only 4 days ($P < 0.05$), but those re-fed for 4 and 8 days still showed significantly fewer vaginal scent marks than those fed *ad libitum* ($P < 0.05$). There was a significant negative correlation between vaginal scent marks and %FOS-ir in GnIH-ir cells (r = -0.314; $P < 0.04$) and a positive correlation between the number of vaginal scent marks and the number of cells that showed GnIH-ir (r = 0.365; $P < 0.02$). Vaginal scent marks were significantly negatively correlated with body weight loss; the more body weight lost, the fewer vaginal scent marks (r = -0.619; $P < 0.0001$), but vaginal scent marks were not significantly correlated with final body weight. Vaginal scent marks were also positively correlated with plasma progesterone concentrations (r = 0.354, $P < 0.02$), but not with leptin, insulin or estradiol concentrations.

The effect of food availability on the number of flank marks was significant ($F(5,42) = 4.70$, $P < 0.002$). The number of flank marks in 4-day food-restricted females was significantly higher than that of females fed *ad libitum* ($P < 0.05$), but the flank marking scores of 8-day food-restricted animals were not significantly higher than those of hamsters fed *ad libitum* (Table 1). There were no significant correlations between flank marks and any other variables (number of GnIH-ir cells, %FOS-ir in GnIH-ir cells, change in body weight, insulin, estradiol, or progesterone concentrations).

Male preference was calculated as (the amount of time females spent with a male – the amount of time spent with food) / the total time in the preference apparatus (Table 1). There was no main effect of food restriction on male preference. There was a significant negative correlation between male preference and change in body weight ($r = 0.352$; $P < 0.01$), but the correlations between male preference and other variables were not statistically significant (body weight, number of GnIH-ir cells, %FOS-ir in GnIH-ir cells, plasma leptin, insulin, estradiol, or progesterone concentrations).

Body weight

Group differences in body weight at the start of the experiment were not statistically significant, but the final body weights after food restriction varied significantly among the groups ($F(5,42) = 4.37$, $P < 0.003$) (Fig. 2). Body weights were significantly decreased at 4 days after the start of food restriction ($P < 0.05$) compared to hamsters fed *ad libitum*. Hamsters fed *ad libitum* throughout the experiment were significantly heavier compared to all other groups except hamsters food-restricted for 12 days and re-fed *ad libitum* for 8 days (Fig. 2). Also, there was a significant positive correlation between body weight and plasma progesterone concentrations ($r = 0.302$; $P < 0.04$) and between body weight and plasma leptin concentrations ($r = 0.285$; $P < 0.05$), but not between body weight and plasma insulin or estradiol concentrations. Body weight was not significantly correlated with either the number of GnIH cells, or the percent of GnIH-ir cells that were positive for FOS.

When the hamsters' final body weights were subtracted from initial body weights, the groups differed significantly in change in body weight ($F(5,42) = 30.92$, $P < 0.0001$) (Fig. 2). Change in body weight was significantly positively correlated with plasma progesterone concentrations ($r = 0.451$; $P < 0.001$) and the number of GnIH cells ($r = 0.459$; $P < 0.002$) and significantly negatively correlated with the percent of GnIH-ir cells that were positive for FOS ($r = 0.570$; $P < 0.0001$).

GnIH immunoreactivity and cellular activation

The percent of FOS-positive GnIH-ir cells was calculated as (the number of cells double-labeled for FOS-ir and for GnIH-ir/ the total number of GnIH-ir cells) * 100. There was a significant main effect of food availability on the percent of GnIH-ir cells that were positive for FOS ($F(5,38) = 3.47$, $P < 0.01$) (Fig. 3). *Post hoc* analysis revealed a significant increase in cellular activation in GnIH-ir cells at 8 and 12 days of food restriction compared to hamsters fed *ad libitum* ($P < 0.05$). There was a significant effect of food restriction on the number of GnIH-ir cells ($F(5,38) = 2.88$, $P < 0.03$), with significant decrease in the number of GnIH cells that were immunoreactive in the females food restricted for 8 and 12 days compared to those fed *ad libitum* and those food restricted for 4 days ($P < 0.05$).

Plasma leptin, insulin, estradiol, and progesterone concentrations

There was a significant main effect of food treatment on plasma leptin concentrations ($F(5,41) = 2.50$, $P < 0.05$) (Fig. 4). *Post hoc* comparisons revealed that plasma leptin

concentrations did not differ between *ad libitum*-fed and food-restricted females after any level of food restriction. However, females food-restricted for 12 days and re-fed *ad libitum* for 4 days had significantly higher plasma leptin concentrations compared to females fed *ad libitum*. In contrast to leptin, there was no significant effect of food restriction or re-feeding on plasma insulin, progesterone, or estradiol concentrations (Fig 4).

Experiment 2: Effect of Metabolic Challenges on NPY Fibers in the DMH

In the first part of Experiment 2, females with either high or low body weight were either fed *ad libitum* or food deprived for 96 hours from day 1 to day 4 of the estrous cycle (Fig. 5). Previous work showed that the lean, food-deprived females would become anestrous, whereas those that were fat at the start of deprivation would be buffered from the effects of deprivation (Schneider and Wade, 1989; 1990). Two-way ANOVA showed a significant main effect of food deprivation on the percent of GnIH-ir cells that were positive for FOS-ir ($F(1,6) = 7.69$, $P < 0.03$), no significant effect of body weight group, and no significant interaction. The more body weight lost, the higher the increase in percent of GnIH-ir cells that were positive for FOS-ir, and this correlation was significant ($r = 0.72$, $P < 0.02$). Body weight loss was significantly negatively correlated with the number of cells that were immunoreactive for GnIH ($r = 0.72$, $P < 0.02$). Neither the percent of GnIH-ir cells positive for FOS-ir nor the number of GnIH-ir cells was significantly correlated with final body weight.

In the second part of Experiment 2, females were sacrificed after either 1.5 or 2.5 days of food deprivation during the follicular phase of the estrous cycle to determine whether there were changes in GnIH that occur in the early stages of metabolic challenge that would be expected to inhibit the GnRH pulse generator in lean, but not fat females (Morin, 1986). One-way ANOVA showed no significant effect of treatment group on the percent of GnIH-ir cells that were positive for FOS-ir, and a significant effect of treatment group on the number of GnIH-ir cells ($F(2,16) = 27.95$, $P < 0.0001$) (Table 2). Both food-deprived groups (30 and 50 hours of deprivation) had significantly fewer GnIH-ir cells than did the *ad libitum*-fed controls ($P < 0.0001$). The percent of GnIH-ir cells that were positive for FOS-ir increased linearly with the amount body weight loss and this correlation was significant ($r = 0.62$, $P < 0.004$). This variable was also significantly positively correlated with final body weight ($r = .576$, $P < 0.01$). The number of GnIH-ir cells was also significantly negatively correlated with the amount of body weight lost ($r = 0.58$, $P < 0.01$) and with final body weight ($r = 0.59$, $P < 0.01$).

Double-labeling for GnIH-ir and NPY-ir revealed that NPY-ir nerve fibers were densely packed in the DMH, and that putative NPY terminals can be observed in close proximity to GnIH cell bodies within this brain area (Fig. 7) at low power light microscopy and confirmed at high power light microscopy and confocal microscopy. An average of 41.46% GnIH-ir cell bodies per animal ($n = 6$) receive contacts from NPY-ir fibers in the DMH.

Discussion

The primary findings were 1) a linear effect of energy availability on ingestive and sex behavior in Syrian hamsters, with appetitive behaviors most sensitive and ovarian steroid secretion the least sensitive to food restriction or deprivation (Fig. 1), 2) a linear effect of energy

availability on cellular activation in GnIH-ir cells in the DMH (Fig. 2) significantly correlated with food hoarding and negatively correlated with vaginal scent marking, 3) no significant effect of food restriction on plasma leptin, insulin, estradiol, or progesterone concentrations (Fig. 4) and no significant correlation among hormone concentrations and cellular activation of GnIH-ir cells, and 4) strong projections of NPY-ir fibers in close apposition to GnIH-containing cell bodies in the DMH (Fig. 7). Together these results are consistent with the idea that a wide range of metabolic deficits, from mild food restriction in fattened females to complete food deprivation in lean females, have linear effects on the GnIH system. Finally, it is plausible that the effects of fuel deficits on behavior are mediated by NPY-containing cells that receive information about the availability of oxidizable fuels and send this information via projections to GnIH neurons in the DMH.

The stimulatory effect of 25% food restriction on the percent of FOS-positive cells in GnIH-ir cells was significant even though food restriction did not significantly increase the number of GnIH-ir cells (and in some groups decreased the number GnIH-ir cells) in the DMH (Fig. 2 and 5). This outcome is consistent with the idea that metabolic deficits cause increases in GnIH secretion or secretion of other peptides without compensatory increases in the synthesis of GnIH. Thus, it might be predicted that these energetic manipulations would increase the release of GnIH at the synapse, but would not increase gene expression or translation to the same extent. Effects of food restriction on GnIH gene expression is not yet known.

In both Experiments 1 and 2a, GnIH parameters were closely associated with body weight loss, rather than with final body weight. Even fat hamsters that were food deprived showed an effect on number of cells that showed GnIH-ir. This is consistent with a role for this peptide in control of behavioral motivation during mild energetic challenges, and does not strongly support a role for this peptide in switching off the HPG system and the estrous cycle after prolonged deprivation.

Food restriction in Experiment 1 and food deprivation in Experiment 2 had significant effects on both cellular activation and on number of GnIH-ir cells, but it is not clear how this information about food availability reaches the DMH. Food restriction, for example, had significant effects on appetitive behaviors without significant effect on plasma concentrations of ovarian steroids, insulin, or leptin, suggesting that information about fuel availability reaches GnIH cells via other means, e.g., via changes in plasma ghrelin or direct information about the availability of oxidizable metabolic fuels detected in periphery, brain stem or hypothalamic areas that project to GnIH cells.

One possibility is that GnIH cells in food-restricted females are more responsive to estradiol than those GnIH cells in females fed *ad libitum*. Plasma estradiol concentrations did not differ significantly among the groups food restricted for different durations (Fig. 4), as would be expected because this level of energy deficit was too low to inhibit ovarian steroid secretion and induce anestrous. This confirms three previous experiments in which 25% food restriction or 48 hours of food deprivation in fat females decreased appetitive sex behavior, increased appetitive ingestive behavior, but failed to significantly decrease plasma estradiol concentrations or inhibit ovarian-steroid-dependent lordosis .(Schneider et al., 2007;Klingerman et al., 2010;Klingerman et al., 2011). Thus, one possible explanation for the decrease in appetitive

estradiol-dependent sexual behavior, independent of changes in circulating estradiol concentrations, is down-regulation of estradiol receptors (ER) on GnIH cells. A similar suggestion has been made regarding downregulation of ER in other brain areas involved in lordosis and ingestive behavior. For example, 48 hours of food deprivation in lean females decreases ER-immunoreactivity (ir) in the ventromedial hypothalamus (VMH), and increases ER-IR in the arcuate nucleus of the hypothalamus (Arc), paraventricular nucleus of the hypothalamus (PVN), and medial preoptic area of the hypothalamus (MPOA) (Li et al., 1994;Panicker et al., 1998). The DMH was not examined in these latter studies. However, ER-α co-localizes with GnIH cells in the Syrian hamsters DMH, and these cells respond to estradiol stimulation with significant increases in cellular activation (Kriegsfeld et al., 2006). Thus, future experiments will determine whether different levels of food restriction (mild to severe) down-regulates ER-α in GnIH cells in the DMH, or whether the effects of food restriction might occur downstream or independent from ER-α-containing GnIH cells.

One such downstream mediator might be GnRH. GnRH and its metabolites have well-documented facilitory effects on sex behavior, in particular, lordosis in rats (Moss and McCann, 1975;Moss and Foreman, 1976;Dudley et al., 1981;Dudley and Moss, 1988;Moss and Dudley, 1990;Dudley and Moss, 1991;Wu et al., 2006), and it is plausible that GnIH-mediated inhibition of GnRH secretion accounts for inhibition of appetitive sex behavior in Syrian hamsters. Furthermore, the appetitive ingestive behavior, food hoarding, was significantly increased at 8 days after the start of restriction and was significantly correlated with cellular activation in GnIH cells (Figs. 1 and 2). This is consistent with mounting evidence that ingestive behaviors are increased by GnIH (Tachibana et al., 2005b;Johnson et al., 2007). GnIH inhibits GnRH secretion in Syrian hamsters (Kriegsfeld et al., 2006), and at least one form of GnRH (GnRH-II) is inhibitory for ingestive behavior (Kauffman, 2004;Kauffman and Rissman, 2004b;a;Kauffman et al., 2005).

Effects of GnIH in Syrian hamsters differs from that of rats, mice and birds, but might be more similar to that of human beings. For example, in male rats, treatment with GnIH increases food intake (Johnson et al., 2007). In white-crown sparrows, the avian homologue of GnIH, stimulates food intake, and in chickens treatment with a GnIH antagonist prevents post-fast hyperphagia (Tachibana et al., 2005a). In our experiment, we saw no direct correlation between food intake and GnIH expression, but we did observe a significant positive correlation between GnIH-ir and food hoarding, an important behavior in the energy homeostasis repertoire of both hamsters and human beings. Syrian hamsters do not increase food intake in response to prior food deprivation and body weight loss (Silverman and Zucker, 1976;Rowland, 1982), even though they show the expected metabolic adjustments (Borer et al., 1979), and exhibit behaviors that reflect increases in hunger (DiBattista and Bedard, 1987). Similarly, in human beings, food intake is not increased after fasting in many circumstances (Hetherington et al., 2000;Al-Hourani and Atoum, 2007;Levitsky and DeRosimo, 2010). Syrian hamsters express increased motivation to engage in ingestive behaviors by increasing the speed of eating, decreasing the latency to eat, increasing the consumption of an otherwise unpalatable diet (DiBattista and Bedard, 1987), and elevated levels of food hoarding (Wong, 1984;Lea, 1986;Phillips et al., 1989). Siberian hamsters also exhibit greater increases in food hoarding compared to food intake following a period of energy restriction (Bartness and Clein, 1994;Day and Bartness, 2004). It has been noted in a particularly lucid review by Bartness et al., that neuroendocrine control of food hoarding in

hamsters is likely to shed light on human ingestive behavior (Bartness et al., 2011). Hamsters, like members of our own species, are more likely to bring food into their home prior to consumption, rather than consume it at the site where it was grown, gathered or hunted, and the amount of food purchased at grocery stores is a function of the hunger and obesity at the time of shopping (Tom, 1983;Beneke and Davis, 1985;Mela et al., 1996). Furthermore, both humans and hamsters are avid food hoarders. Many species of hamsters have been reported to hoard pounds of food in their burrows (Vander Wall, 1990), and humans are well known to store food in their cabinets, refrigerators, freezers, and smoke houses, not to mention grain silos and food warehouses. Thus, understanding the peptides that control healthy and disordered eating and body weight gain might require attention to effects of GnIH on food hoarding.

A large body of research implicates NPY in metabolic control of reproduction and ingestive behavior. NPY is a potent orexigenic peptide (Clark et al., 1984;Kulkosky et al., 1988;Corp et al., 2001;Clarke et al., 2005), inhibits sex behavior (Clark et al., 1985;Thornton et al., 1996), and inhibits LH secretion in the presence of low circulating levels of estradiol (Khorram et al., 1987;Sahu et al., 1987;Malven et al., 1992). NPY has greater effects on appetitive compared to consummatory behaviors. Treatment with NPY has a far greater effect on food hoarding than on food intake in Siberian hamsters (Day et al., 2005;Keen-Rhinehart and Bartness, 2007). Similarly, NPY treatment increases the number of approaches to a bottle of sucrose while decreasing the passive consumption of sucrose applied in drops to the palate, and NPY delays copulatory behavior but only in males offered the opportunity to approach and drink from a bottle of sucrose (Ammar et al., 2000). Furthermore, NPY cell bodies in the DMH and from other brain areas have long been implicated in control of energy intake. NPY mRNA is overexpressed in the DMH during the hyperphagia of lactation (Smith, 1993) and in various models of obesity (Kesterson et al., 1997;Guan et al., 1998a;Guan et al., 1998b;Tritos et al., 1998). Adenoassociated virus (AAV)- mediated increases in NPY gene expression in the DMH of lean rats increases food intake and body weight, and accelerates the development of high-fat diet-induced obesity (Yang et al., 2009). Decreased NPY expression in the DMH by AAV-mediated RNA interference prevents the hyperphagia, obesity and diabetes of Otsuka Long-Evans Tokushima Fatty (OLETF) rats (Yang et al., 2009). Thus, we were compelled to examine the proximity of NPY projections to GnIH neurons in the DMH. Food-deprived females were used to maximize identification of NPY cells. NPY terminals showed strong projections to the DMH and were seen in close apposition to GnIH neurons (Fig. 7). It is possible that these NPY cells originate in the arcuate nucleus of the hypothalamus, the brain stem, or from within the DMH, all areas where NPY gene expression has been identified and from which NPY cells project to the DMH in other rodents (Bai et al., 1985;Sahu et al., 1988;Bi et al., 2003).

These results show a clear correlation between neural activation in GnIH-ir cells and ingestive and sex behavior. It is not known, however, whether GnIH secretion *causes* changes in behavior. GnIH might be a causal factor for increased hunger and food hoarding, decreased sexual motivation, or both, but it might be a nonfunctional correlate of other causal factors (metabolic events, other hormones, and other neuropeptides such as kisspeptin, NPY, alpha-melanocyte stimulating hormone or orexin). GnRH, for example, might influence ingestive and sex behavior by virtue of its direct link to metabolic cues, since recent evidence shows that GnRH neurons receive dendritic input from outside the blood-brain barrier (Herde et al., 2011). Further work is necessary to determine whether changes in GnIH cells are a correlate or a causal

factor in control of behavior. Nevertheless, these results are consistent with the idea that GnIH in the DMH, and possibly NPY cells that project to the DMH are part of a system that prioritizes sex and ingestive behavior in order to optimize reproductive success in environments where energy availability fluctuates. Current experiments are underway to examine if central or systemic treatment with GnIH, NPY, and antagonists to their receptors have the expected influences on appetitive and consummatory sex and ingestive behavior in Syrian hamsters.

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Figure legends

1. Mean and standard error of the mean of A. the amount of food hoarded and B. the number of vaginal marks produced in 15 min on day 3 of the estrous cycle. Food-restricted females were fed 75% of their *ad libitum* intake for their designated length of restriction. Hamsters that were re-fed were food-restricted for 12 days and re-fed *ad libitum* for 4 or 8 days. * Number of vaginal marks different from hamsters fed *ad libitum* at $P < 0.05$.

2. Mean and standard error of the mean for body weight change of female Syrian hamsters either fed *ad libitum* or food restriction to 75% of their *ad libitum* intake for 4, 8, or 12 days or food restricted for 12 days and then re-fed for 4 or 8 days. * Significantly different than *ad libitum* at $P < 0.05$.

3. Mean and standard error of the mean for A. the number of GnIH-ir cells, B. the percent of GnIH-ir cells that showed FOS in the DMH of female Syrian hamsters, and C. photomicrographs of cells double-labeled for GnIH (red) and FOS-ir (green) following food restriction and refeeding. Food-restricted females were fed 75% of their *ad libitum* intake for 4, 8 or 12 days or were food restricted for 12 days and then re-fed for 4 or 8 days. * Significantly different than *ad libitum* at $P < 0.05$.

4. Mean and standard error of the mean for plasma concentrations of A. progesterone, B. estradiol, C. leptin, and D. insulin in female Syrian hamsters either fed *ad libtum* or food-restricted to 75% of their *ad libitum* intake at 4, 8, or 12 days after the start of food restriction or after 12 days of restriction and either 4 or 8 days of re-feeding. * Significantly different than *ad libitum* at $P < 0.05$.

5. Mean and standard error of the mean for A. body weight of *ad libitum*-fed or food deprived female hamsters that were lean or fat prior to the start of food deprivation and B. body weight change of the same females after food deprivation. * Significantly different than *ad libitum* at $P < 0.05$.

6. Mean and standard error of the mean for A. the number of GnIH-ir cells in the DMH and B. the percent of GnIH cells that showed FOS-ir in the DMH of female Syrian hamsters that were food-deprived or fed *ad libitum*, and half of the food-deprived hamsters were lean and the other half were fattened prior to the start of food deprivation. * Significantly different than *ad libitum* at $P < 0.05$. Photomicrographs of GnIH/FOS-ir in the groups described.

7. Representative photomicrographs of cells double-labeled for GNIH (red) and NPY (green). A cluster of GnIH cells receive extensive NPY projections at 250x in this confocal scan (A) and a single GnIH-ir cell with presumptive NPY boutons at 1000x at the conventional (B) and confocal (C) microscopic levels.

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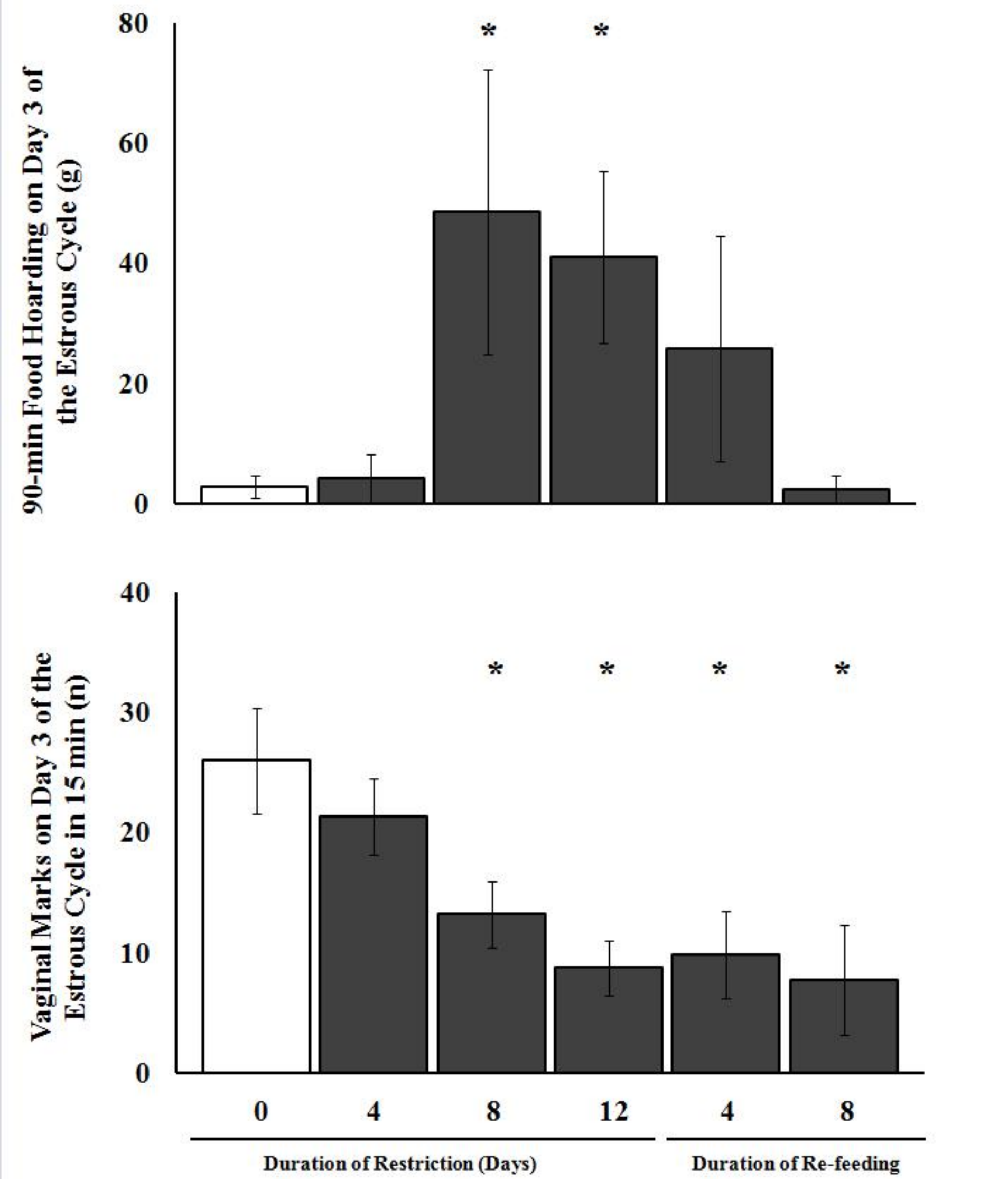
Figure 1.JPEG



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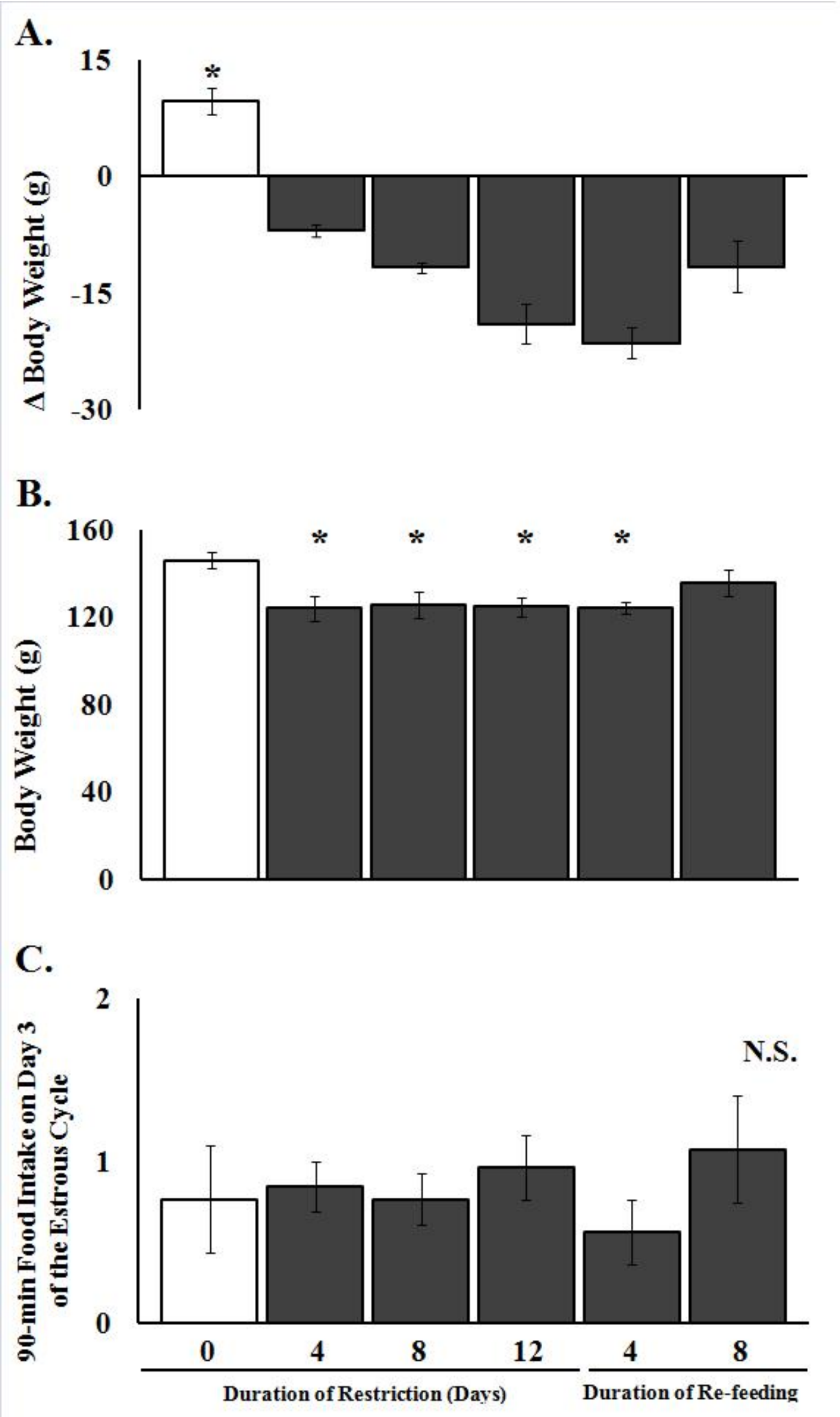

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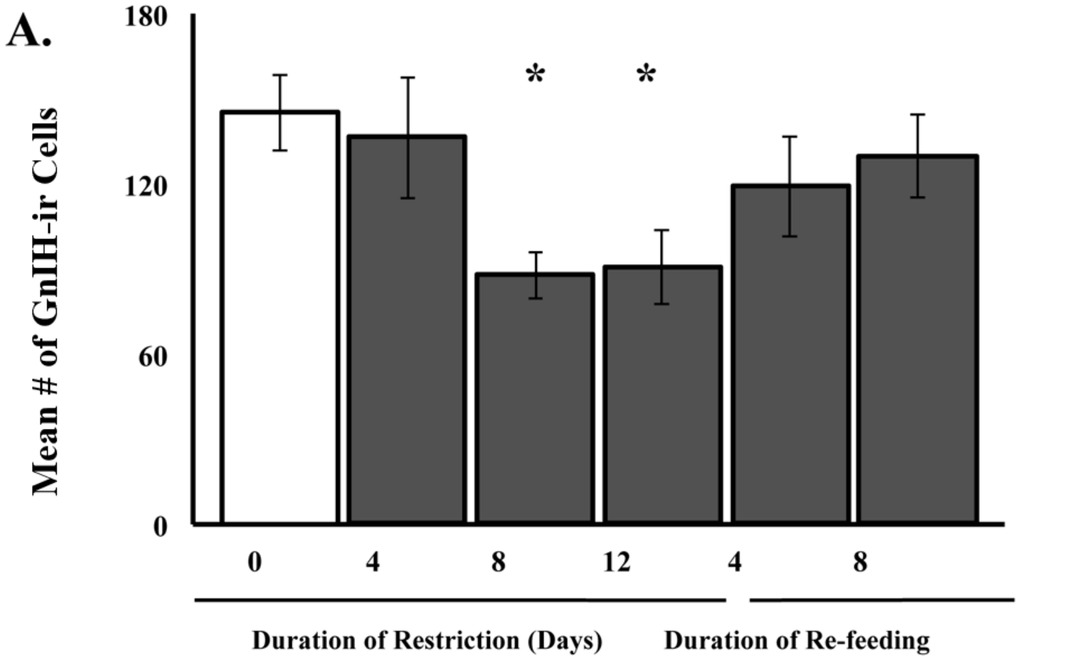
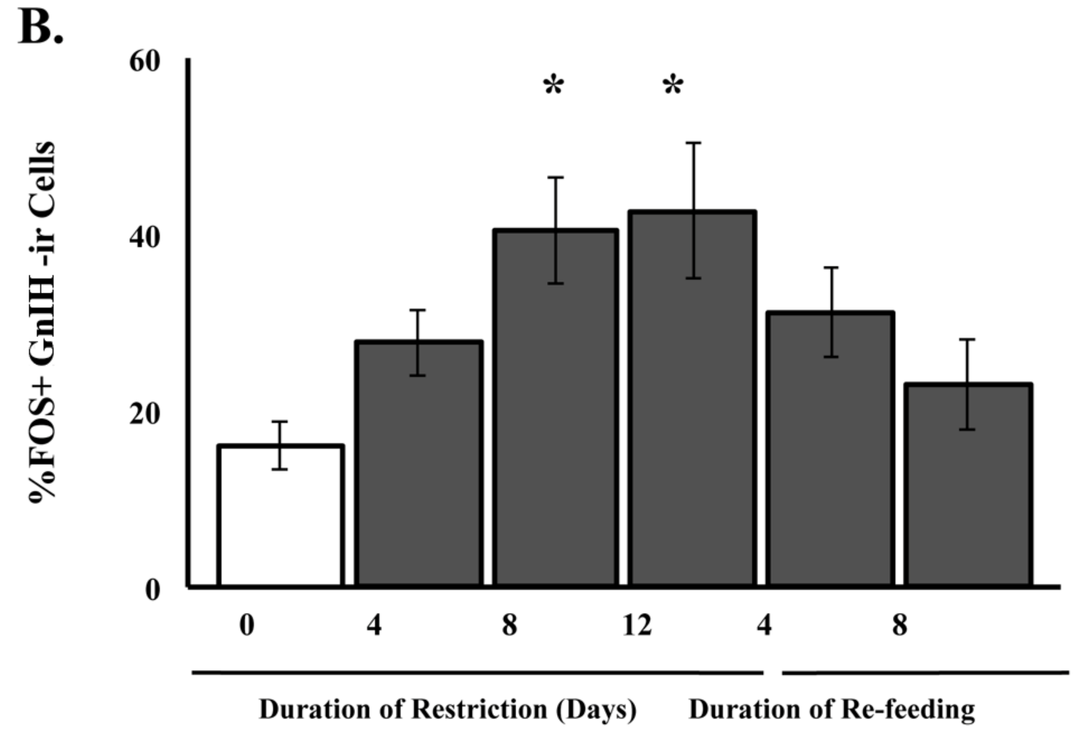

A.
Mean # of GnIH-ir Cells
180
120
60
0
*
*
0
4
8
12
4
8
Duration of Restriction (Days)
Duration of Re-feeding


B.
%FOS+ GnIH -ir Cells
60
40
20
0
*
*
0
4
8
12
4
8
Duration of Restriction (Days)
Duration of Re-feeding

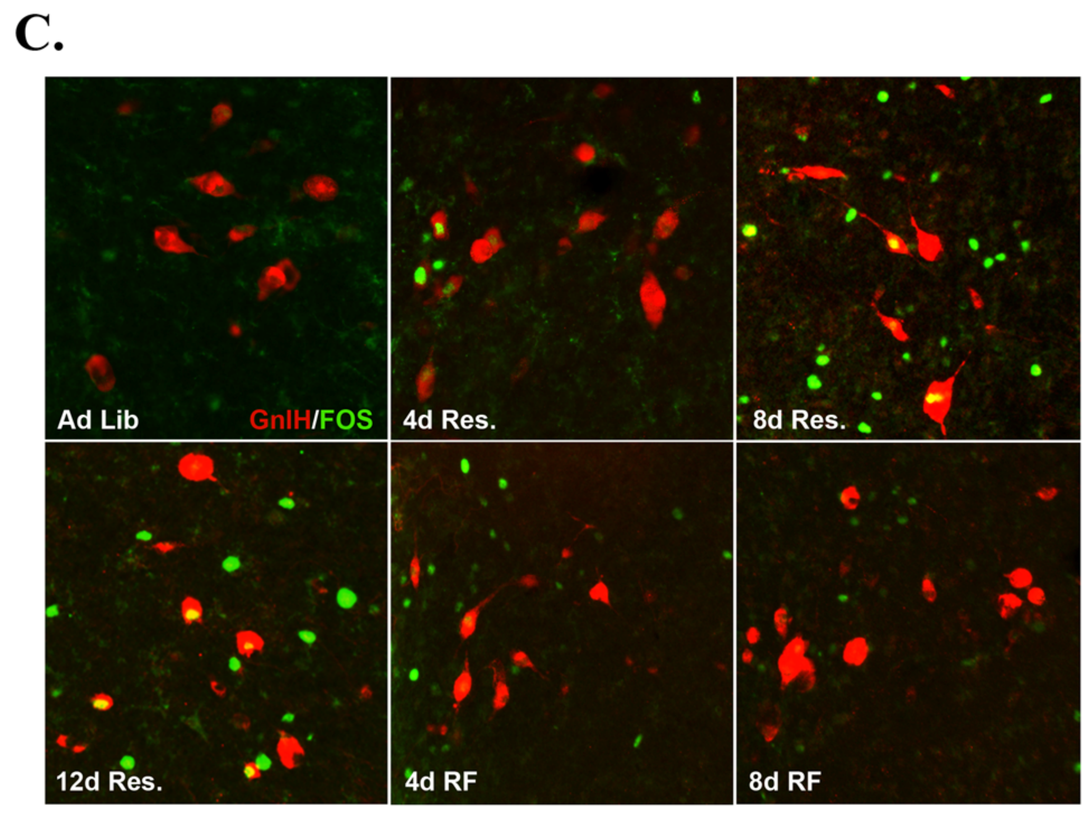
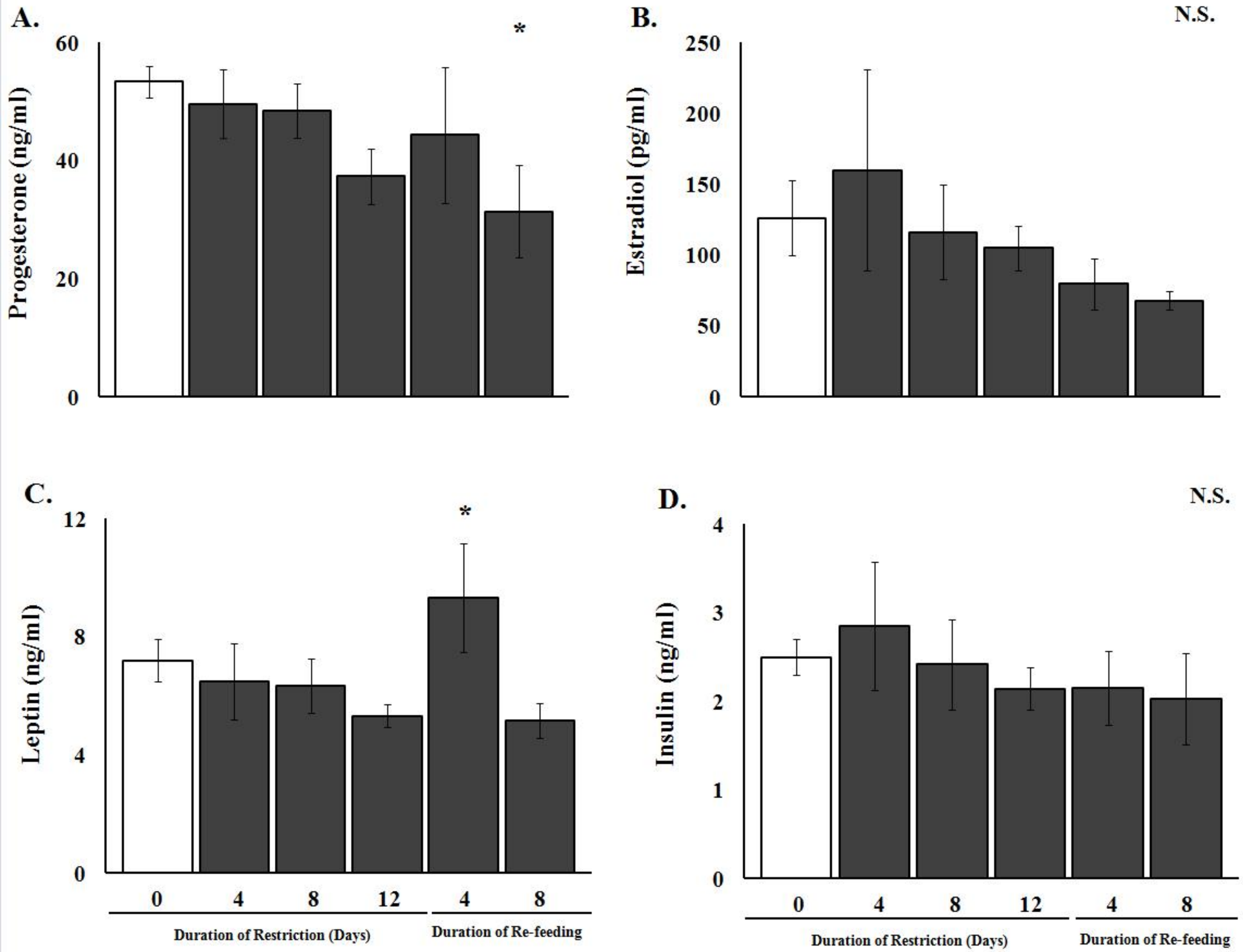

C.
Ad Lib
GnIH/FOS
4d Res.
8d Res.
12d Res.
4d RF
8d RF

Figure 4.JPEG



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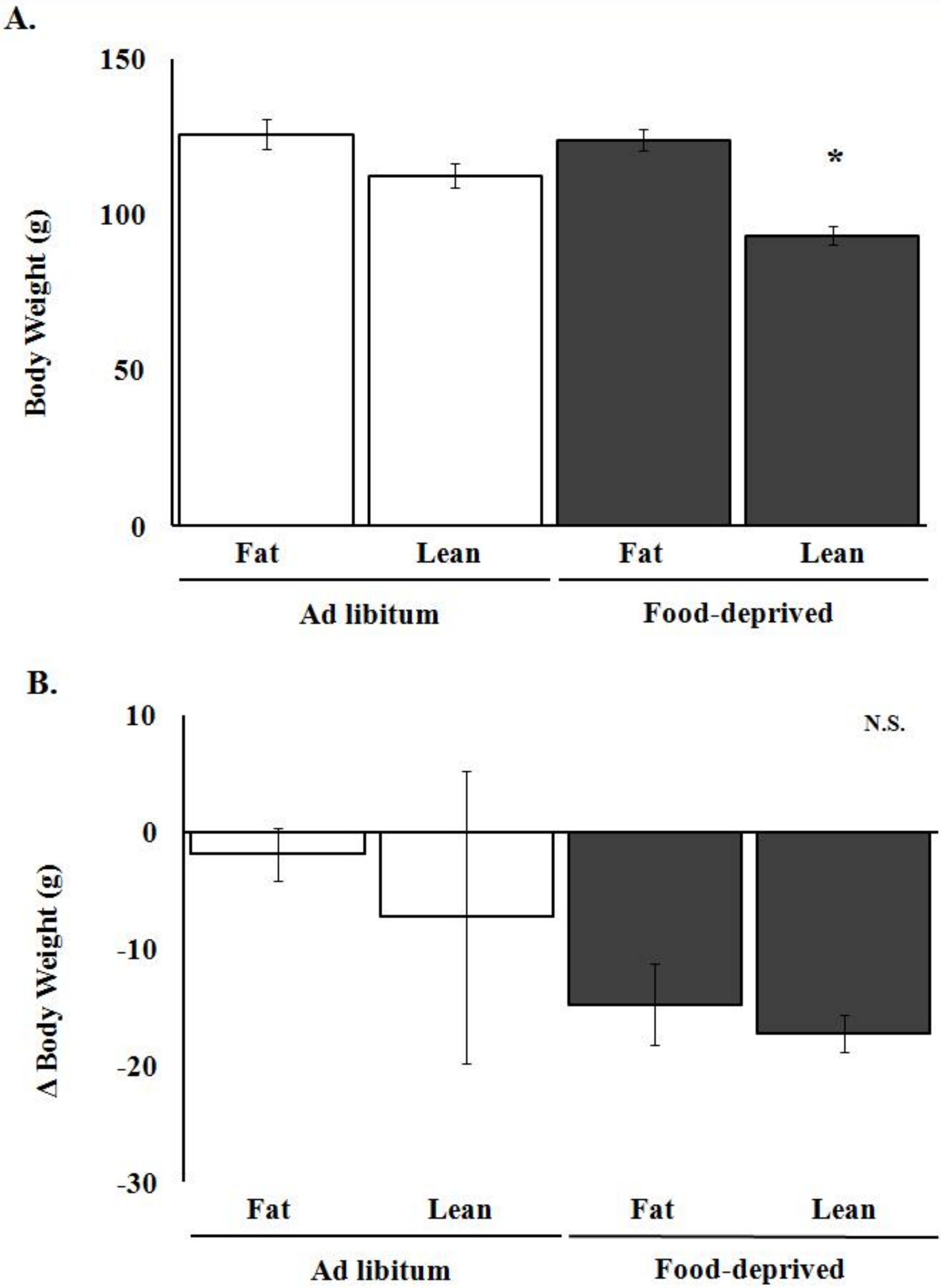

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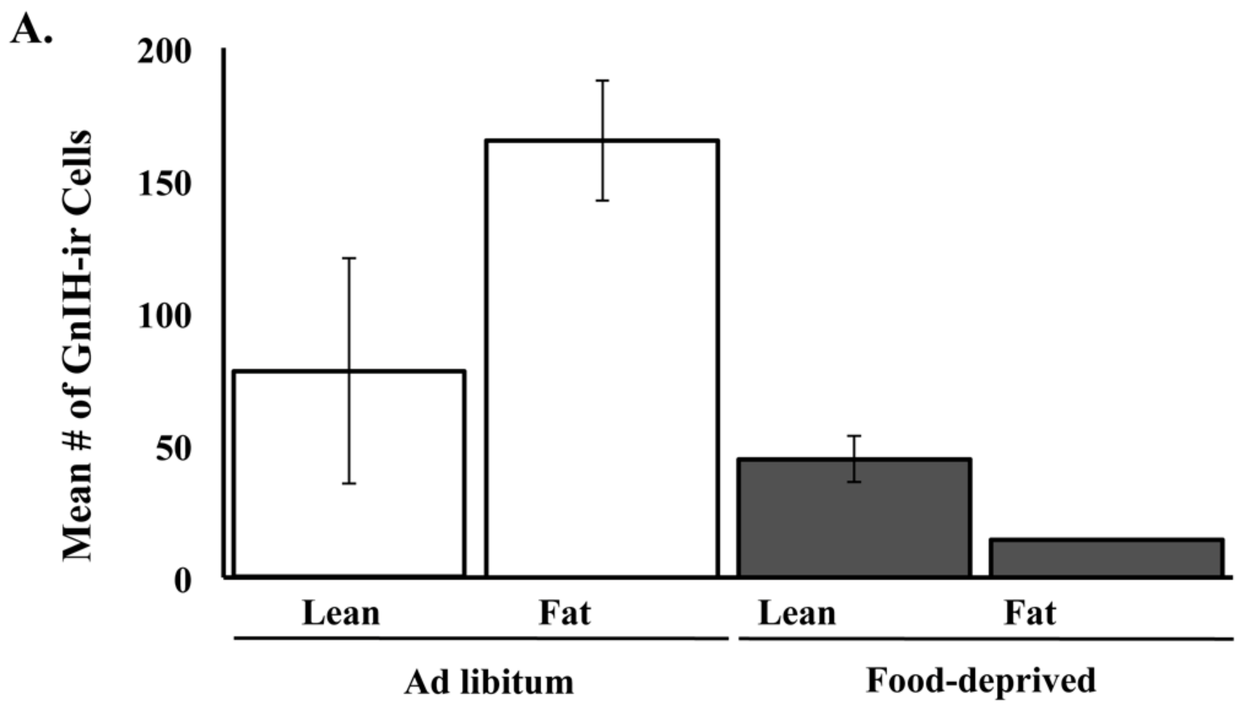

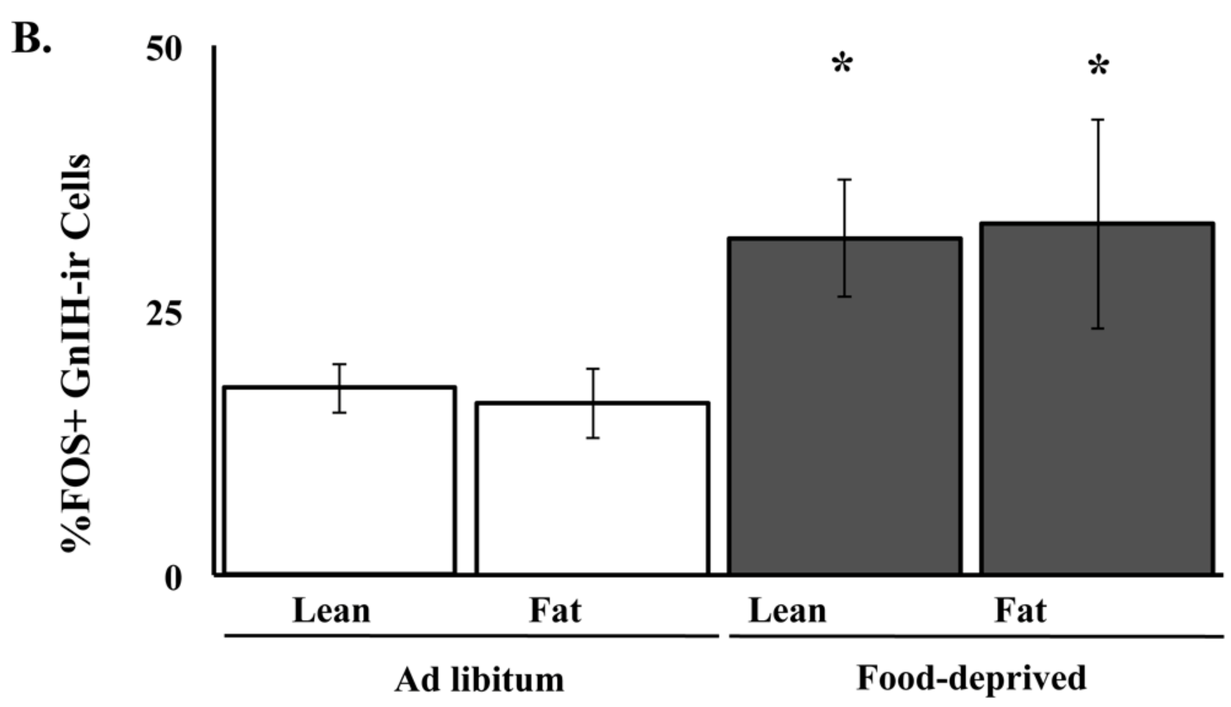

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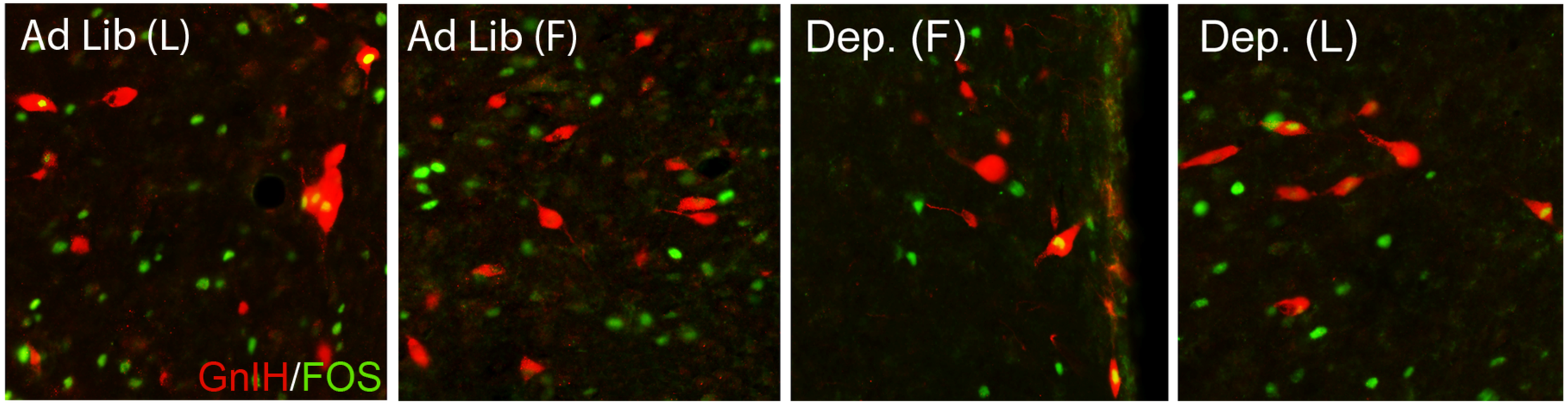

Figure 7.TIFF