Author/s:
Anversa, RG;Campbell, EJ;Ch'ng, SS;Gogos, A;Lawrence, AJ;Brown, RM

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Brown Robyn (Orcid ID: 0000-0003-4167-3634)

A MODEL OF EMOTIONAL STRESS-INDUCED BINGE EATING IN FEMALE MICE WITH NO HISTORY OF FOOD RESTRICTION

Anversa, RG[1,2], Campbell, EJ[1], Ch'ng SS[1], Gogos, A[1,2], Lawrence, AJ[1,2], *Brown, RM[1,2]

[1] The Florey Institute of Neuroscience and Mental Health, Mental Health Division, Parkville, Melbourne, Australia.

[2] The Florey Department of Neuroscience and Mental Health, University of Melbourne, Parkville, Melbourne, Australia.

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***Correspondence:**

Robyn M Brown

Florey Institute of Neuroscience and Mental Health

University of Melbourne

30 Royal Parade

Parkville, VIC, 3052

AUSTRALIA

Ph: +61 3 9035 6592

Email: robyn.brown@florey.edu.au

FAX: +61 3 9035 3107

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Abstract

Overeating is a major contributing factor to obesity and related health complications. For women in particular, negative emotions such as stress strongly influence eating behavior and bingeing episodes. Modelling this type of binge eating in rodents presents challenges: firstly, stress-induced anorexia is commonly observed in rodents therefore a mild stressor is required in order to observe an orexigenic effect. Second, many studies report using calorie restriction to observe the required behavior; yet this does not necessarily reflect the human condition. Thus, the aim of this study was to develop a model of emotional stress-induced bingeing independent of caloric restriction. Female and male C57BL/6J mice were divided into *ad libitum* (n=20 per sex) and food-restricted (n=20 per sex) groups which were both further split into a control group and a group exposed to frustration stress (n=10 per group). All mice were provided intermittent access to a highly palatable food in 2 cycles. At the end of each cycle the stress group was subjected to a 15-min frustration episode where highly palatable food was within the home cage but inaccessible. Both groups were then given free access for 15min. Frustrated female mice from the *ad libitum* displayed binge-like behavior compared to controls ($p = 0.0001$). Notably, this behavior was absent in males. Ovariectomy had no impact on binge-like behavior. Collectively, these data validate a novel model of emotional stress-induced binge eating specific to female mice which does not require caloric restriction and is not driven by ovarian hormones.

Introduction

Overeating is a major contributing factor to obesity and related health complications. Negative emotions such as stress can influence bingeing episodes, compulsive seeking and eating, especially of highly palatable foods. Bingeing is characterized by an excessive intake of food in a short period of time even when not in a negative energy balance and is characteristic of binge eating disorder (BED) and bulimia nervosa, conditions that affect millions worldwide[1]. Eating disorders are more prevalent in the female population and are often comorbid with depression, substance abuse, anxiety, and stress. As such, eating disorders are multifactorial and complex conditions, challenging to model and treat[2,3,4].

The majority of previous studies examining binge-like eating in animal models have included cyclic food restriction. These paradigms are potentially suboptimal when the aim is to investigate reward-based food seeking and bingeing behavior driven by hedonic processes independent of the metabolic memory of negative energy balance[5,6,7]. Furthermore, despite the higher incidence of binge eating in the female population and the established role that stress and negative affect plays in this behavior, many studies to date have used male subjects and often do not specifically examine stress-related overeating. When a stressor is used it is often a physical stressor such as tail-pinch or foot-shock which does not accurately model the mode of stress experienced by human subjects (i.e. emotional)[8,9,10,11]. Indeed, stress-related disorders are positively correlated with bingeing episodes and are more prevalent in females, underscoring the importance of designing ethologically relevant animal models to investigate the interplay between stress and bingeing behaviors[12,13,14,15]. In 2009 Cifani and colleagues reported an elegant model of emotional-stress induced binge eating in

female rats where repeated cyclic caloric restriction induced binge eating of highly palatable food when exposed to a frustrative stressor[5,7,16,17]. This protocol models the impact of 'yo yo dieting' on binge eating behavior. We wanted to extend these findings to establish whether this behavior can be observed in the absence of a history of caloric restriction and could be readily modelled in mice.

Given the prevalence of binge eating in women, it is possible that ovarian sex hormones are implicated in bingeing behavior. Many studies have investigated the role of ovarian hormones in binge eating, in both animals[18,19] and humans[20,21,22,23]. For example, changes in hormones, such as progesterone, that occur during the menstrual cycle in women, may contribute to binge eating[21,22,23]. Yet, there is substantial heterogeneity in the presentation of binge eating in both humans and animals, and stress is another factor that may modulate this behavior. Importantly, stress can influence ovarian hormone levels and dysregulation of the menstrual cycle and estrous cycle, in humans[24] and animals[7], respectively. Thus, there is a complex interaction between stress, ovarian hormones, and physiological responses underpinning binge eating behavior. As such, improved models of stress-induced overeating are needed to better understand how stress differentially affects bingeing phenotypes both within and between sexes.

Herein, we present the validation of a stress-induced binge eating model in female mice that does not depend on cyclic caloric restriction and that is highly reproducible. We demonstrate that the elicited binge-like eating behavior is not driven by ovarian hormones.

Materials and methods

Ethics statement

All procedures were conducted in adherence to the Prevention of Cruelty to Animals Act (2004), under the guidelines of the National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia (2013) and were approved by The Florey Institute of Neuroscience and Mental Health Animal Ethics Committee (AEC 16-017).

Animals and diet

A total of 72 female and 40 male 2-month-old C57BL/6J mice (Animal Resource Centre, Perth, Australia) were used. Mice were singly housed in different rooms according to their sex and allowed to habituate for 10 days prior to the beginning of experimentation. The rooms were maintained on a reverse 12-h light/dark cycle (lights off from 0900 to 2100). All experiments were performed 2 h after the beginning of the dark cycle. Room temperature was maintained at 21-22.5°C and humidity at 45-60%. Mice received *ad libitum* access to standard chow (4.5% fat; Barastoc, Ridley Corporation, Melbourne, Victoria, Australia) and water unless otherwise specified.

Emotional frustration-induced binge eating behavior

Animals were divided into food-restricted (n = 20) and non-food-restricted (n = 20) groups. Within each group, animals were split into a control group that was not submitted to frustration (n = 10) and a frustration stress group (n = 10). The frustration stress model comprised two 8-day cycles. For the first 4 days of each cycle, animals had either free access to chow or were chow restricted (68% of normal chow intake[5]). Both groups were then permitted 2 hours of free access to highly palatable food high in fat and sugar (Reese's® peanut butter drops and Nestlé® chocolate drops) at the beginning of their dark cycle (1000-1200) over 2 days. Animals had 1 day of free feeding on chow before being submitted to frustration stress. On the last day of cycle 1 and 2, stress group animals were submitted to the frustration episode, which consisted of an enclosed tea strainer containing the highly palatable food placed within the home cage. Mice had visual and olfactory access to the food but were not allowed to consume it for 15 min. Subsequently, the highly palatable food was placed in their home cages, and all mice were allowed to freely consume the food for the next 15 min. The amount of highly palatable food given was weighed before and after the experiments to observe how much was consumed post-stress. For the control group, an empty tea strainer was placed in the home cage. The experimental timeline was adapted from Cifani *et al.*[5] (Figure 1).

Ovariectomy (OVX) and sham surgeries

OVX was performed in a new cohort of female mice according to the protocol previously described by Gogos and Van den Buuse[25]. Mice (n = 32) were randomly divided into sham (ovaries not removed, n = 8), OVX plus empty implant (vehicle) (n = 8), OVX plus estrogen implant (n = 8), and control (no surgical procedures or frustration, n = 8) groups. Mice were anesthetized under isoflurane (5% v/v in air) in an induction chamber until they lost the righting reflex. Anesthesia was maintained at 1.5–2% v/v in air. Mice were injected with meloxicam (3 mg/kg, i.p.; Ilium, Glendenning, New South Wales, Australia) and Baytril (25 mg/kg, i.p, Bayer, Pymble, New South Wales, Australia) for perioperative analgesia and antibacterial protection, respectively. A dorsal midline incision was made in the skin caudal to the posterior border of the ribs. An incision was made in the muscle, and the fat pad located just beneath the muscles was extricated to expose the ovaries. The ovaries were removed, and muscle and skin were sutured (Surgical Specialties Corporation, Tijuana, Baja California, Mexico). This process was performed bilaterally. The procedure for the sham group was identical to that for OVX, excluding removal of the ovaries. During surgery, mice received a subcutaneous silastic implant prepared as described previously[25]. Briefly, implants were either empty (sham and OVX + vehicle) or filled with 100% crystalline 17β-oestradiol (OVX + estrogen implant; 5-mm implants containing approximately 25 mg per implant; Sigma-Aldrich, St Louis, Missouri, USA). The dose used for 17β-oestradiol implants was based on previous dose-finding studies[25,26]. Animals were allowed to recover for 10 days prior to behavioral testing. Verification of complete bilateral OVX was performed at the conclusion of the study by dissection and uterine weight observations. Animals that had a successful bilateral ovariectomy were expected to have smaller and lighter in weight uteri, due to the physical absence of the ovaries, when compared to animals that did not have their ovaries removed[25,26].

Statistical analysis

To assess differences in highly palatable food intake following frustration in both males and females, 3 way ANOVA with repeated measures and Bonferroni's multiple comparisons test was performed to compare food consumption between subjects from the control and frustrated groups in the first 15 min following frustration stress on cycle 1 and cycle 2. Highly palatable food consumption post stress, sex and cycle were factors. *Ad libitum* and food restricted

groups were analyzed separately. 2 way ANOVA with Bonferroni's post hoc test was also used to analyze highly palatable food consumption post-stress in cycle 2. For the OVX experiments, 2 way ANOVA with repeated measures and Bonferroni's multiple comparisons test was used to compare differences in highly palatable food intake in cycle 1 and 2, and subsequently cycle 2 data was analyzed by 1 one way ANOVA. 1 way ANOVA and Bonferroni's multiple comparisons test was used uterine weight analysis between groups. *p*-values <0.05 were considered statistically significant. Body weight was monitored daily by weighting the animals between 0900-1000. Chow consumption was analyzed by calculating the amount of chow added and amount of chow consumed daily. Palatable food intake was recorded during habituation days and frustration stress days of cycle 1 and 2 by subtracting the amount consumed from the amount given. Data are presented as means ± SEM.

Results

Binge eating behavior induced by frustration stress

The amount of highly palatable food consumed following frustration stress for the first 15 min was recorded to evaluate possible binge eating behavior. Consumption after 30 and 60 min was also recorded, but no differences were observed among any of the groups (data not shown). Data on body weight, chow consumption, and highly palatable food consumption during the 2 days of habituation per cycle were collected for males, females, and OVX cohorts. Animals did not gain significant weight on the days where 2 h access to highly palatable food was provided, and no significant differences were observed within or between subjects.

Palatable food intake in males and females after frustration stress

Figure 2 depicts the cycle 1 (2a) and cycle 2 (2b) data for highly palatable food intake in males and females from the *ad libitum* cohort 15 min immediately after the frustrative episode. In cycle 1 (Figure 2a), no differences in the amount of highly palatable food consumption were observed between non-stressed and frustration stress males and females ($F_{(1,36)} = 1.250$; $p = 0.2709$). At the end of cycle 2 (Figure 2b), non-stressed males and stressed males from the *ad libitum* group did not show differences in highly palatable food consumption after 15 min ($p = 0.7625$). In contrast, females from the *ad libitum* group subjected to frustration stress ate

72% more than the control group. These females were never food restricted and were sated when behavioral experiments were performed. As soon as the food was made available, non-food restricted frustrated females displayed pronounced binge-like eating for 15 min. Analysis by repeated measures 3 way ANOVA showed main effects of sex ($F_{(1,36)}$ = 4.414; p = 0.0427), cycle ($F_{(1,36)}$ = 41.960; p < 0.0001) and stress ($F_{(1,36)}$ = 24.92; p < 0.0001) and significant interactions between both sex and stress ($F_{(1,36)}$ = 4.391; p = 0.0432) and cycle and stress ($F_{(1,36)}$ = 10.74; p = 0.023). Bonferroni multiple comparisons that the 72% increase in consumption showed by stressed *ad libitum* females was significantly higher than control *ad libitum* females (p < 0.0001).

In food restricted groups (Figure 3a,b), 3 way ANOVA with Bonferroni's *post hoc* analysis revealed a main effect of cycle ($F_{(1,36)}$ = 67.43; p <0.0001) and stress ($F_{(1,36)}$ = 6.488; p = 0.0153) and a significant interaction between these two factors ($F_{(1,36)}$ = 4.431; p = 0.0402) but no main effect of sex (p = 0.1672). Post hoc comparisons revealed that no differences were observed in either sex between control and stressed animals in the amount of highly palatable food consumed post-stress for cycle 1 (see Figure 3a). Given our findings with the *ad libitum* group in cycle 2 we proceeded to analyze food restricted group data for cycle 2 only using 2 way repeated measures ANOVA and found a main effect of stress ($F_{(1,36)}$ = 7.301; p = 0.0104) which suggests that stress increases palatable food consumption in both food restricted males and females at this time point (see figure 3b).

For the OVX cohort (Figure 4) 2 way repeated measures ANOVA revealed a main effect of cycle ($F_{(1, 28)}$ = 12.79; p =0.0013) and treatment ($F_{(3,28)}$ = 6.785; p = 0.0014) and a trend towards an interaction between these two factors ($F_{(3,28)}$ =2.366; p =0.0923). Bonferroni multiple comparisons revealed no differences between groups on cycle 1. In cycle 2, similar results were observed to those in the previous cohort, where frustrated mice consumed almost double the amount of highly palatable food compared to controls (control vs sham, p = 0.0194; control vs OVX + vehicle, p = 0.0008; control vs OVX + estrogen p = 0.0006, Figure 4). Given our focus on cycle 2 data we proceeded to analyze cycle 2 data only by 1 way ANOVA and found a main effect of group ($F_{(3,28)}$ = 9.169; p = 0.0002) and post hoc comparisons revealed all stress groups were significantly different to control (control vs sham; p = 0.0136; control vs OVX + vehicle; p = 0.0007; control v OVX + estrogen; p = 0.0006). For both 2 way and 1 way ANOVA analyses no significant differences were found between any of the stressed groups.

Uterine weight comparisons (Figure 5) by 1 way ANOVA demonstrated that ovariectomized mice had significantly lower uterine weight than shams, attributed to the removal of ovaries ($F_{(2,19)}$=82.55; $p < 0.0001$, main effect of treatment). This specific comparison was revealed by Bonferroni post hoc analysis ($p = 0.0004$). Multiple comparisons also revealed ovariectomized mice that received estrogen treatment had significantly higher overall uterine weight than sham and vehicle-treated ovariectomized mice ($p < 0.0001$; figure 5).

Discussion

In female humans, negative emotions strongly affect eating behavior and bingeing episodes[27,28] , however there are few rodent models which successfully model this behavior. Here, we report the validation of a stress-induced binge eating model using an acute and mild frustrative episode that does not depend on caloric restriction in female mice. The frustration paradigm consisted of olfactory and visual exposure but lack of access to highly palatable food for 15 min. Following this period, animals were provided access to the palatable food and permitted free consumption for 15 min. This protocol employs a repeated frustrative episode to induce binge-like eating in sated female mice with no history of food restriction. Indeed, we find the extreme bingeing behavior observed in female mice is not as pronounced when mice have a history of food restriction.

Females from the *ad libitum* groups displayed a compulsive-like phenotype towards highly palatable food once it was made available for consumption following frustration stress, as evident from the large amount of food consumed (around 1.5 g, which is more than half their daily chow intake) in only 15 min. The large amount of palatable food consumed within a short period of time is 72% higher than control animals and is reminiscent of an acute emotional binge, lending face and construct validity to this model[1,29,30,31,32]. It should be noted that 2 cycles of intermittent access to the highly palatable food was required in order to see this effect with no differences being observed after one cycle. This observation supports the longer protocol chosen by Cifani and colleagues where multiple cycles of intermittent access are employed.

Furthermore, our results demonstrate that the repetition of the stressful situation led to an increased binge-like behavior and highly palatable food consumption, which is agreement

with rodents literature on how sub-chronic and chronic can induce hyperphagia and susceptibility to food addiction[6,33,34]. This data is also supported by the human literature on vulnerability to obesity, eating disorders and addiction in those submitted to chronic stressful episodes[35,36,37].

A notable finding of this study was the sexual dimorphism observed in food consumption following frustrative stress. In food-restricted males, only those that were food restricted displayed binge-like eating after exposure to a stressor. This is in agreement with previous studies performed in rats[10,29,30]. Conversely, in male mice that were not food restricted no stress-induced binge behaviour was observed. This may be attributed to the predominance of homeostatic drivers responding to metabolic challenges (history of food restriction) over the hedonic or rewarding characteristics of the palatable food in male compared to female animals[38,39].

In contrast, we observed that females responded differently to the frustration stress when compared to males. *Ad libitum* fed female mice displayed binge-like behavior after the frustrative stressor, suggesting that these animals may have binged on highly palatable food to negate the aversive effects of stress, rather than to fulfil a homeostatic drive. These data are in accordance with the human literature demonstrating that women respond differently to stress-related bingeing which can be partly attributed to factors such as the rewarding effects of highly palatable food consumption. Compared to males, females have been reported to be more motivated to obtain food rewards (especially sugary rewards) in both animals models[31,40] and humans[32], where in humans this is more prevalent during the luteal phase[41,42]. Our findings support the concept that females may exhibit distinct behaviors towards highly palatable food and present with bingeing episodes independently of homeostatic need when compared to males[2,43,44,45].

The same binge-like behavior was observed in the OVX female cohort independent of estrogen supplementation, suggesting that ovarian hormones were not necessary to drive binge-like behavior in our model. Our results indicated that OVX was not able to explain the sex differences in highly palatable food consumption post-stress in females. A potential reason for this is the short nature of our paradigm which comprised 2 h of intermittent access to highly palatable food for only 4 days throughout cycle 1 and 2. These conditions differ from those used in chronic experiments examining the escalating changes in eating behavior caused by

OVX[18]. Further, the literature on stress-induced bingeing mainly employed OVX female rats and not mice and used stressors longer than 15 min or more aggressive stressors such as tail-pinch or foot shock[10,46]. Given the heterogeneous presentation of binge eating behavior, it is unlikely that a simple relationship between ovarian hormones and bingeing can fully explain divergent bingeing phenotypes. In conclusion, this study provides a model of emotional stress-induced binge eating which is specific to females and does not depend on caloric restriction or ovarian hormones. The highest response to the stressor was observed after a 2nd repeated cycle of exposure.

Some advantages of this model include its use of a mild acute stressor and replicability across experimental cohorts. The pronounced binge-like eating by sated female mice suggests that this acute bingeing behavior was motivated by reward, where the highly palatable food in this case acted as "comfort food" and was not consumed out of simple metabolic need. Our findings strengthen the validity of this animal model for investigating the physiological and behavioral substrates underpinning binge eating of highly palatable food induced by a mild emotional stressor in females.

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Figure legends

Figure 1. Behavioral testing timeline. Timeline for the behavioral experiments on restricted and *ad libitum* access to food mice groups. Abbreviations: 15': 15 min; *Ad lib.*: *ad libitum*; HFHS: high fat/high sugar.

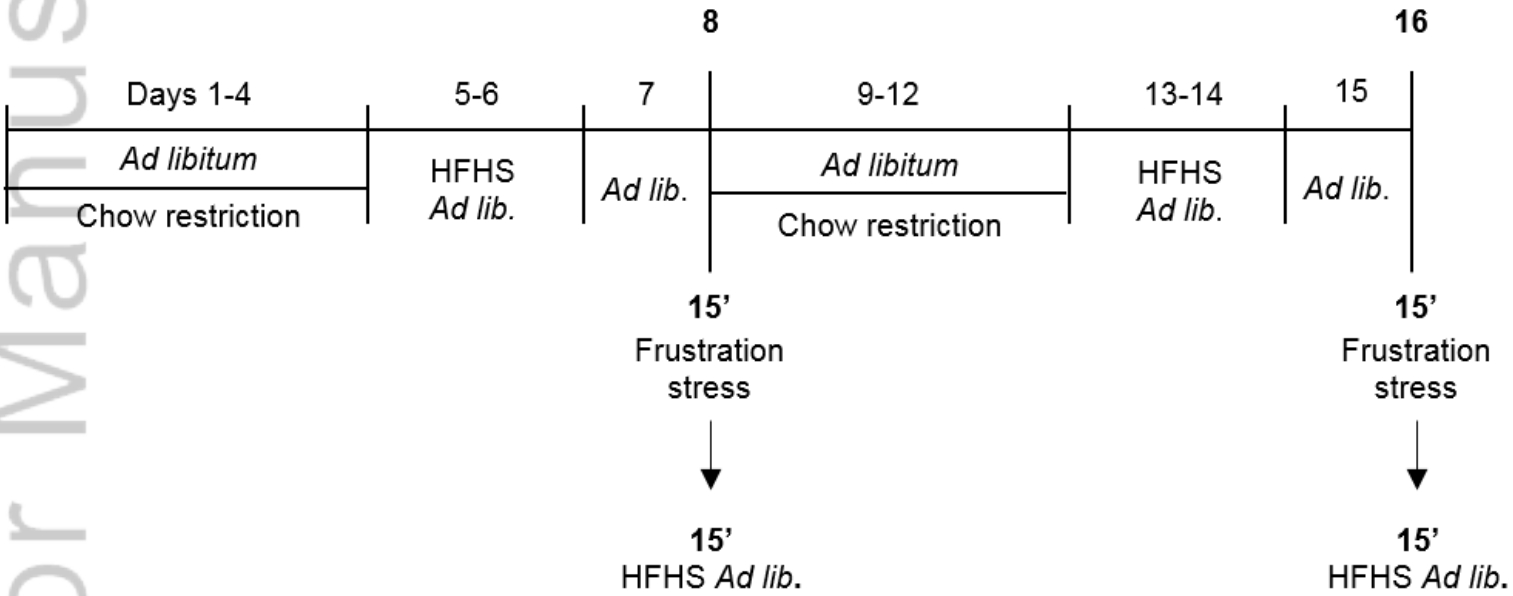
Figure 2. Highly palatable food intake following frustration stress in *ad libitum* male and female mice after both cycle 1 (a) and cycle 2 (b). Data shown as mean ± SEM highly palatable food intake (g) recorded 15 min after stressor. **** $p < 0.0001$ as compared to non-stress group (repeated measures 3 way ANOVA with Bonferroni post hoc analysis; $n = 10$ per group).

Figure 3. Highly palatable food consumption post-frustration stress in food restricted male and female mice after both cycle 1 (a) and cycle 2 (b). Data shown as mean ± SEM highly palatable

food intake recorded 15 min after stressor. * $p < 0.05$ main effect of stress (repeated measures 3 way ANOVA with Bonferroni post hoc comparisons; $n = 10$ per group).

Figure 4. Highly palatable food consumption post-frustration in ovariectomized (OVX) female cohort with no history of food restriction. Data shown as mean ± SEM highly palatable food intake (g) recorded 15 min after stressor. * $p < 0.05$, *** $p < 0.001$ as compared to control group for that cycle (2 way repeated measures ANOVA with Bonferroni post hoc analysis; $n = 8$ per group).

Figure 5. Uteri weight from OVX females in grams. Data shown as mean ± SEM. *** $p > 0.01$, **** $p > 0.001$, as compared to sham weight; #### $p < 0.0001$ as compared to OVX + vehicle (1 way ANOVA with Bonferroni's multiple comparison tests; $n = 7$-8 per group).



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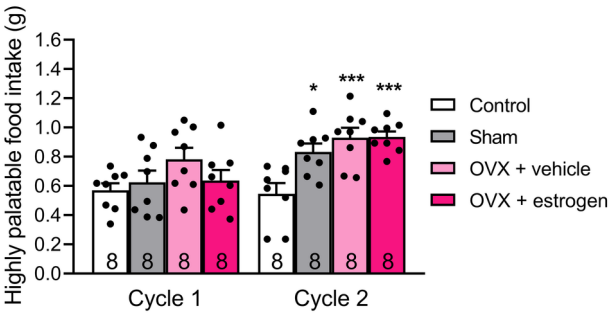

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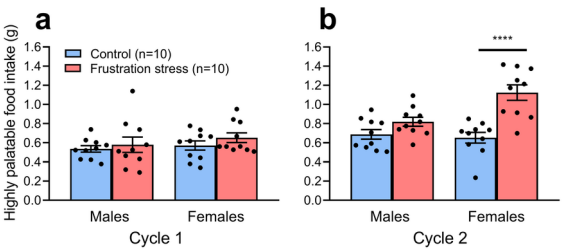

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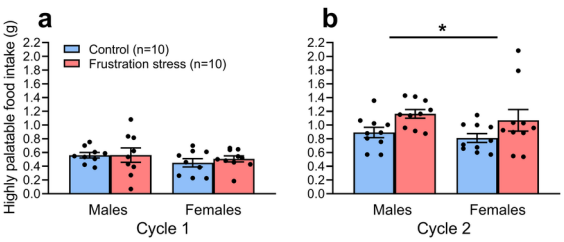

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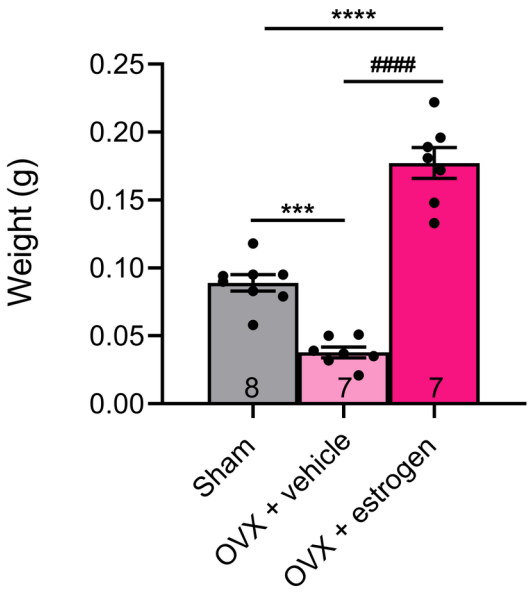

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