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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Effects of SIRT1 mutation or over-expression in astrocytes
on metabolism and reproduction.

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Irene Su Jeen Choi

Committee in charge:

Professor Nicholas Webster, Chair
Professor James Golden, Co-Chair
Professor Madeline Butler

2013

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Co-Chair

Chair

University of California, San Diego

2013

I dedicate this thesis to my spiritual family for being so loving, supportive and encouraging in the midst of my breakdowns and breakthroughs.

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LIST OF ABBREVIATIONS

AAC	Area above the curve
AUC	Area under the curve
BW	Body weight
CONT	Control
FSH	Follicle stimulating hormone
GFAP	Glial fibrillary acidic protein
GnRH	Gonadotropin-releasing hormone
GTT	Glucose tolerance test
HFD	High-fat diet
HPG	Hypothalamic-pituitary-gonadal axis
ITT	Insulin tolerance test
LFD	Low-fat diet
LH	Lutenizing hormone
MUT	Mutant SIRT1 in astrocytes
NC	Normal chow
OX	Over-expression of SIRT1 in astrocytes
SIRT1	Sirtuin 1
WAT	White adipose tissue

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ACKNOWLEDGMENTS

I would like to thank Dr. Nicholas Webster for taking me in and giving me the opportunity to explore research. I appreciate all the helpful criticisms and guidance in directing my project.

I would also like to thank my post-doc and mentor Emily Rickert for training me, investing in my future, and fueling my passion for science. For the past 2 years, she's been with me from someone with absolutely no training in lab research to someone who can now independently perform experiments, analyze results, and speak in public. And this process is largely attributed to her. So I am forever grateful and appreciative of her knowledge and teachings.

Next, I would like to thank all the members of the Webster Lab for their support and contributions, including Marina Fernandez, Jung Bu, Supriya Sen, Katherine Hsueh, and Vicky Hwang.

Lastly, I would like to thank my family and friends for being the emotional support that I needed in working towards my graduate degree.

ABSTRACT OF THE THESIS

Effects of SIRT1 mutation or over-expression in astrocytes
on metabolism and reproduction.

by

Irene Su Jeen Choi

Master of Science in Biology

University of California, San Diego, 2013

Professor Nicholas Webster, Chair

Sirtuin 1 (SIRT1) is an NAD⁺-dependent histone deacetylase, activating and inhibiting gene-expression in several organs, such as the liver, pancreas, adipose tissue, intestine, and brain. Whereas the impact of SIRT1 in these tissues has been extensively studied, its role in astrocytes and effect on reproduction is still elusive. SIRT1 over-expressing mice show phenotypes resembling mice in caloric restriction – inhibited adipogenesis, lowered levels of blood cholesterol, improved glucose tolerance, and

increased metabolic activity. Furthermore, during times of caloric restriction, SIRT1 levels increase and reproductive events such as gonadotropin-releasing hormone (GnRH) pulses, luteinizing hormone (LH) pulses, and ovulation cease. I hypothesize that SIRT1 in astrocytes may contribute to this altered reproductive function and also play a role in metabolism and inflammation. To evaluate this, our laboratory has developed conditional over-expressing (OX) and mutant (MUT) SIRT1 using a tamoxifen-inducible cre-recombinase under the control of the glial fibrillary acidic protein (GFAP) promoter, which expresses in astrocytes. The two transgenic lines include either a floxed stop over-expression of SIRT1 or a floxed exon 4 of SIRT1, mimicking a SIRT1 knockout. To prevent any affects on mouse reproductive development, the normal chow (NC) infused tamoxifen diet was not started until 10 weeks of age, maintained for 2 weeks, and a recovery period for 2 weeks before the start of all studies. Differences were seen in weights, food intakes, glucose and insulin tolerance, hormone levels and reproductive cyclicity. High-fat diet (HFD) and 24hr fast were performed to evaluate the effect of stress-induced diets. Reproductive changes were evaluated by monitoring estrous cyclicity, endocrine and steroid hormone levels, as well as gonadal histology.

INTRODUCTION

Reproduction is a tightly regulated function, driven by the hypothalamic-pituitary-gonadal (HPG) axis. GnRH in the hypothalamus stimulates the secretion of LH and follicle-stimulating hormone (FSH) in the anterior pituitary, which in turn stimulates the gonads. Fluctuations in LH and FSH hormones cause changes in the hormones produced by each gland and have widespread as well as local effects on the body. In males, FSH stimulates Sertoli cells to produce inhibin and also stimulate spermatogenesis. LH in males, stimulates the Leydig cells to produce testosterone. In females, FSH stimulates the maturation of ovarian follicles and together with LH stimulates the ovaries to produce steroids such as estradiol and progesterone. In females, FSH and LH regulate the reproductive cycle, called the menstrual cycle in humans, which lasts 28 days; in rodents this cycle, called the estrous cycle, lasts approximately 4-5 days. Vaginal smear cytology was used to determine the estrous cycle phases – diestrus, proestrus, estrus, and metestrus. Each stage was determined by the proportion of distinctly shaped epithelial cells, cornified cells, and leukocytes. Each stage is characterized by different levels of estradiol, FSH, and LH. During diestrus, estradiol levels start to increase in order to prepare for ovulation. Estradiol secretion reaches a peak, GnRH neurons are stimulated and consequently, during the evening of proestrus, FSH and LH surge and ovulation occurs.¹ The following stage, estrus, refers to the phase when the female is sexually receptive, or “in heat”.

Due to the elevated energy requirements of reproduction, metabolic factors such as under-nutrition and over-nutrition exert a pivotal role in the control of the HPG

axis. Energy balance, appetite and reproductive function are tightly linked and alterations in energy and appetite have been shown to affect feedback loops in the HPG axis.² Both HFD and caloric restriction have been associated with a decrease in reproductive function. Consumption of HFD impairs reproduction via suppression of HPG axis, infertility in females, and impairment of reproductive function in males.³ During times of caloric restriction reproductive cycling stops, steroid production is halted and ovarian function is suppressed.³

Glucose energy deficit during caloric restriction is detected and conveyed to the hypothalamus where GnRH secretion is disrupted, and LH and FSH pulse cease.² SIRT1 is a protein that has been implicated in the adaptation to metabolic starvation. The sirtuins, SIRT1-SIRT7, are a family of highly conserved NAD⁺-dependent histone deacetylases with homology to the yeast SIR2 gene. SIRT1, the most extensively studied member of this family, plays an important role in several processes ranging from cell cycle regulation to energy homeostasis and is expressed in the brain,⁴ liver,⁵ pancreas,⁶ adipose tissue,⁷ muscle,⁸ and heart⁹. SIRT1 has been shown to target and deacetylate lysine residues of histones, to decrease transcriptional activity, and protein substrates, to decrease protein activity. Mammalian SIRT1 inhibits the activity of proteins that are important for apoptosis (p53, Ku70), the cell cycle (FOXO), circadian rhythms (CLOCK), inflammation (NF- κ B), mitochondrial function (UCP2), and metabolism (PPARgamma, PGC-1a, PTB1B).¹⁰ SIRT1 can repress protein activity by binding to its promoter to repress transcription in the nucleus or by deacetylating the protein in the cytosol.¹⁰

In metabolism, SIRT1 acts by deacetylating proteins that induce catabolic processes while inhibiting anabolic processes; SIRT1 increases cellular energy stores and ultimately maintains cellular energy homeostasis.¹¹ Upon HFD feeding, SIRT1 levels decrease, resulting in various metabolic disorders such as glucose intolerance, increased body weight (BW), and oxidative stress.¹⁰ In response to food deprivation, SIRT1 proteins increase, resulting in decreases in adiposity and serum cholesterol, with improved insulin sensitivity.¹² Increase in SIRT1 protein has also shown decrease in reproductive function.¹²

The majority of interactions between metabolic and reproductive systems takes place in the brain, specifically the hypothalamus.¹³ Metabolic affects of neuronal SIRT1 knockout in male mice¹⁴ as well as affects in female reproduction have been shown. But no SIRT1 studies have been done on astrocytes. Astrocytes are the most abundant cells in the brain; they segregate, maintain, and support neurons.¹⁵ Although the function of SIRT1 in several organs has been studied, the role of SIRT1 in the brain, particularly astrocytes, is still elusive. Like SIRT1, astrocytes also have anti-inflammatory and pro-inflammatory properties. During HFD feeding, astrocytes become reactive and they respond by producing a variety of inflammatory mediators that help maintain brain homeostasis.¹⁶ The brain's high energy requirement and its energy supply are tightly regulated by astrocytes.¹⁶ There is also growing evidence that SIRT1 changes inflammation by inhibiting inflammatory pathways in macrophages.¹⁷ In the hypothalamus, astrocytes express estrogen receptors to modulate GnRH release in GnRH neurons; thus, astrocytes have also been implicated to play a role in

reproduction.¹⁵ I hypothesize that over-expressing or mutation of SIRT1 in astrocytes alters metabolism and reproduction in male and female mice.

Our laboratory has developed conditional over-expression (OX) and mutant (MUT) SIRT1 mice, using a tamoxifen-inducible cre-recombinase under the control of the GFAP promoter, since GFAP is only expressed in astrocytes. Control (CONT) groups are all age-matched and litter-matched and contain loxP alleles but no cre-recombinase or contain loxP alleles and cre-recombinase but not given tamoxifen. Normal chow (NC) mixed with tamoxifen was given at the post-pubertal age of 10 weeks, and the animals were on the diet for 2 weeks. Mice were given at least 2 weeks to recover before any experiments were performed. The n>5 for all lines – OX, MUT, and CONT.

On NC, weights were monitored weekly and food intakes were monitored daily. The mice were tested for metabolic changes in glucose, insulin, leptin sensitivity, and reproduction was evaluated via estrous cyclicity, and FSH and LH levels.

Evidence suggests that the effects of SIRT1 mimic the beneficial effects of calorie restriction, the only known physiological intervention that promotes a longer, healthier lifespan across species.¹⁸ To study the effects of astrocyte SIRT1 on diet restricted mice, a 24 hour fast was done on 5 month old mice on NC. Food was removed from single-housed mice at 9AM and a measured amount was given back 9AM the next day. Glucose, food intake, and weights were measured before and after the 24 hour fast.

While caloric restriction leads to an increase in SIRT1, HFD has been associated with low SIRT1 activity.¹⁹ At 4 months of age, mice were randomly selected for either

HFD or low-fat diet (LFD) and were on diet for 10 weeks while BW and food intake was measured weekly. At 7 months of age, mice were harvested for tissues.

Previous studies showing SIRT1's various effects on metabolism and reproduction provides a solid foundation to further study the role of SIRT1 in astrocyte-specific mutation or over-expression.

RESULTS

Breeding Scheme

MUT x GFAP mice were generated by cross-breeding a C57BL/6 SIRT1-floxed mouse²⁰ with a BALB/c GFAP-Cre transgenic mouse²¹. The SIRT1-floxed mice contain loxP sequences flanking the exon 4 of the SIRT1 gene, which encodes the SIRT1 catalytic domain. When bred with the GFAP-Cre mice, the deleted SIRT1 allele transcribes a mutant protein that has no apparent functional SIRT1 activity (Fig1a). OX x GFAP mice were generated by cross-breeding C57BL/6 SIRT1-floxed stop mouse²² with a BALB/c GFAP-Cre transgenic mouse²¹ (Fig1b). Excision of stop site by cre results in over-expression of SIRT1 under a constitutively active CAGGS promoter.²²

Normal Chow Metabolism

OX weigh more and consume more food.

MUT weigh less and consume less food.

BW and food intake were monitored daily for 6 weeks starting at 14 weeks of age. The OX females gained significantly more weight than the MUT or CONT mice (Fig2a,b). The increase in BW was associated with significantly increased food intake (Fig2g,h). Despite the increased BW, the inguinal and gonadal adipose tissue was not significantly heavier in the OX females compared to the other groups (Fig2c,d,e,f). Overall, OX females were heavier and consumed more food than CONT and MUT on NC.

MUT males trended to gain more weight over time but results were not significant (Fig5a,b). The increase in BW was associated with significantly increased food

intake (Fig5g,h). Although no difference was detected in weight gain for MUT males on NC, MUT males had significantly higher inguinal white adipose tissue (WAT) percentage compared to the OX (Fig5c,d,e,f).

OX are glucose intolerant and insulin sensitive.

MUT are glucose tolerant and insulin insensitive.

Significant differences were seen in food intake and BW, so we wanted to see if the mice were metabolizing glucose and responding to insulin differently. At 5 months of age, mice were subjected to a glucose tolerance test (GTT) and an insulin tolerance test (ITT) the following week. For both experiments, mice were fasted for 6hrs and injected intraperitoneally (IP) with 2g/kg of glucose for the GTT and 0.4U/kg of insulin for ITT. OX females have higher 6hr fasted insulin and glucose levels than MUT (Fig3a,b). During the GTT, OX females continued to have elevated levels of glucose at every time point, making them glucose intolerant (Fig3c). AUC values confirm a significantly better preserved glucose tolerance of MUT mice on NC (Fig3d). In contrast, an ITT proved MUT females insulin insensitive and OX females insulin sensitive (Fig3e,f). A serum analysis showed MUT females to have a significantly lower basal insulin level (Fig3b).

Like the females, OX males were also glucose intolerant and insulin sensitive (Fig6c,e). OX males had higher 6hr fasted insulin and glucose levels than MUT (Fig6d,f). Both CONT and MUT males on NC were comparable throughout the study, indicating normal glucose and insulin tolerance for MUT male mice. A serum analysis showed MUT males to have a significantly lower insulin level (Fig6b).

OX are leptin sensitive.

Significant differences are seen in BW, food intake, and GTT and ITT with no clear explanation or reasonable answer. Leptin, a key regulator of appetite and metabolism, is an appetite suppressant. A leptin sensitivity test was performed to see if differences in BW and food intake could be explained by possible differences seen in a leptin test. To test leptin sensitivity, mice were injected with 0.5mg/kg of leptin twice a day at 6am and 6pm for 2 days, while food intake was measured.

There is no significant difference in 6 hour fasted leptin levels in females (Fig4a). OX females trended to be more leptin sensitive, seen by a higher reduction of food intake, but no significant difference between genotypes was detected (Fig4b,c).

MUT males have a lower 6 hour fasted leptin level (Fig7a). At day 2 of leptin injection, OX males have significantly reduced food intake compared to MUT males, making OX males leptin sensitive (Fig7b,c).

Normal Chow Reproduction

MUT females spend more time in estrus.

OX females cycle more frequently.

Hypothalamic astrocytes have been implicated to play a role in reproduction by expressing estrogen receptors¹⁵ and SIRT1 has been shown to play a role in estrogen receptor-mediated signaling²³. To evaluate the reproductive role of SIRT1 in astrocytes, over the course of 6 weeks, estrous cyclicity was observed and analyzed in post-pubertal

females, from 14 to 20 weeks of age. Because estrous cyclicity is driven by the HPG axis, it is a good indicator of whether or not female reproduction is functioning normally. Compared to the CONT, MUT spend significantly more time in estrus, or “in heat”, while OX spend less time in estrus (Fig8a). And as a result of spending less time in estrus, OX complete more estrous cycles (Fig8b). The extra days spent in estrus by MUT, is offset by the decrease in the days spent in diestrus. In both lines, no difference was observed in the percent days spent in proestrus or metestrus. When looking at FSH and LH levels in each estrous stage, MUT possessed higher FSH levels in diestrus and morning of proestrus (Fig9a,b). To determine whether or not a mouse surged, diestrus values from all lines were averaged (240pg/mL) and any value that was two standard deviations above the average (612pg/mL) was considered a surge (Fig10a). As a result, significantly more OX surged than MUT (Fig10b).

According to the data, LH surge is abnormal in MUT females. GnRH stimulates the pituitary gland to synthesize and secrete LH. So we artificially gave GnRH to test whether or not the gonadotropes in the pituitary gland were responding any differently. There was no difference in female LH levels, before and after the 10min GnRH stimulation (Fig11a). Compared to CONT and OX, MUT females had elevated levels of FSH before and after 10min GnRH stimulation (Fig11b).

After a GnRH stimulation in males, slightly elevated levels of FSH and a significant increase in LH levels can be seen (Fig12a,b). There is no significant difference in FSH or LH levels between genotypes.

24hr Fast Metabolism

OX maintain higher glucose levels.

MUT maintain lower glucose levels.

SIRT1 has been implicated to mimic the beneficial effects of calorie restriction.

To explore the phenotypes associated with a calorie restriction, individually housed, 5 month old mice were subjected to a 24hr fast. BW, food intake, and glucose were measured before and after the fast. After a 24hr fast, female glucose levels drop and recover 24hrs after redistribution of food (Fig14a). OX females had consistently elevated glucose levels compared to MUT females before, during and after the fast. After the fast, OX females maintain same glucose level prior to the fast, while MUT and CONT glucose levels overshoot levels after the fast (Fig14a). All mice overeat after the fast (Fig14b). No difference is seen in BW loss and gain before and after the fast (Fig13a,b).

After a 24hr fast, male glucose levels drop and recover 24hrs after redistribution of food (Fig16a). Consistent with previous food intake and GTT results, OX males consume more food and have consistently higher levels of glucose (Fig16a,b). After the fast, OX males maintain same glucose level prior to the fast, while MUT and CONT glucose levels overshoot levels after the fast (Fig16a). All mice overeat after the fast (Fig16b). No changes in weight or percent change in weight is seen (Fig15a,b).

HFD and LFD Metabolism

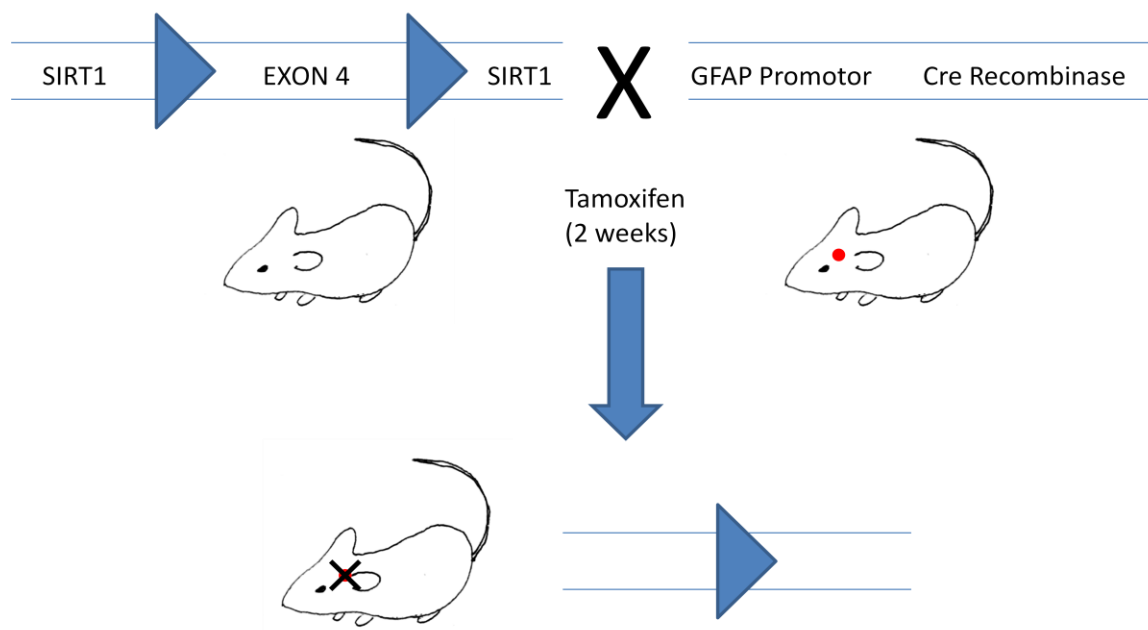
OX weigh more and consume more food and energy.

MUT weigh less and consume less food and energy

Upon HFD feeding, SIRT1 levels decrease and anti-inflammatory responses are also activated in astrocytes. After seeing differences in metabolic phenotypes on NC and a 24hr fast, at 17 weeks of age, mice were randomly selected for either HFD or LFD. Weekly BWs and food intakes were measured for 10 weeks and mice were harvested at 27 weeks. Females maintained a steady weight on LFD, with no significant interaction between genotypes (Fig17a). On HFD, OX females weighed significantly more than MUT (Fig17b). The gap between OX and MUT female weights were further exacerbated after 4 weeks on the HFD. Increase in BW gain in OX females was associated with significant increases in food and energy consumption (Fig18a,b,c,d,e,f). In addition, females of all genotypes, consumed more HFD than LFD. Overall, OX females were heavier and consumed more food than CONT and MUT on both HFD and LFD.

There is no difference in weight gain for MUT males on LFD or HFD (Fig19a,b). OX males consume significantly more food and energy on HFD, while MUT males consume significantly less food and energy on HFD (Fig20a,b,c,d,e,f). This data shows consistency with female results.

a)



b)

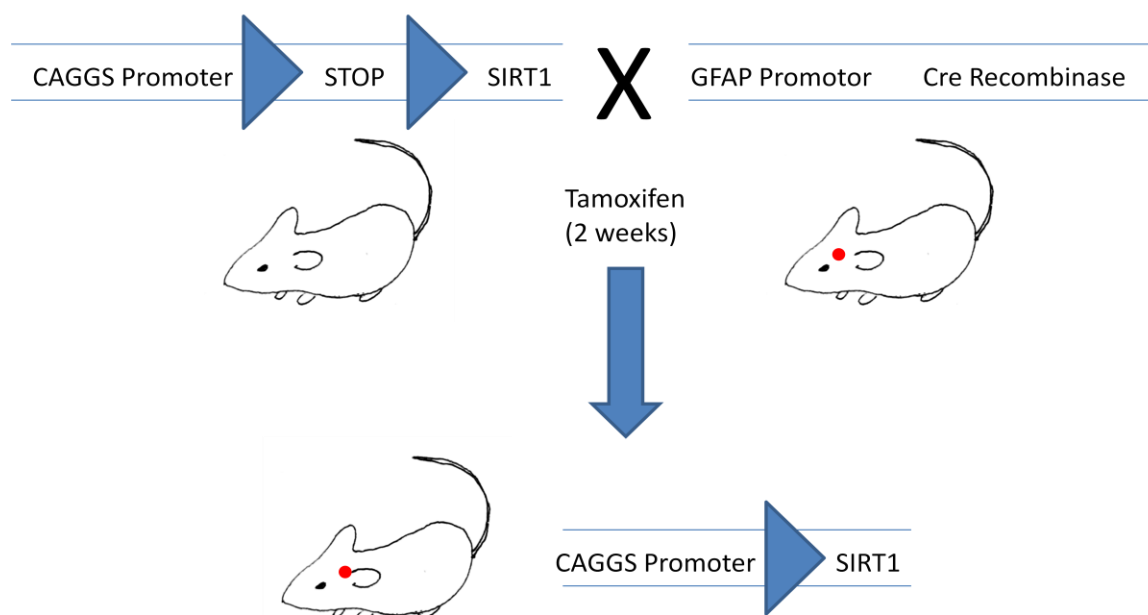
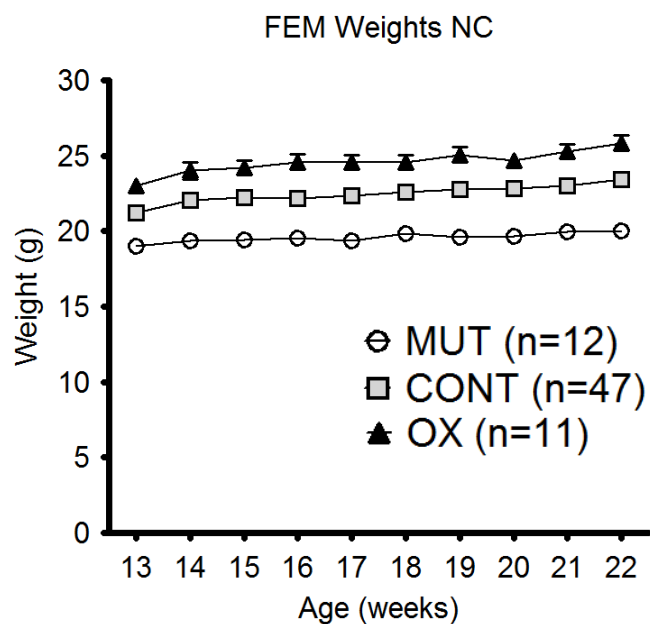


Figure 1: Mutant SIRT1 (MUT) and Over-Expressing SIRT1 (OX) breeding scheme.

a)



b)

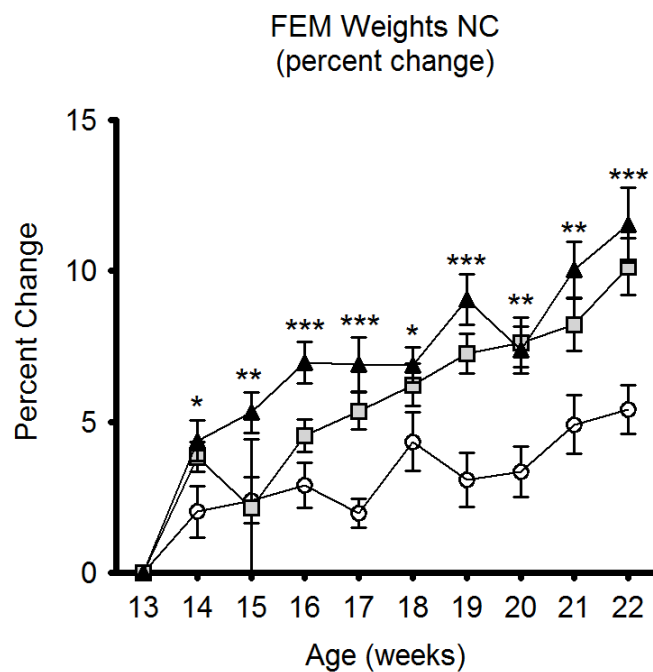


Figure 2: OX Females weigh more and consume more food. MUT Females weigh less and consume less food. No significant difference in inguinal or gonadal WAT weight.

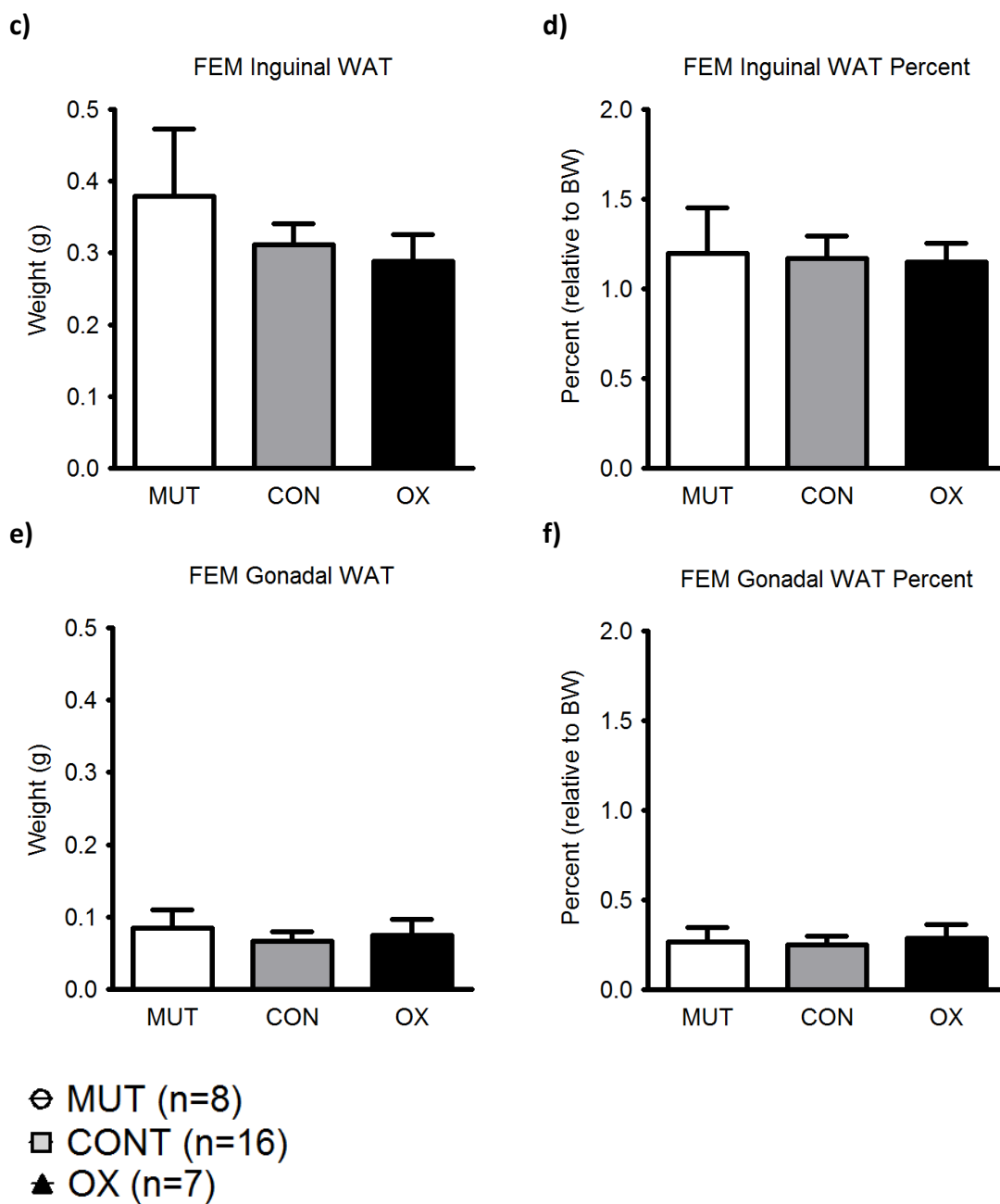
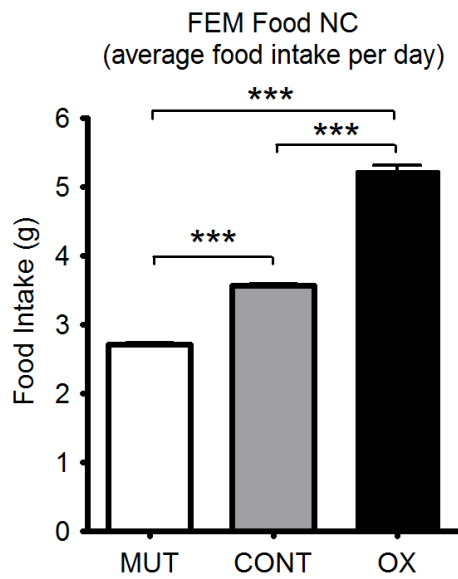


Figure 2: OX Females weigh more and consume more food. MUT Females weigh less and consume less food, continued. No significant difference in inguinal or gonadal WAT weight, continued.

g)



⊖ MUT (n=12)
 □ CONT (n=47)
 ▲ OX (n=11)

h)

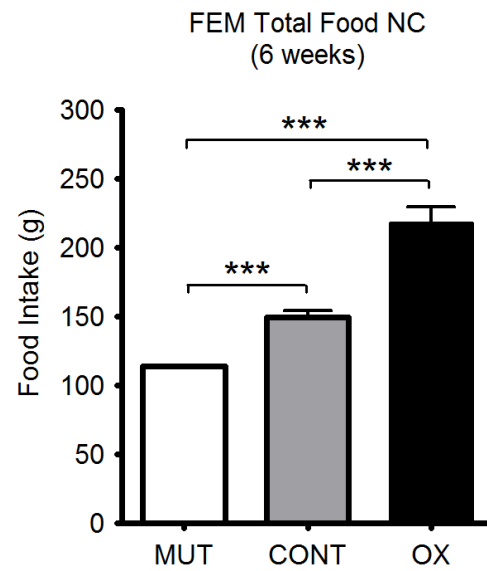
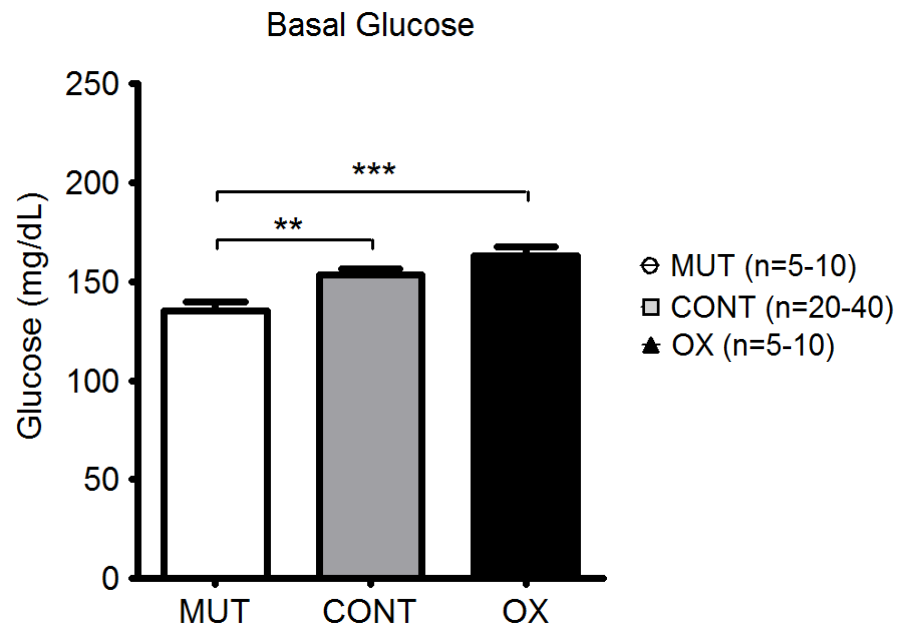


Figure 2: OX Females weigh more and consume more food. MUT Females weigh less and consume less food, continued. No significant difference in inguinal or gonadal WAT weight, continued.

a)



b)

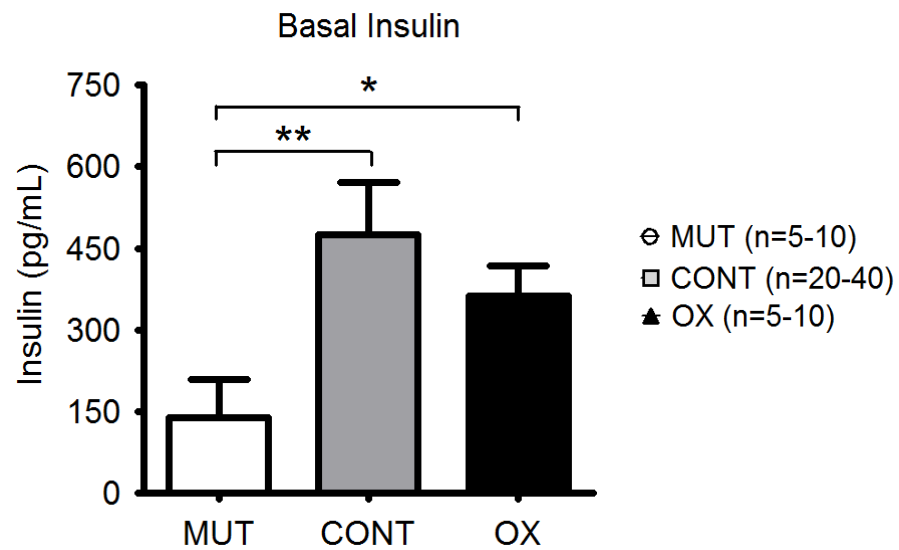
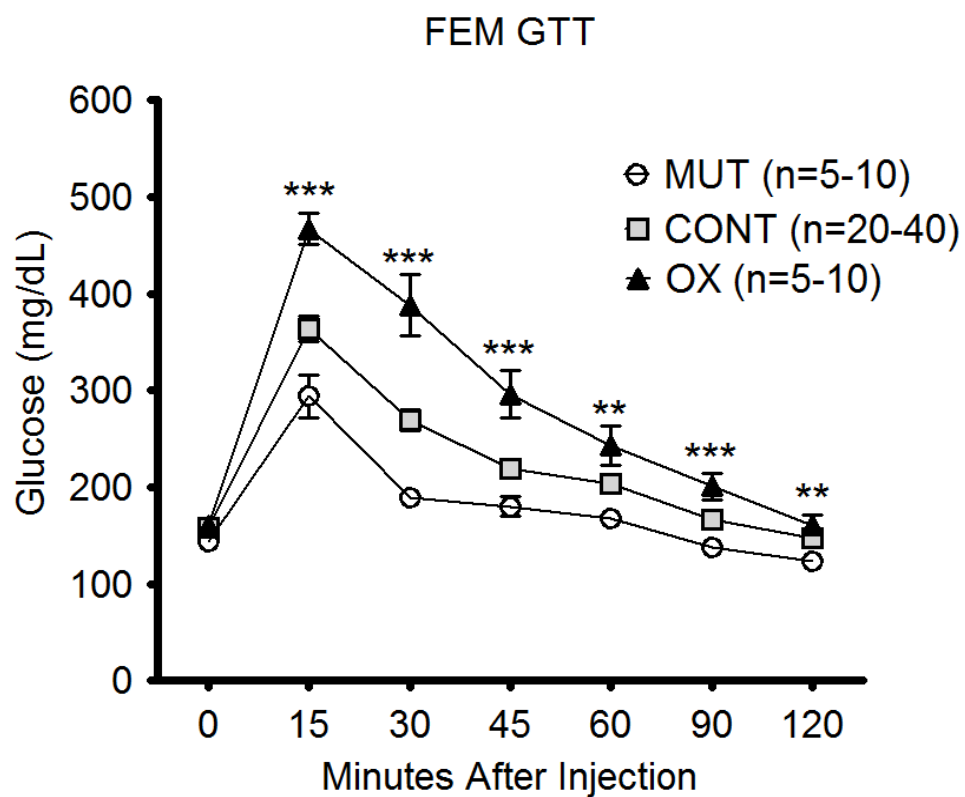


Figure 3: OX Females have higher 6 hour fasted, basal glucose and insulin levels and are glucose intolerant and insulin sensitive. MUT Females have lower 6 hour fasted basal glucose and insulin and are glucose sensitive and insulin intolerant.

c)



d)

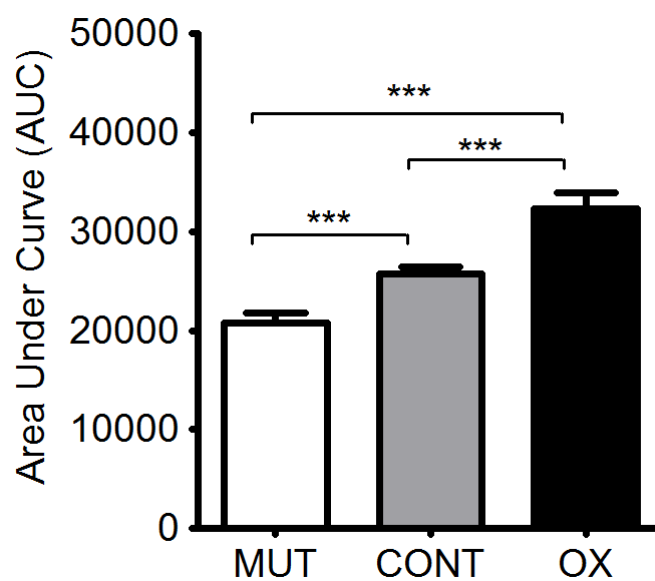
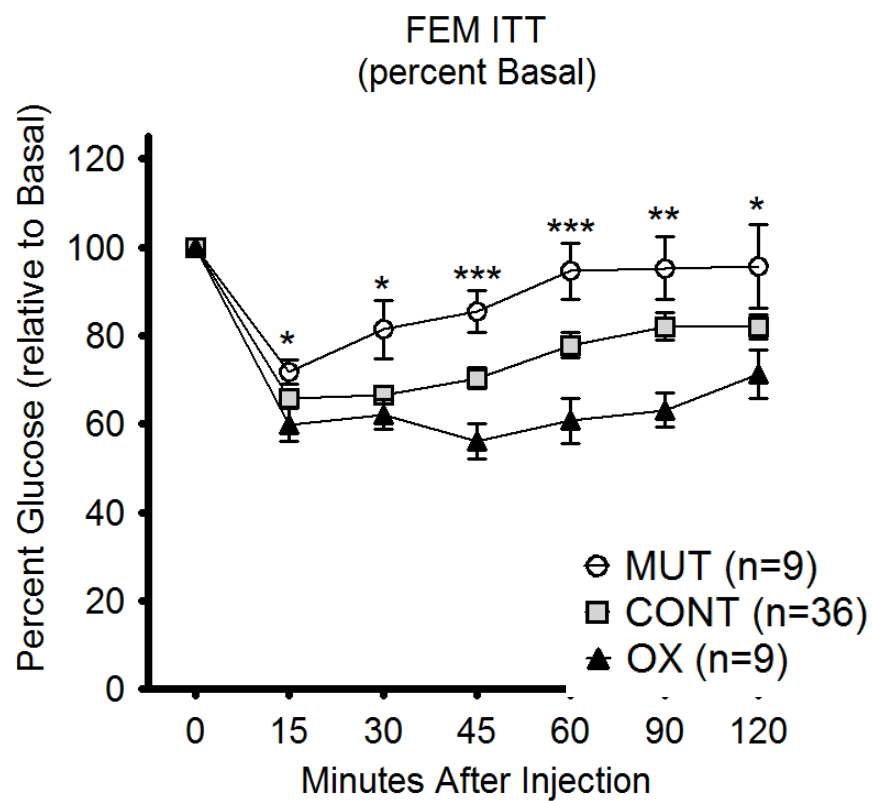


Figure 3: OX Females have higher 6 hour fasted, basal glucose and insulin levels and are glucose intolerant and insulin sensitive. MUT Females have lower 6 hour fasted basal glucose and insulin and are glucose sensitive and insulin intolerant, continued.

e)



f)

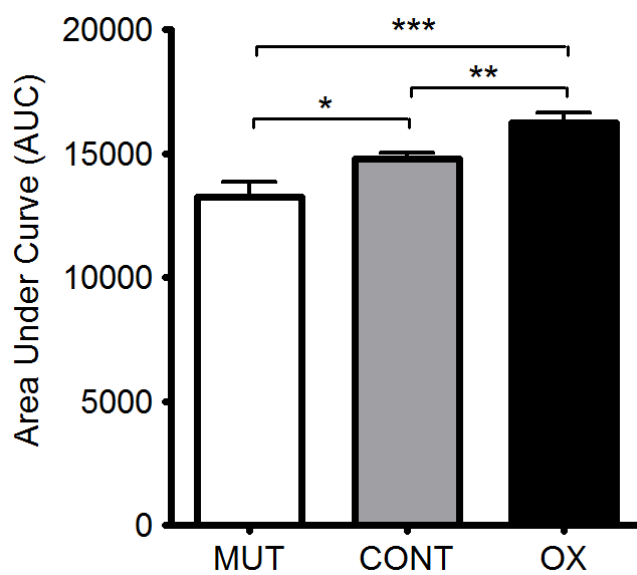


Figure 3: OX Females have higher 6 hour fasted, basal glucose and insulin levels and are glucose intolerant and insulin sensitive. MUT Females have lower 6 hour fasted basal glucose and insulin and are glucose sensitive and insulin intolerant, continued.

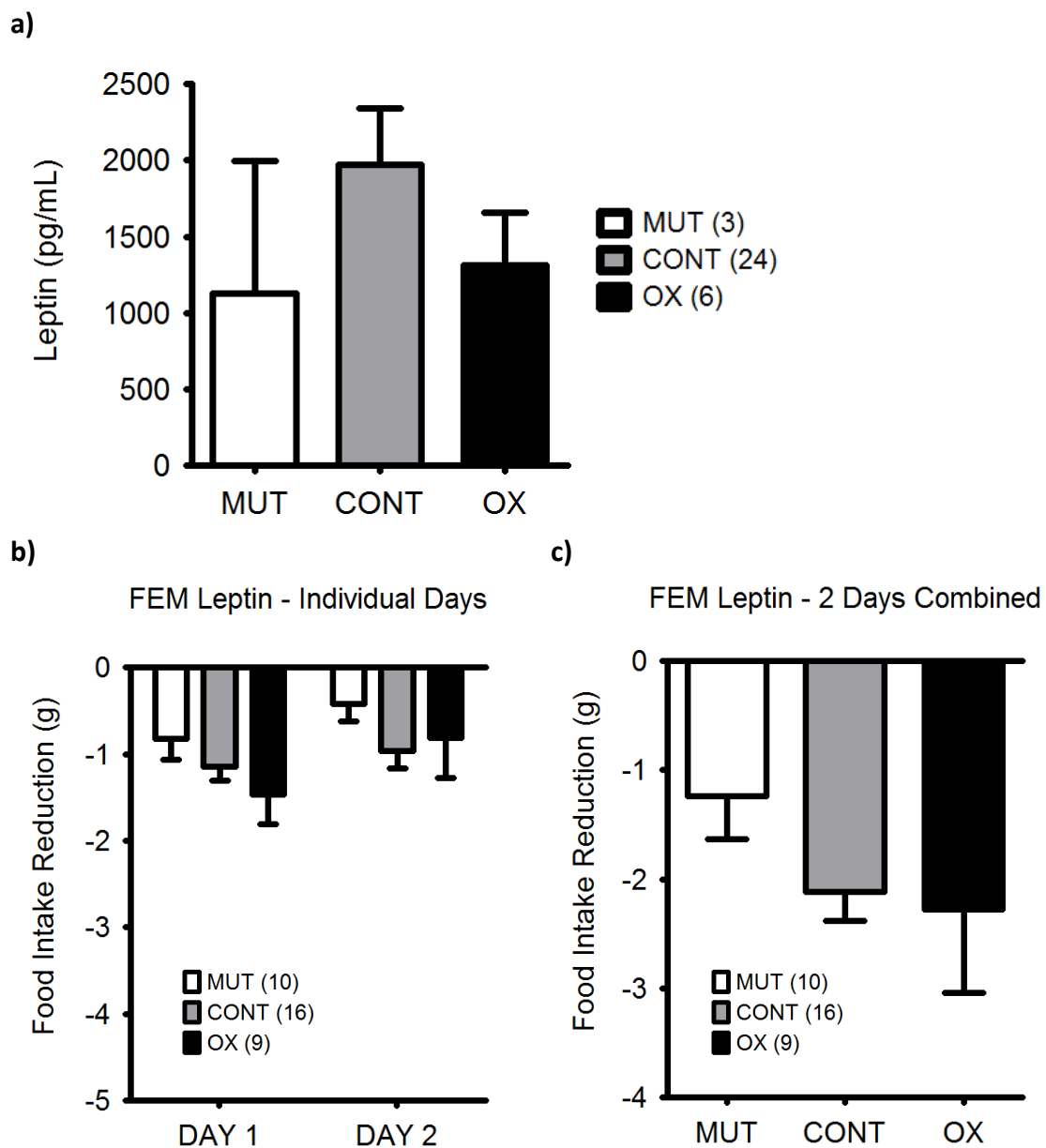
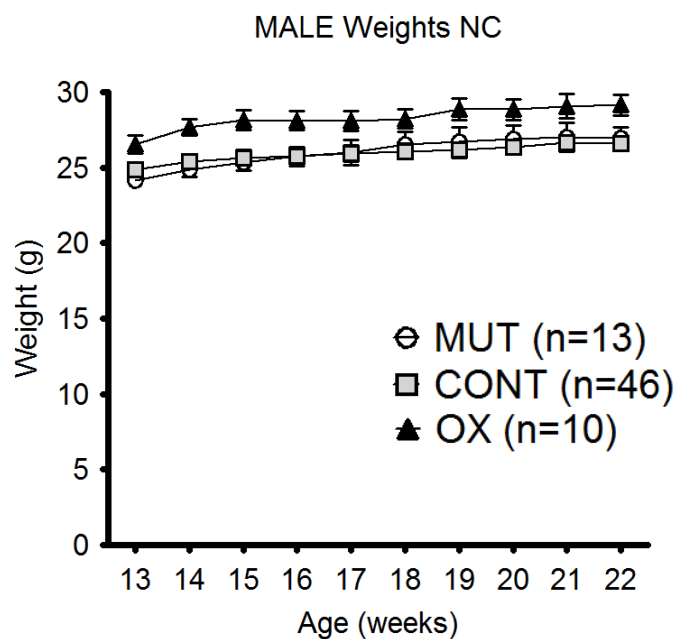


Figure 4: No difference in Female 6 hour fasted leptin or Female leptin sensitivity.

a)



b)

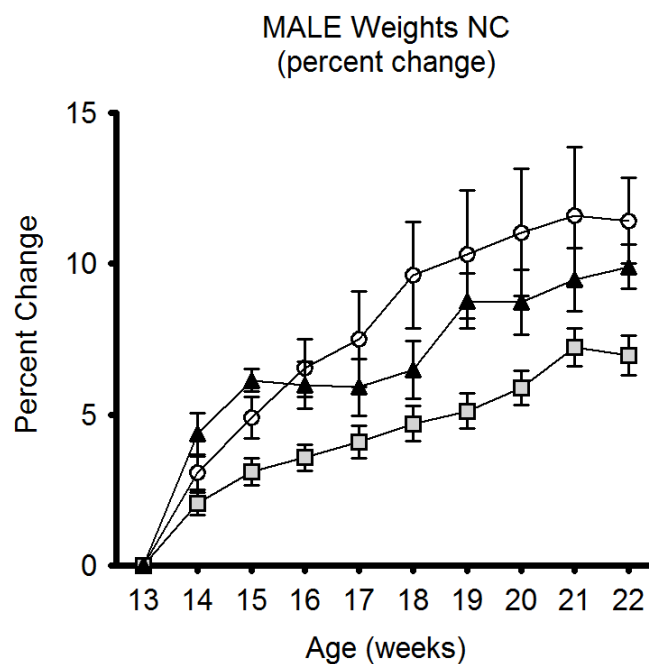


Figure 5: MUT Males have more inguinal WAT and consume less food. OX Males consume more food. OX have significantly less percentage of inguinal WAT.

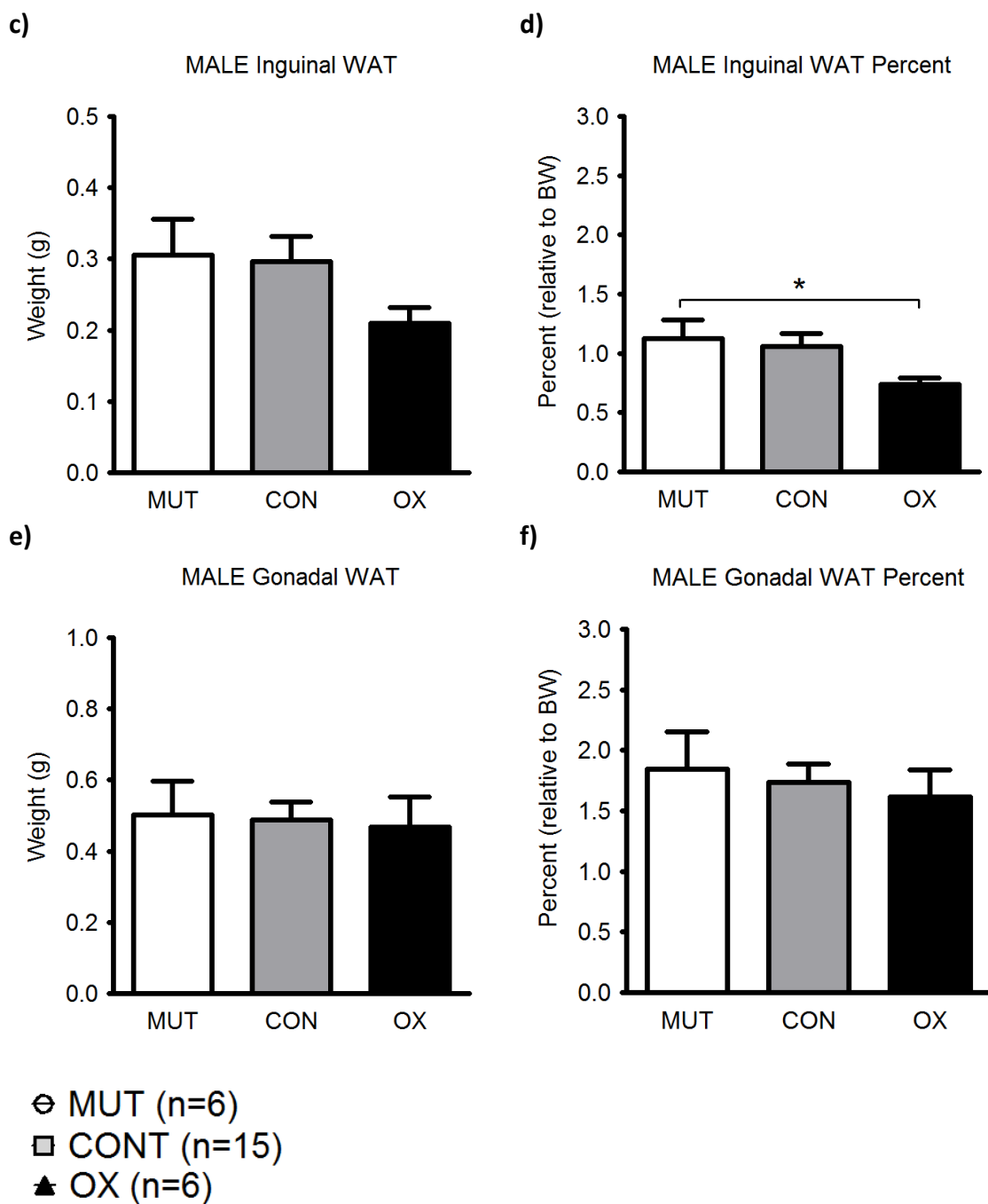
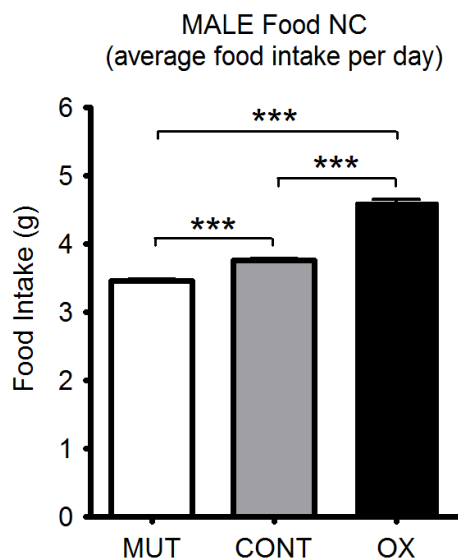
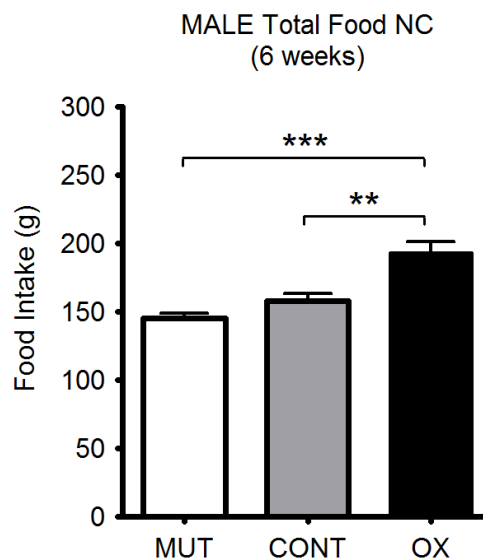


Figure 5: MUT Males have more inguinal WAT and consume less food. OX Males consume more food. OX have significantly less percentage of inguinal WAT, continued.

g)



h)



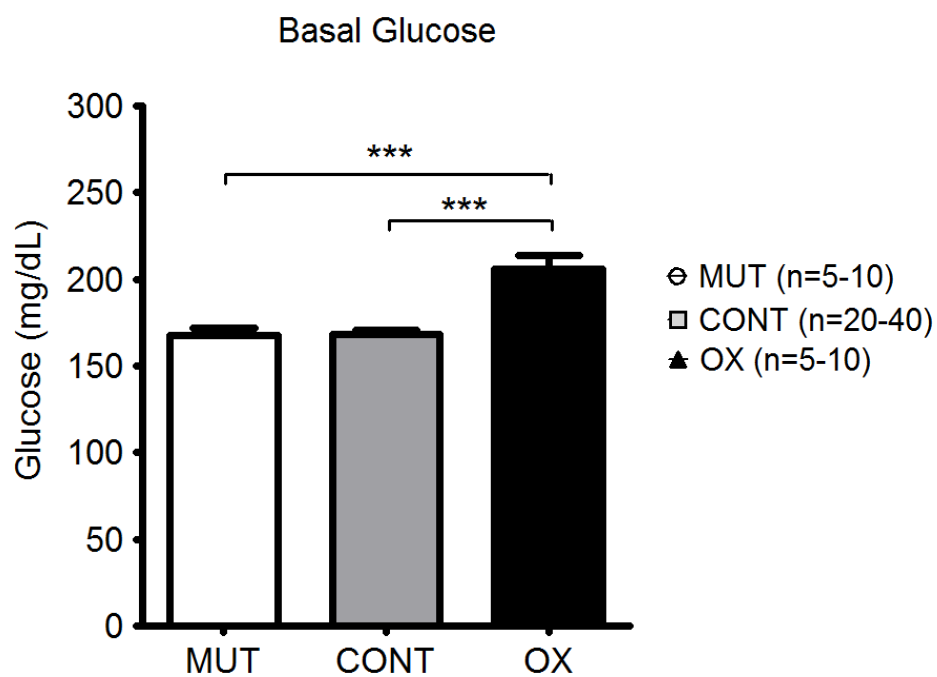
⊖ MUT (n=13)

□ CONT (n=46)

▲ OX (n=10)

Figure 5: MUT Males have more inguinal WAT and consume less food. OX Males consume more food. OX have significantly less percentage of inguinal WAT, continued.

a)



b)

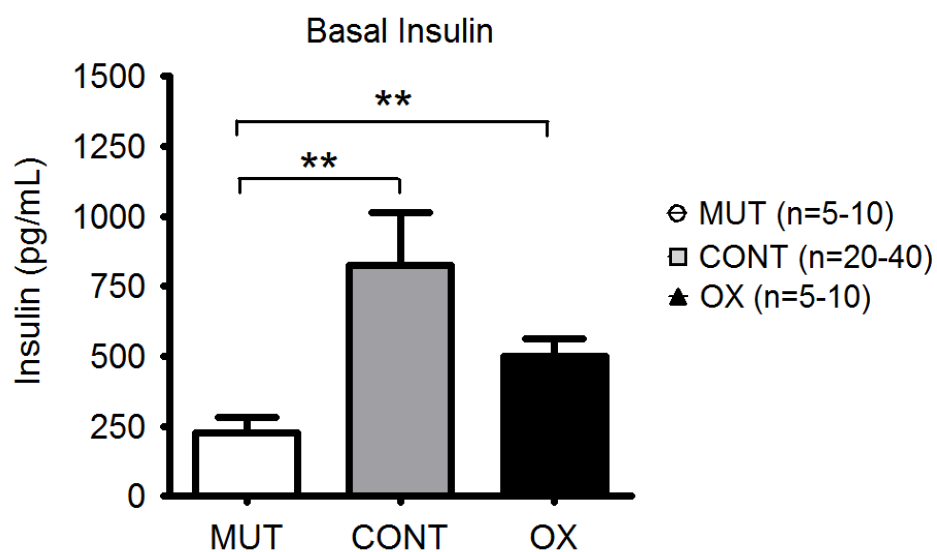
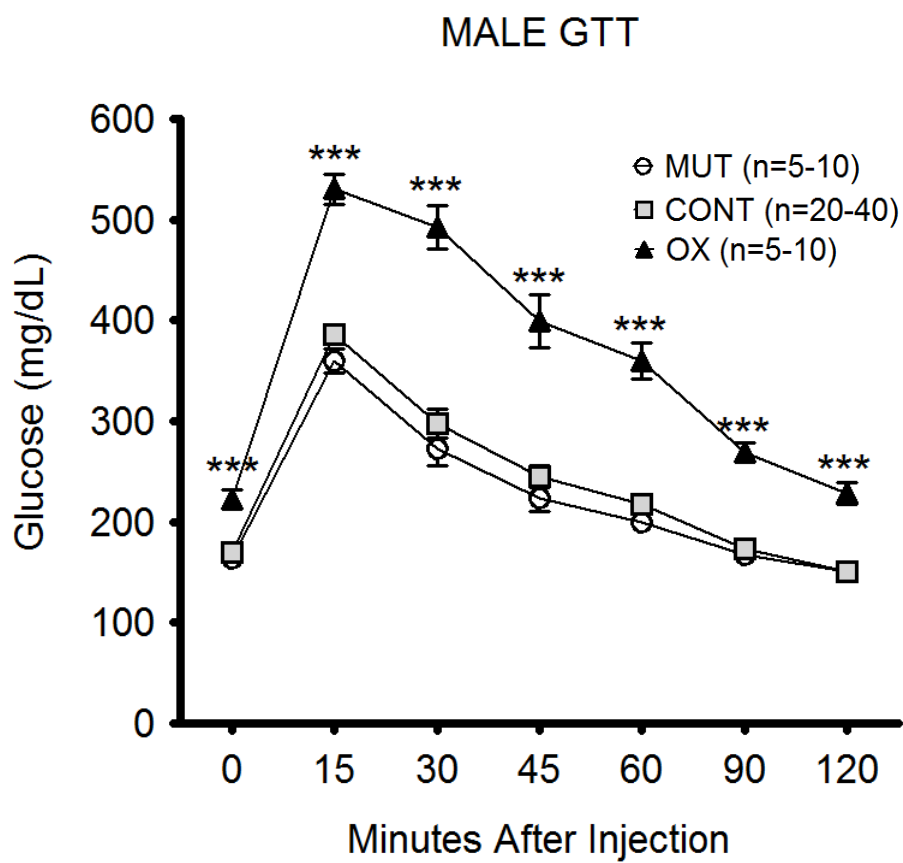


Figure 6: OX Males have higher 6 hour fasted basal glucose and insulin and are glucose intolerant and insulin sensitive. MUT Males have lower insulin.

c)



d)

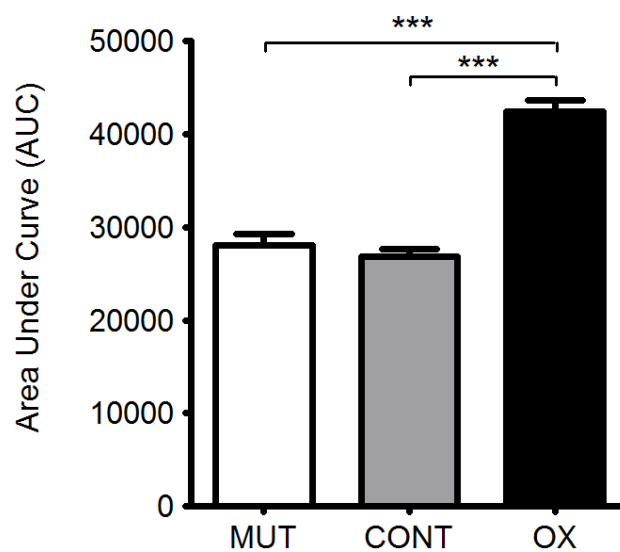
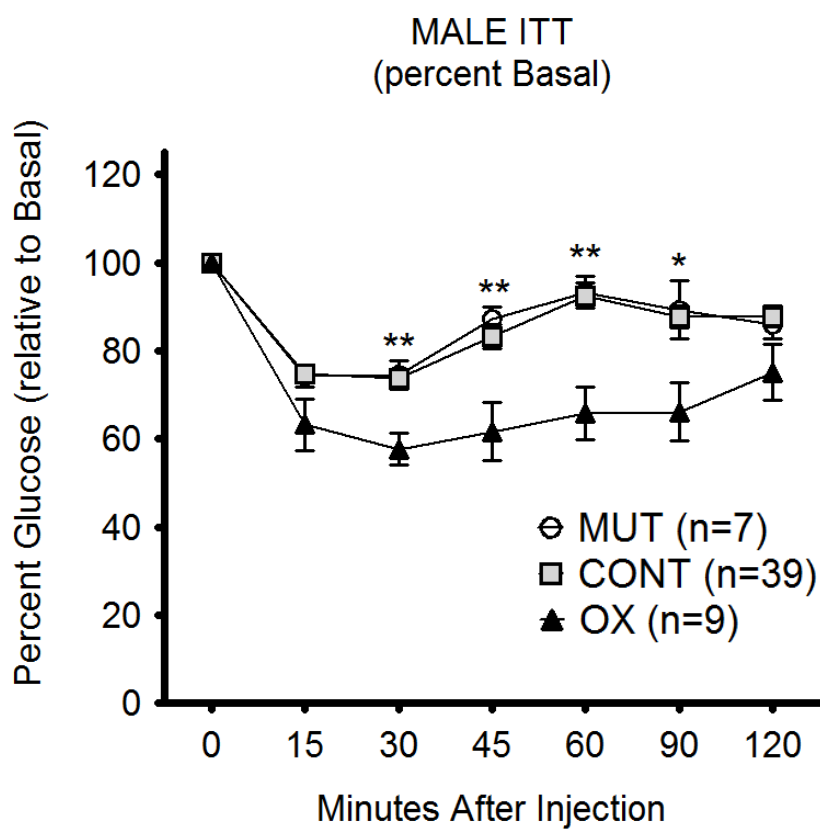


Figure 6: OX Males have higher 6 hour fasted basal glucose and insulin and are glucose intolerant and insulin sensitive. MUT Males have lower insulin, continued.

e)



f)

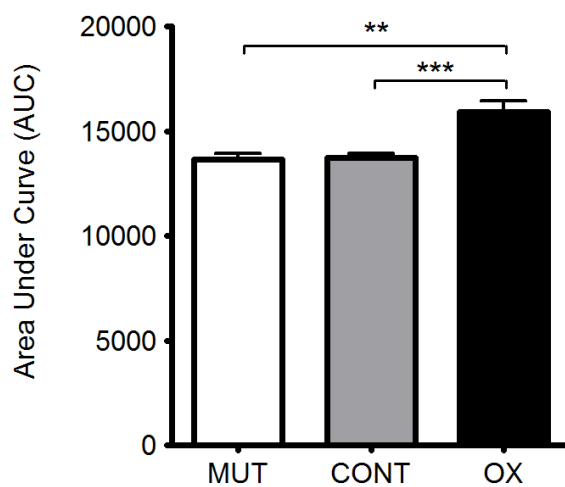


Figure 6: OX Males have higher 6 hour fasted basal glucose and insulin and are glucose intolerant and insulin sensitive. MUT Males have lower insulin, continued.

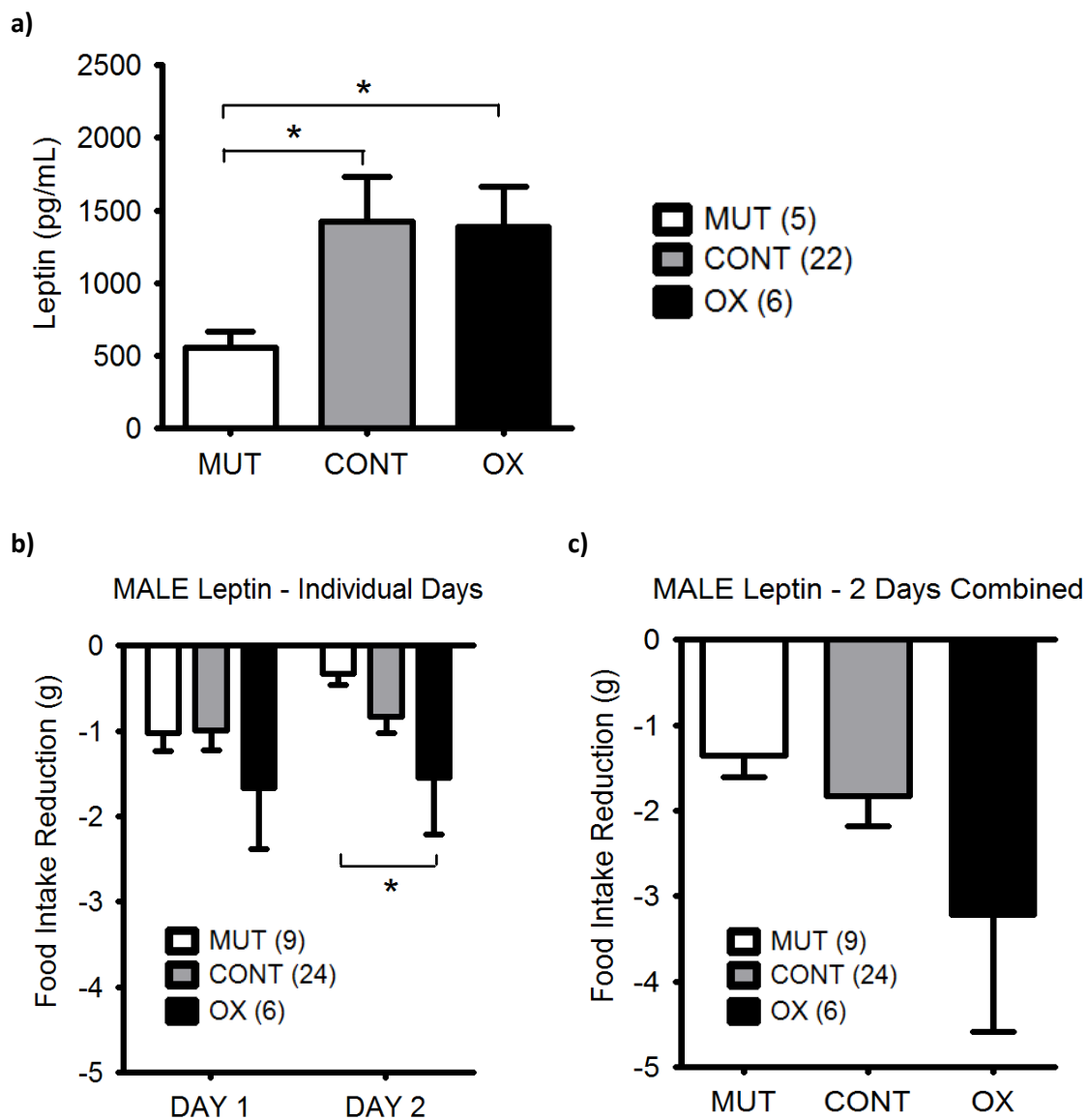
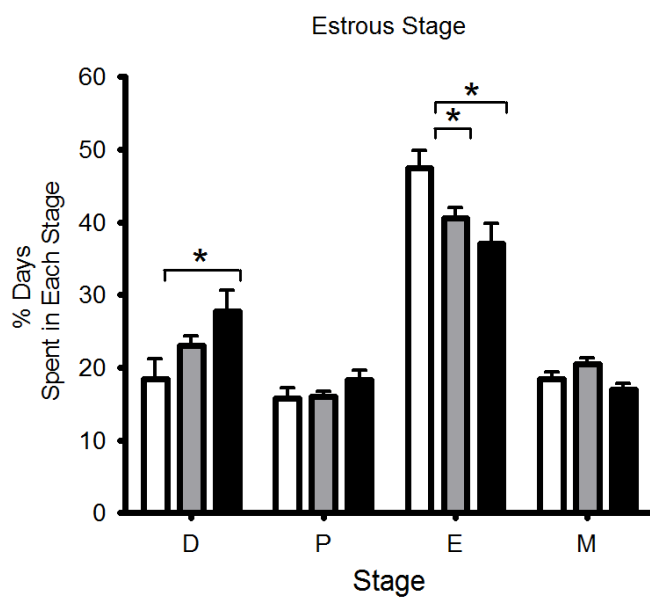


Figure 7: MUT Males have lower leptin levels. OX Males are leptin sensitive.

a)



b)

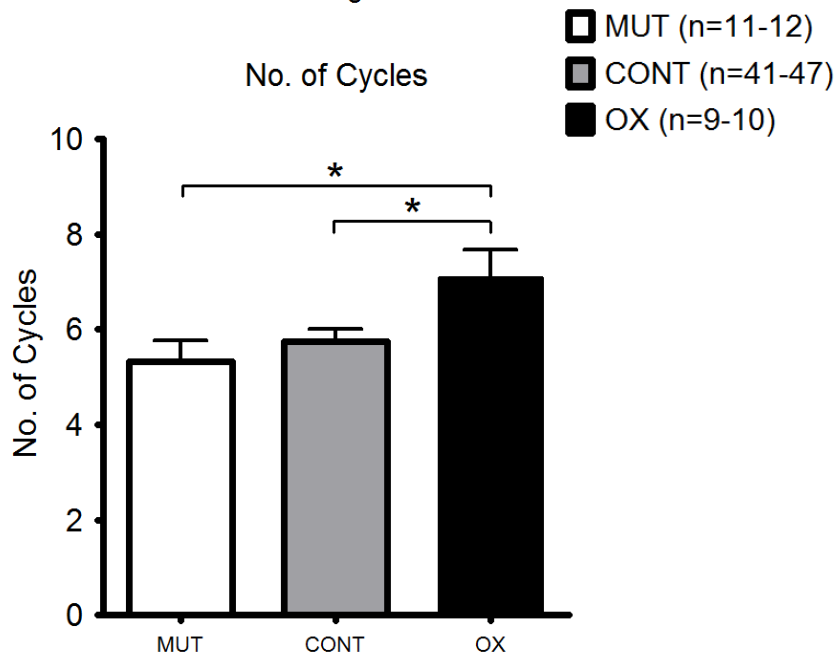
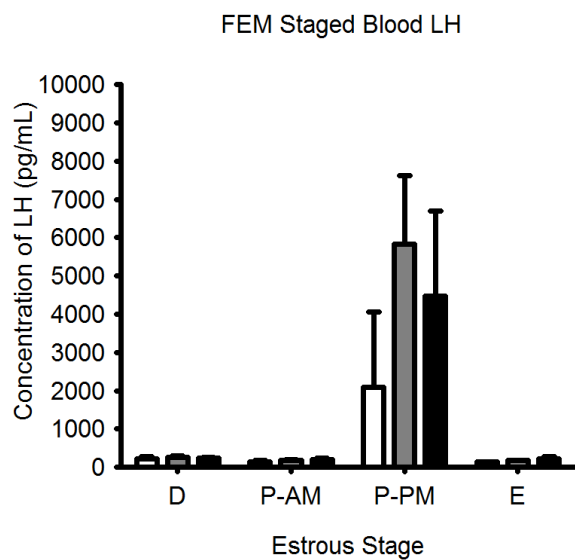


Figure 8: MUT Females spend more days in estrus and less days in diestrus. OX Females cycle more frequently.

a)



b)

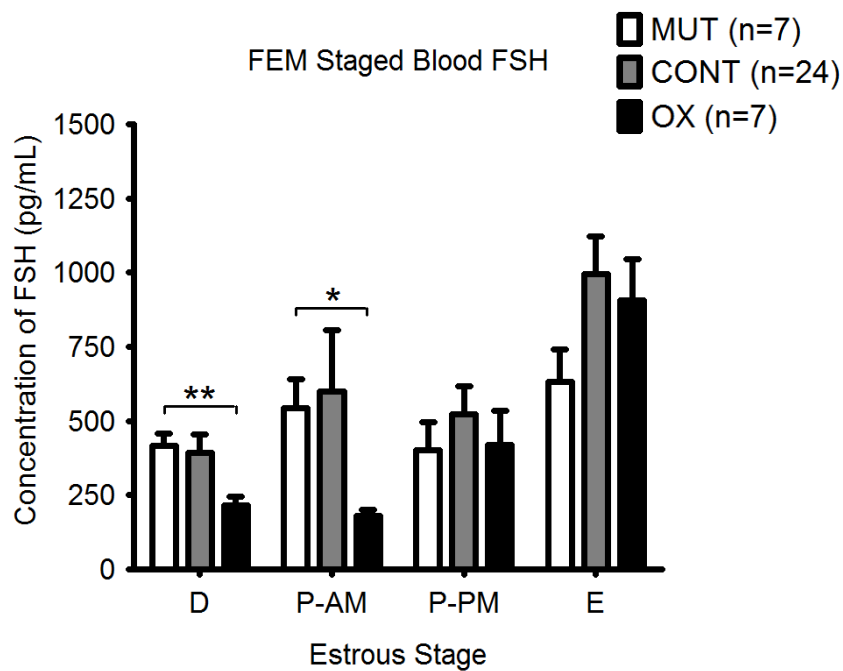
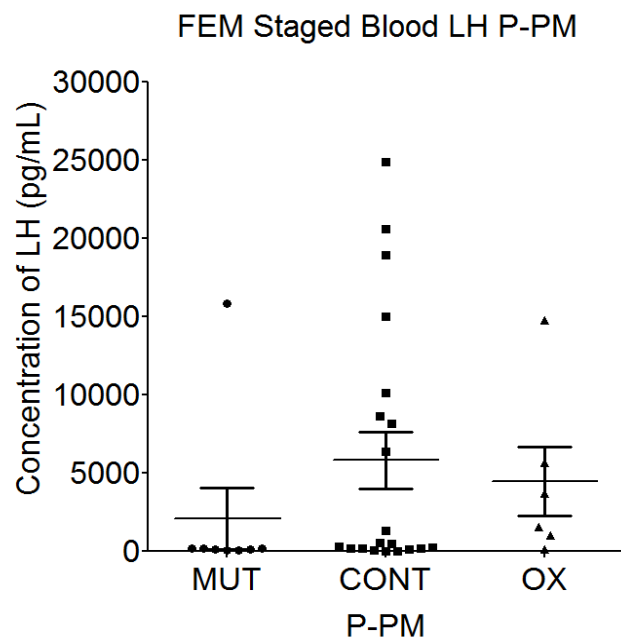


Figure 9: OX Females have lower FSH levels in diestrus and morning of proestrus.

a)



b)

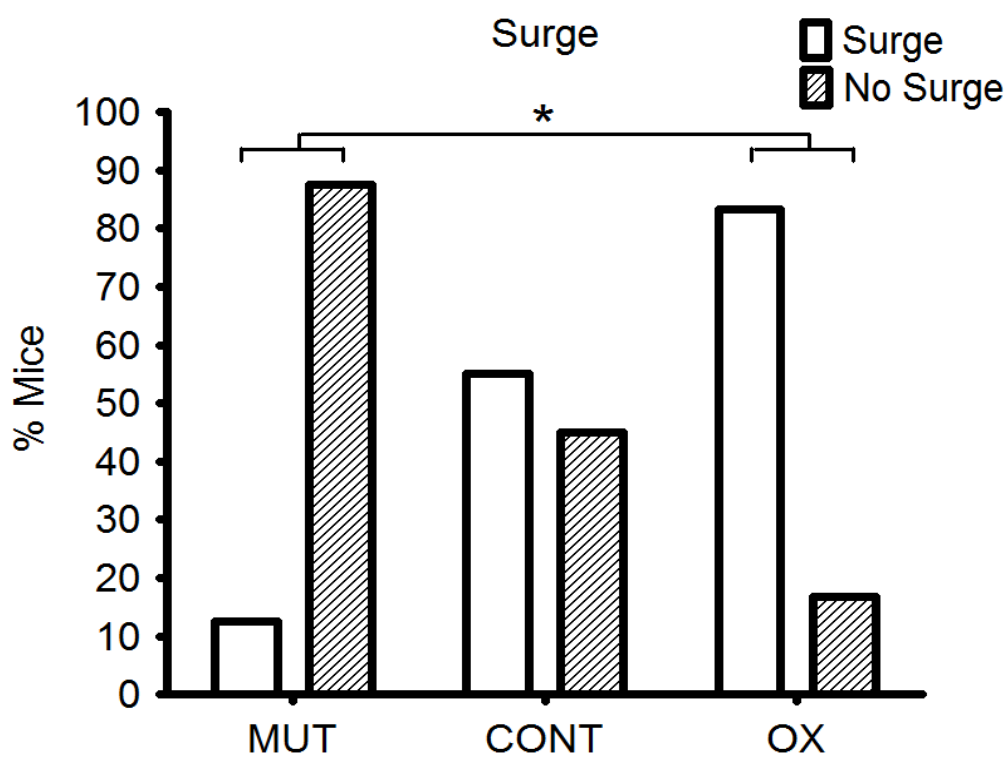
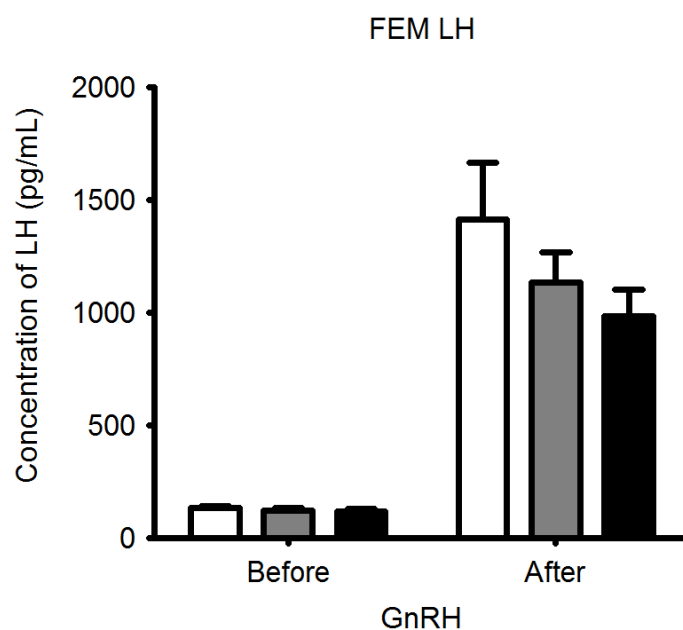


Figure 10: More OX Females surge. Less MUT Females surge.

a)



b)

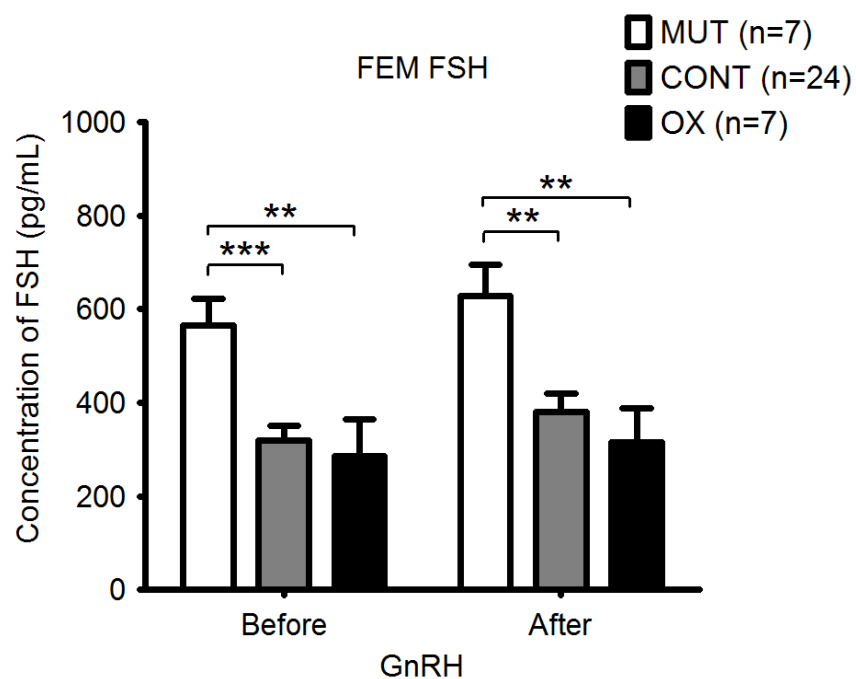
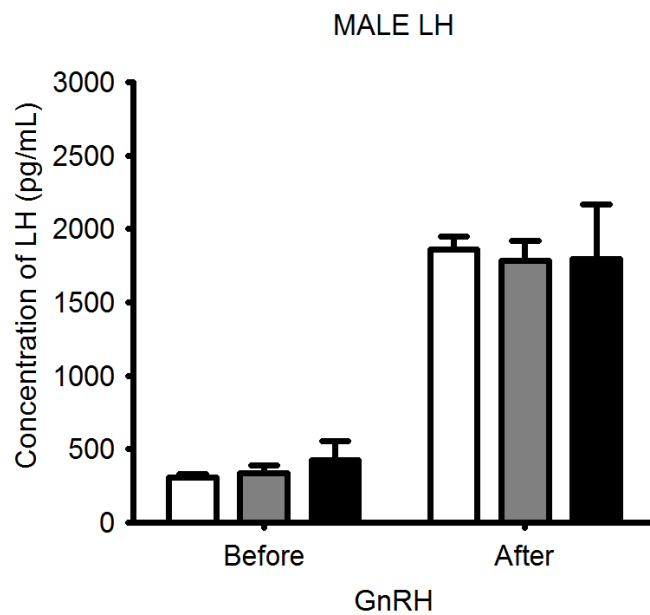


Figure 11: MUT Females have higher FSH levels.

a)



b)

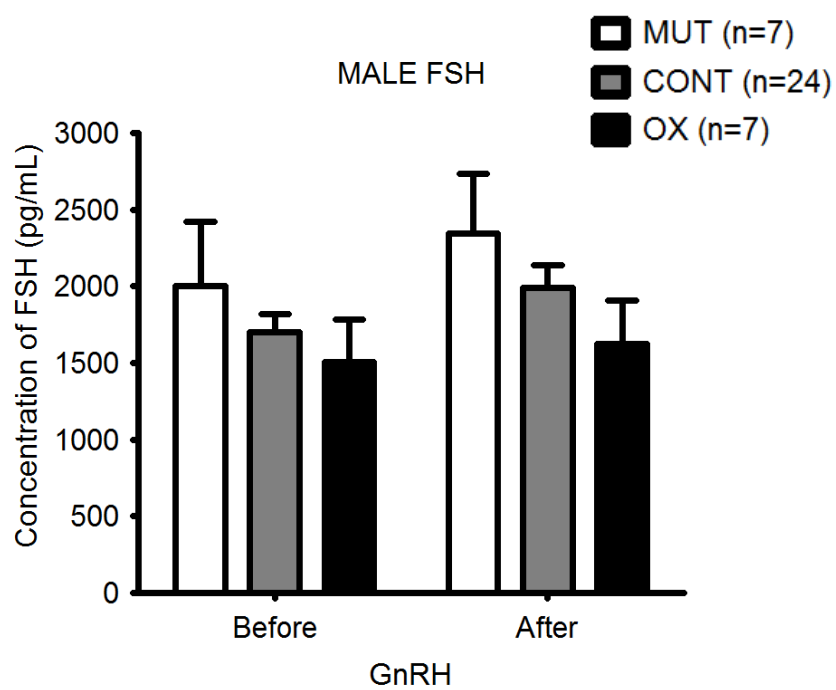
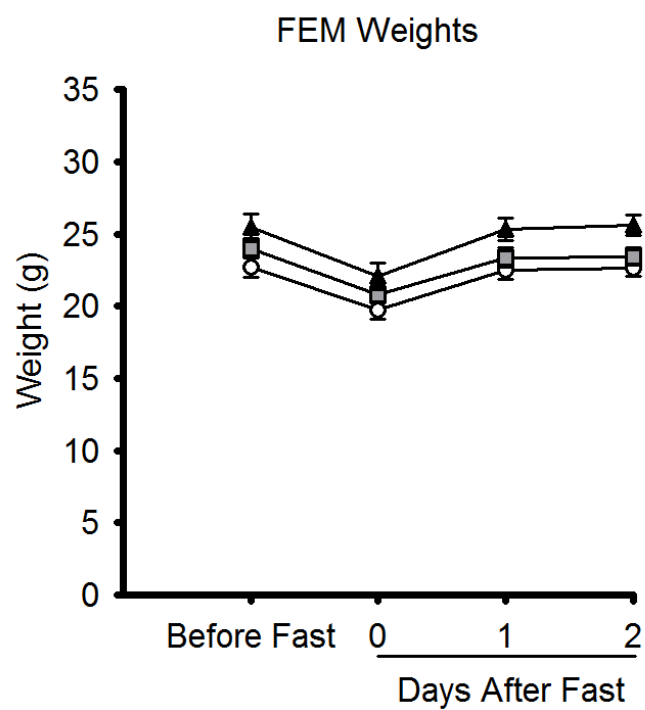


Figure 12: No difference in Male FSH and LH levels.

a)



b)

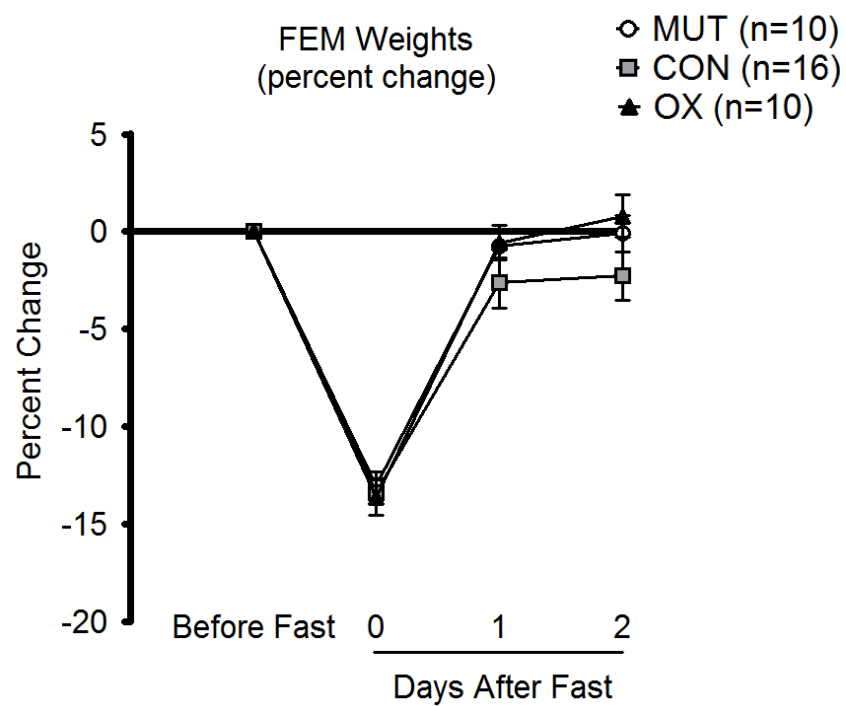
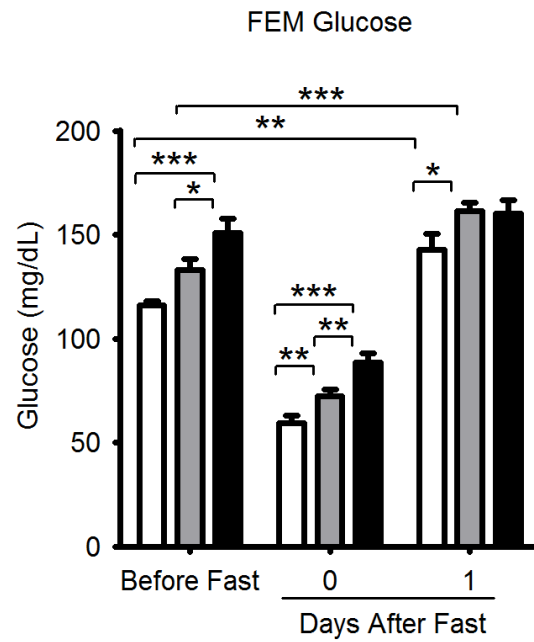


Figure 13: No difference in Female fasted weights.

a)



b)

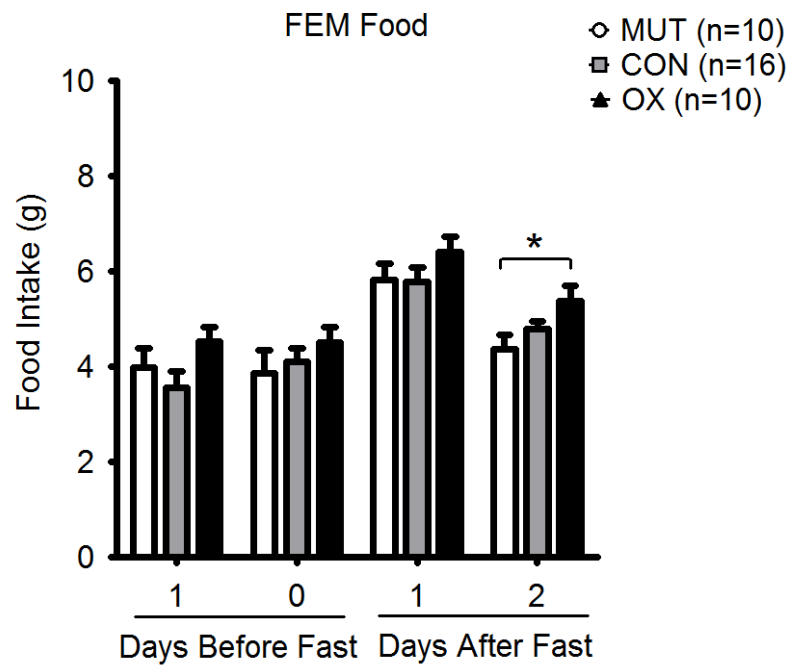
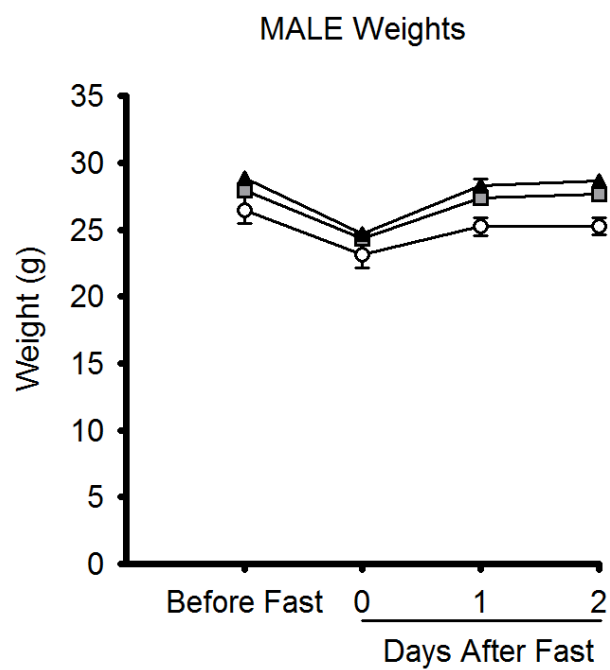


Figure 14: OX Females have higher glucose levels and MUT Females have lower glucose levels.

a)



b)

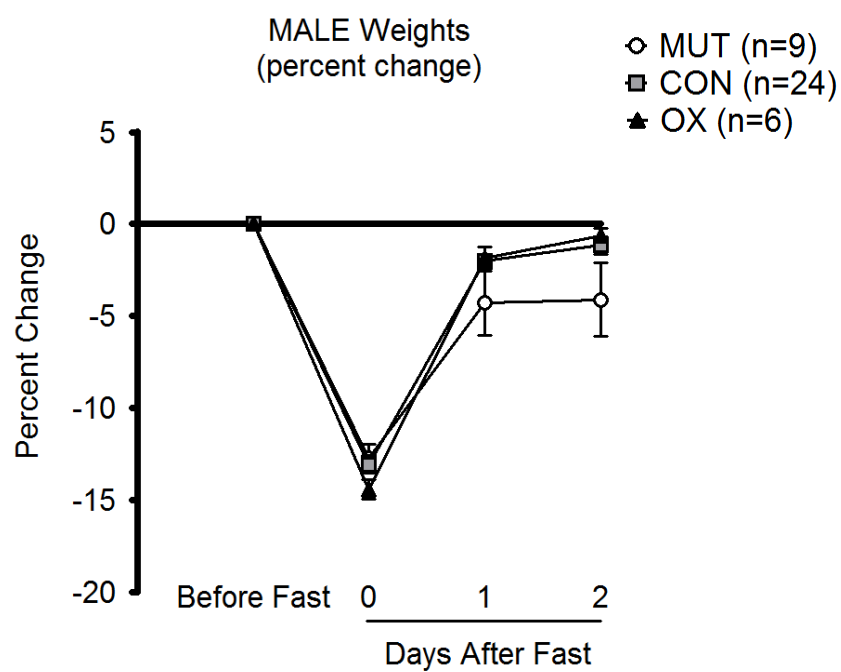
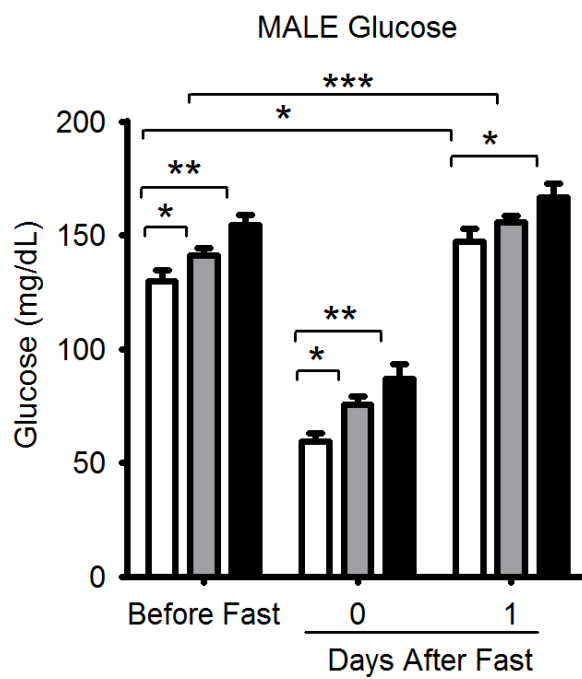


Figure 15: No difference in Male fasted weights.

a)



b)

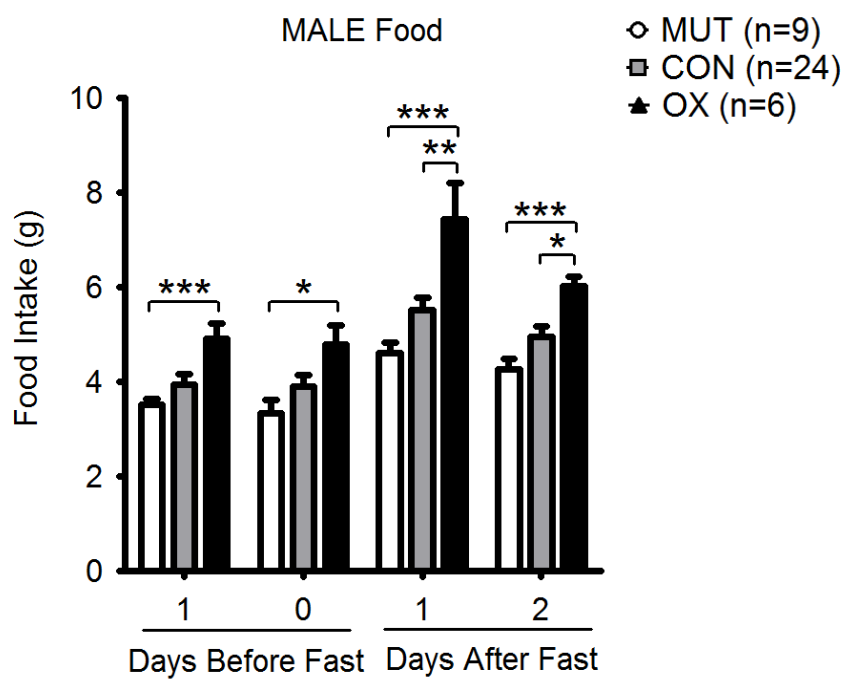
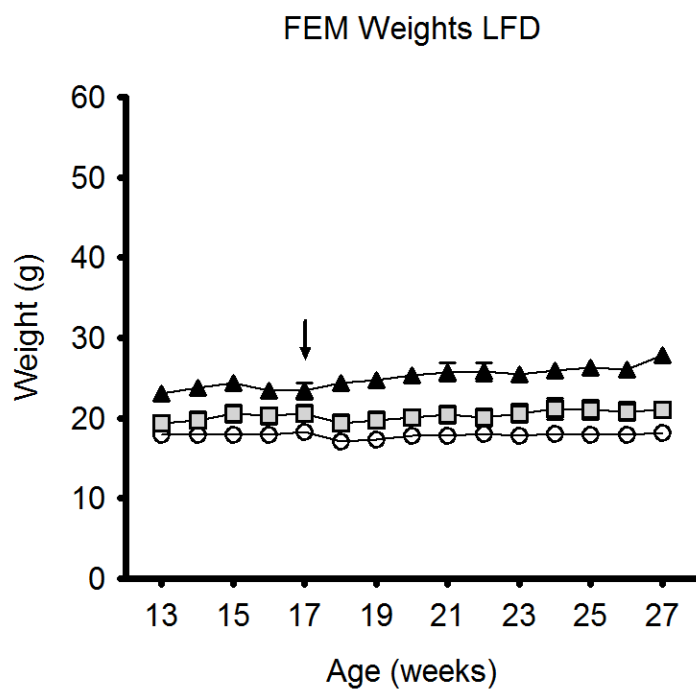


Figure 16: OX Males consume more food.

a)



b)

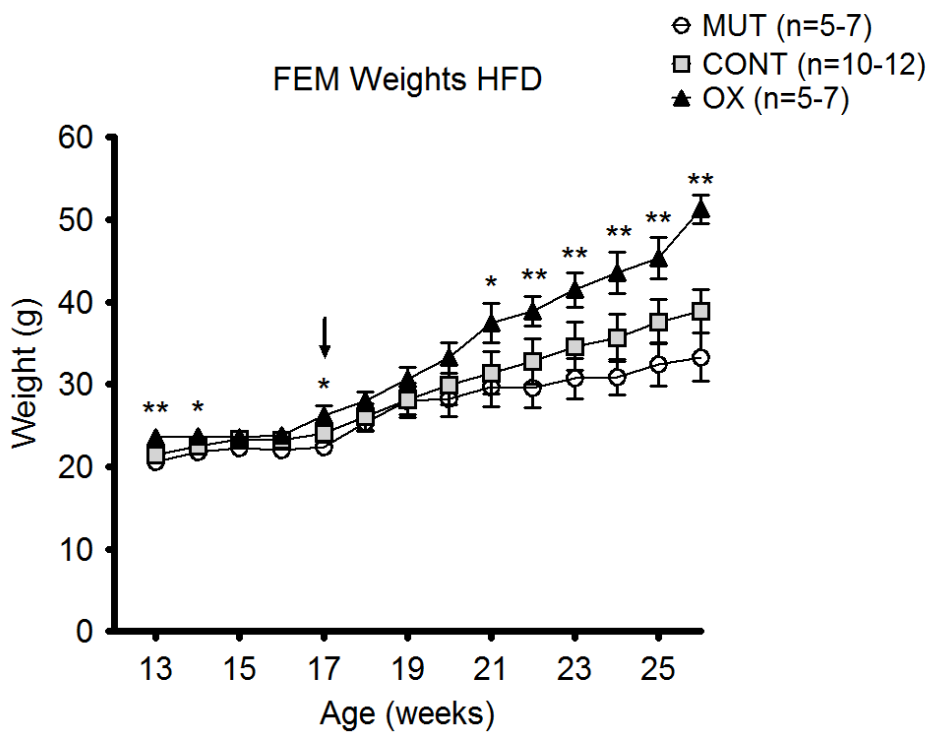


Figure 17: On HFD, OX Females weigh more and MUT Females weigh less.

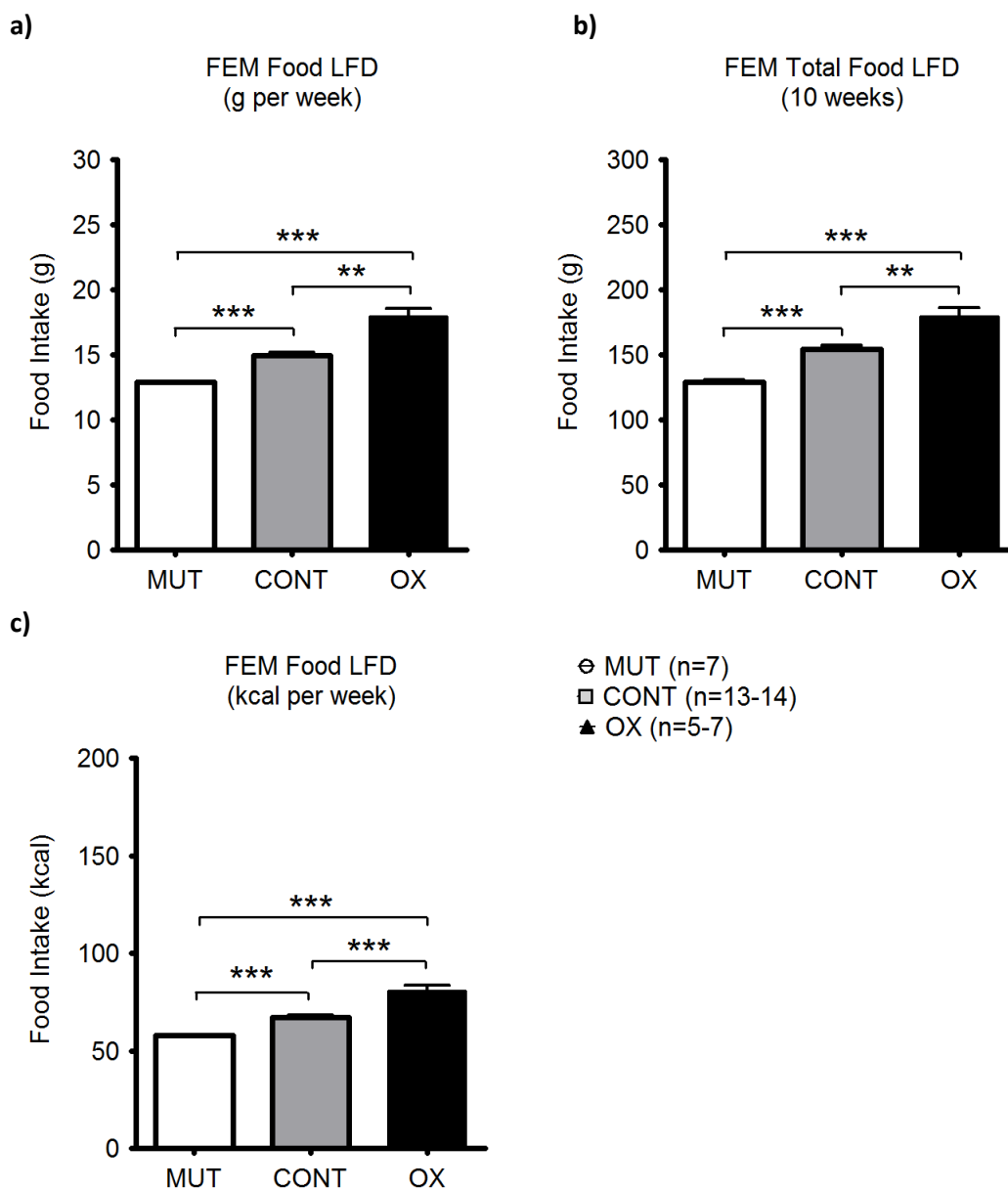


Figure 18: On HFD and LFD, OX Females consume more food. MUT Females consume less food.

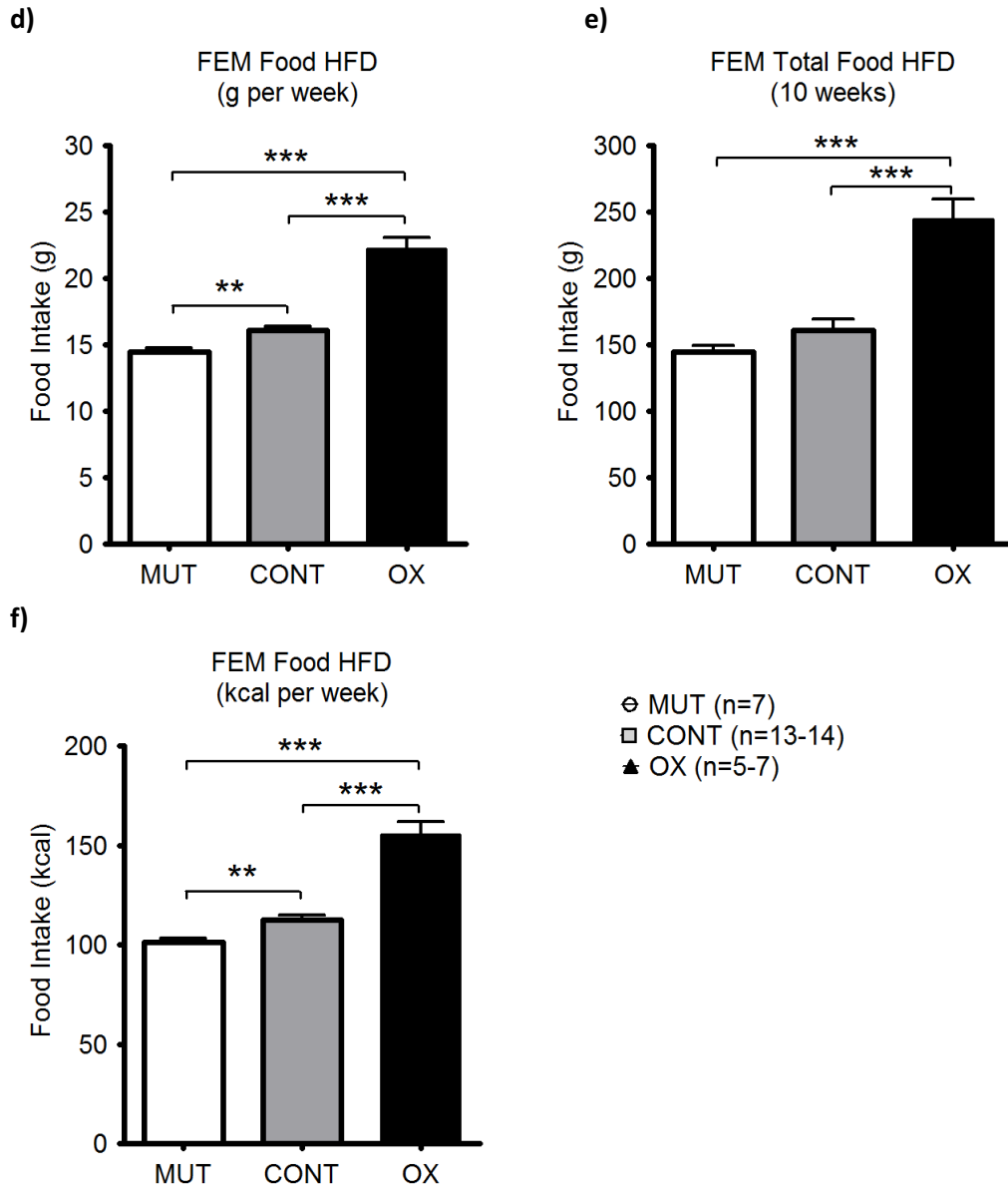
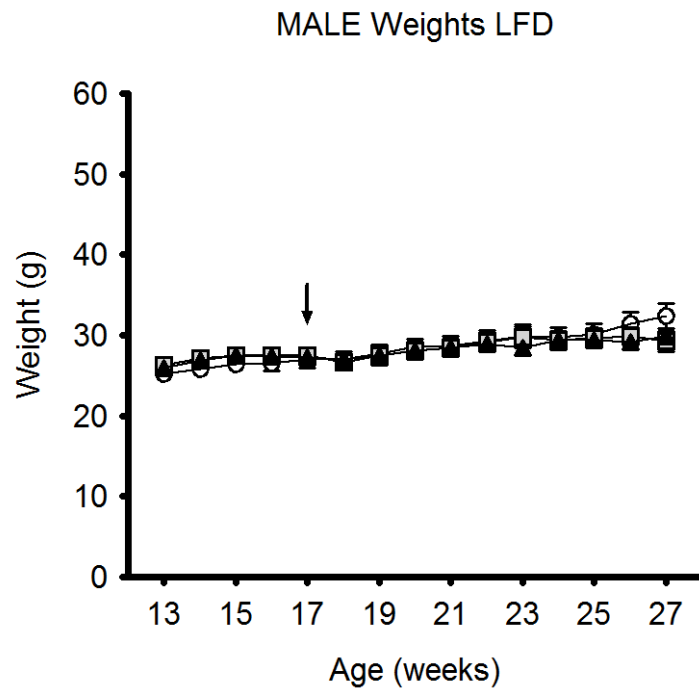


Figure 18: On HFD and LFD OX Females consume more food. MUT Females consume less food, continued.

a)



b)

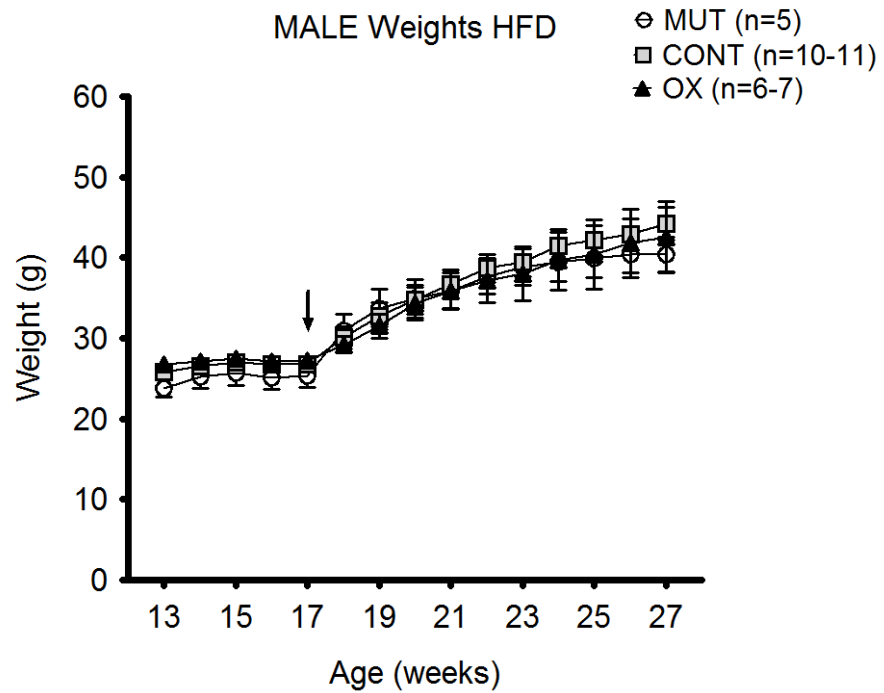


Figure 19: On HFD and LFD, no difference in Male weights.

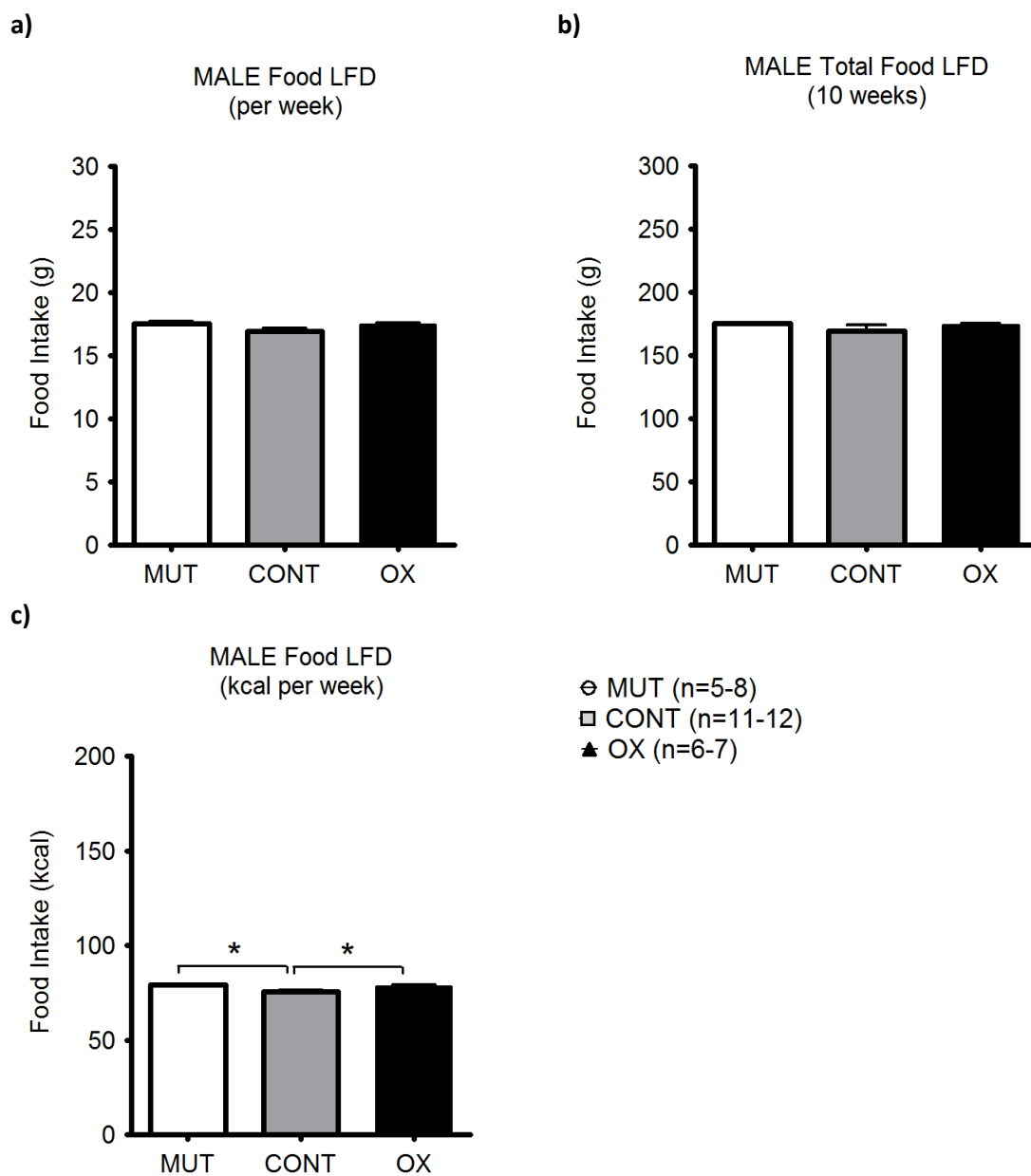


Figure 20: On HFD, OX Males consume more food. MUT Males consume less food.

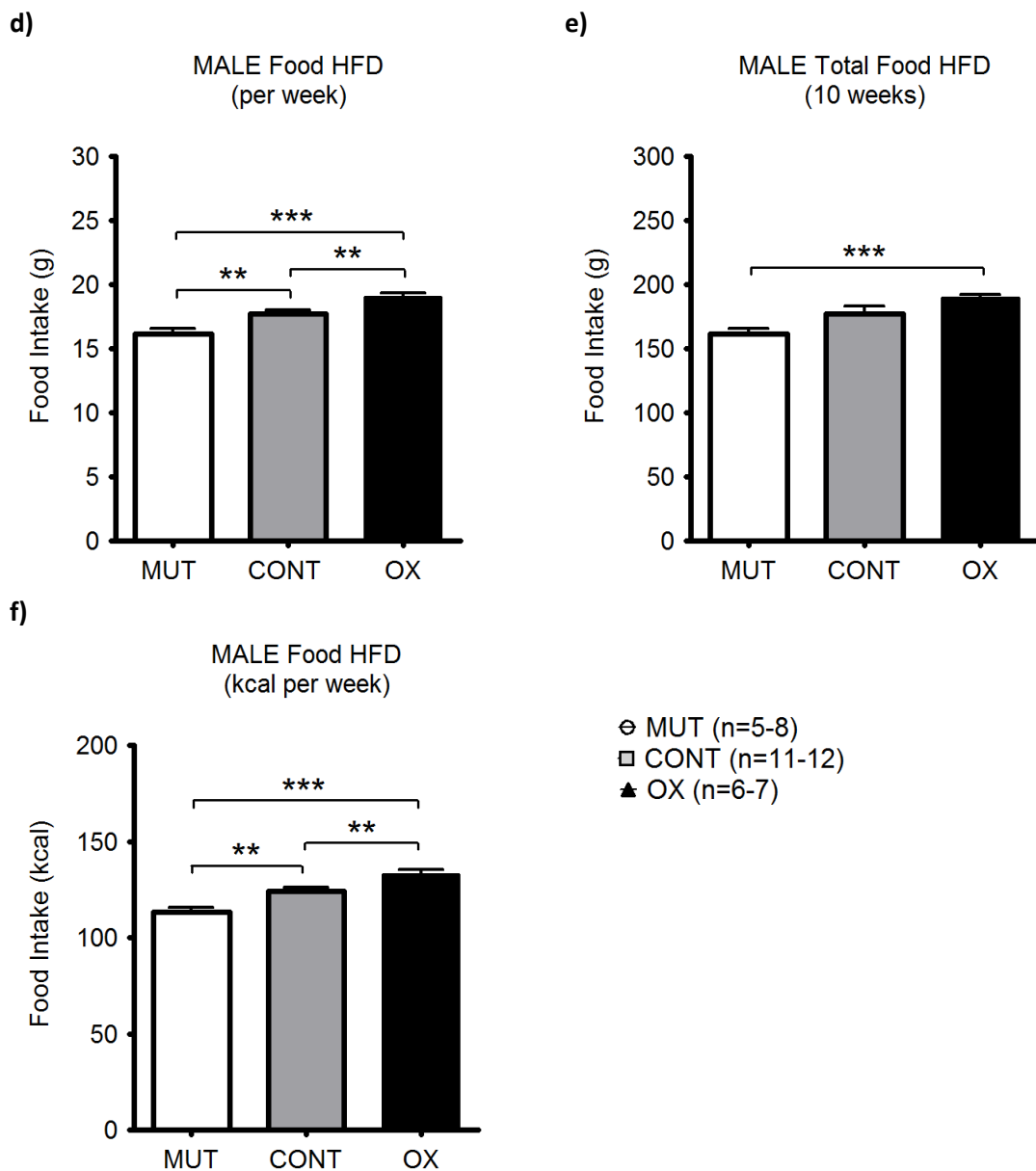


Figure 20: On HFD OX Males consume more food. MUT Males consume less food, continued.

DISCUSSION

In this study, I present data supporting the hypothesis that SIRT1 in astrocytes plays a role in metabolism and reproduction.

Previous studies have shown that a whole-body heterozygous SIRT1 knockdown results in increased obesity.²⁴ In contrast, we found that a SIRT1 mutation in astrocytes leads to a decrease in weight gain. SIRT1, known as the master metabolic regulator, is able to modify and control transcription factors and co-factors involved in system metabolic homeostasis.¹¹ Whereas, SIRT1 activation in the liver leads to increased fatty acid oxidation and improved glucose homeostasis,¹¹ SIRT1 over-expression in astrocytes results in an increase in food intake and BW. These results suggest alternative metabolic roles of SIRT1 in astrocytes divergent from that of the liver. SIRT1 in astrocytes also plays a metabolic role in controlling food intake. OX mice consume almost double the amount of food consumed by MUT. But OX mice consuming two times as much food, does not translate over to a significantly higher BW. There was no difference in WAT weight. Although not measured, there might have been a difference in lean body mass. But lean body mass alone would not explain the incredibly pronounced difference in food intake. While the OX are consuming significantly more food, weight may not be gained due to increase in energy expenditure or thermogenesis. These are just a few options to explore that may explain this finding.

Both astrocytes and SIRT1 are major contributors to metabolism. SIRT1 has been shown to regulate hepatic glucose metabolism by interacting with the FOXO family of transcription factors.¹⁰ Astrocytes also play an essential role in the deliverance of

glucose and oxygen to neurons.²⁵ SIRT1 in astrocytes may play a role in glucose metabolism and insulin responsiveness. To assess the impact of NC on glucose and insulin homeostasis, the mice were subjected to a GTT and ITT at 5 months of age. Studies have shown that heterozygous mutant SIRT1 mice show better glucose tolerance²⁶ but here we found that MUT males display no difference in glucose tolerance or insulin sensitivity. Previous studies show improvement of glucose tolerance in over-expression of SIRT1¹⁰, but here we see that OX results in glucose intolerant mice and MUT females glucose tolerant. During a GTT, glucose is injected and the mouse relies on endogenous insulin secretion to uptake glucose. Perhaps there is a defect in endogenous insulin secretion from the pancreatic beta cells. The consistently high glucose levels in OX could be due to a lack of insulin production, resulting in intolerance to glucose.

Endogenous insulin was measured to possibly explain this phenomenon but results show MUT to have significantly lower insulin levels than CONT and OX. So we gave exogenous insulin to look at glucose uptake response in muscle and adipose tissue. OX are glucose intolerant and based on basal glucose and insulin, they might be more insulin resistant. But an ITT showed OX females and males significantly more responsive to insulin and MUT females insulin insensitive. This is the complete opposite of what you might expect. Astrocytes could be affecting the production of insulin through the neurons that signal to the pancreas. A measurement of C-peptide, a byproduct created when insulin is produced, is a good measure of insulin secretion. If the amount of C-peptide is the same there could be a difference in clearance of insulin. If the amount of

C-peptide differs, there is a difference in insulin secretion, which would explain the difference seen in the GTT and ITT. Further studies would have to be done to explain this phenomenon.

OX mice trended to be more leptin sensitive and MUT leptin resistant. While OX mice consumed more food and energy, they were more leptin sensitive. There seems to be no issue in leptin production as leptin levels were actually higher in OX mice. A viable hormone to explore would be to look at ghrelin levels. Ghrelin, an appetite stimulate, specifically triggers a central SIRT1 pathway that is essential for its action.²⁷ Higher levels of ghrelin might be a possible explanation for OX consuming more food.

LH and FSH are essential hormones for reproduction. After a 10min GnRH stimulation, all mice respond with an LH surge. Because all mice are responsive to the GnRH stimulation, we know the pituitary gland is functioning and secreting FSH and LH normally. FSH requires more time than LH to surge; therefore, the FSH surge isn't as prominent. Higher levels of FSH and LH in MUT females support the finding for the increased number of days spent in estrus. Much like the females, MUT males trended to have higher levels of FSH. But when looking at the proportion of female mice that surge and don't surge, significantly more OX mice surge than MUT. This opens up to the possibility that OX females may be more sexually receptive. We know that all the females go through proestrus and estrus, as seen in the vaginal cytology data, but most of the MUT are not surging. To further explore this, if you do timed matings in the evening of proestrus and check females for plugs the following day, you can count how

many of those matings were successful and how many of those plugs led to successful pregnancies.

Caloric restriction, such as a 24hr fast, acts as an additional activator of SIRT1. The first priority of metabolism in starvation is to provide sufficient glucose to the brain and other tissues dependent on glucose. Fasting provokes a catabolic state, nearly depleting liver glycogen stores in mice. Studies have shown that a hypothalamic SIRT1 knock-out results in decreased food intake and BW gain on HFD.²⁴ When chronically challenged with a HFD, studies have also shown that a whole-body SIRT1 heterozygous male mouse becomes obese and has more fat mass.²⁸ Under a HFD, we see that OX male mice consume significantly more food and energy than the MUT yet there is no difference in BW. A process to explore would be energy expenditure or thermogenesis. While there is no difference in BW, the difference in food intake could be accounted for by differences seen in activity levels. A more thorough HFD study measuring glucose and insulin tolerance, WAT weight, lean body mass weight could also be done.

Given the role of SIRT1 in astrocytes, potential therapies centered on SIRT1 regulation might provide promising therapies in the treatment of metabolic diseases or reproductive ailments.

MATERIALS AND METHODS

Generation of astrocyte-specific SIRT1 mutant and over-expressing mice

Our laboratory has developed conditional OX and MUT SIRT1 mice, using a tamoxifen-inducible cre-recombinase under the control of the GFAP promoter, which is expressed in astrocytes. MUT x GFAP mice were generated by cross-breeding a C57BL/6 SIRT1-floxed mouse²⁰ with a BALB/c GFAP-Cre transgenic mouse²¹. The SIRT1-floxed mice contain loxP sequences flanking the exon 4 of the SIRT1 gene, which encodes the SIRT1 catalytic domain. When bred with the GFAP-Cre mice, the deleted SIRT1 allele transcribes a mutant protein that has no apparent functional SIRT1 activity. OX x GFAP mice were generated by cross-breeding C57BL/6 SIRT1-floxed stop mouse²² with a BALB/c GFAP-Cre transgenic mouse²¹. CONT contain loxP alleles but no cre-recombinase.

Weaning and genotyping were performed at 21 days after birth. The mice were randomly housed. MUT were maintained in the same cages littermate CONT. Likewise, OX were maintained in the same cages as their littermate CONT.

NC containing sugar(C&H)-supplemented Tamoxifen (Harlan) was fed at 10 weeks of age for 2 weeks. Any experiments done, were performed 2 weeks after Tamoxifen diet. And all subsequent experiments have been done to both lines with CONT.

All protocols have been reviewed and approved by the Institutional Animal Care and Use Committee.

Genotyping

PCR analysis of mouse tail DNA was used to determine genotype.

Primers Cre For: 5'-GCATTACCGGTCGATGCAACGAGTG-3' and Cre Rev: 5'-GAACGCTAGAGCCTGTTTTGCACGTTC-3' were used for PCR. Presence of Cre was scored by amplification of a 400bp PCR product.

Primers Mut For: 5'-GCCCATTAAAGCAGTATGTGTGG-3' and Mut Rev: 5'-GGAATCCCACAGGAGACAGA-3' were used for PCR. Presence of homozygous floxed alleles were scored by amplification of a 250bp PCR product, whereas the amplification of a 216bp fragment corresponds to the non-floxed sequence.

Primers OxA: 5'-GCACAGCATTGCGGACATGC-3', OxB: 5'-CCCTCCATGTGTGACCAAGG-3', and OxC: 5'-GCAGAAGCGCGGCCGTCTGG-3' were used for PCR. Presence of homozygous floxed stop alleles were scored by amplification of a 551 bp PCR product with OxB and OxC primers and the absence of PCR product with OxA and OxB primers.

Insulin and Glucose Tolerance Test

Tolerance tests were performed on 6hr fasted mice at 5 months of age, by intraperitoneal injection of 0.4U/kg body weight human insulin (Novolin R) for insulin test or 2g/kg body weight 50% dextrose (Hospira) for glucose test. Blood glucose was measured by tail bleeding using an ACCU-Check glucometer at 0, 15, 30, 45, 60, 90, and 120 minutes after glucose administration.

Leptin Test

At 5 months of age, individually housed mice were injected intraperitoneally 2 times a day for 2 days, once at 6am and once at 6pm, with 0.5mg/kg of leptin (NHPP) for one week. For the second week, the same process was repeated except with PBS. Food intake monitored prior to, during, and after the test.

GnRH Test

At 5 months of age, 1ug/kg GnRH (Sigma) was injected intraperitoneally. Serum collected before injection and 10 minutes after injection, for comparison.

Vaginal Cytology

Vaginal smear cytology was used to determine the estrous cycle phases. This method predicts the estrous cycle according to the proportion of three cell types observed in the vaginal smear: epithelial cells, cornified cells, and leukocytes.¹

At 3 months of age, vaginal cytology was examined daily for 6 weeks. Females were cycled in the AM, vaginal fluid was transferred onto slide, slide was stained with methylene blue (0.5% methylene blue and 1.6% potassium oxalate in water) and then visualized under a light microscope to assess reproductive stage. Females were bled at the beginning of diestrus, pro-estrus, estrus, and metestrus for hormone analysis.

Endocrine Profile Analysis

Serum was collected by nicking the tail. Plasma obtained after centrifugation and stored at -80°C. GnRH and staged blood plasma were used to measure LH and FSH levels

via Luminex Assay. 6 hour fasted blood plasma was used to measure leptin and insulin levels via Meso Scale Discovery.

Gene Analysis

At 5 months of age, 6 hour fasted blood serum was collected by nicking the tail. Plasma obtained after centrifugation and stored at -80°C. Plasma was used to measure gene expression via Fluidigm BioMark.

24 Hour Fast

At 5 months of age, individually housed mice on NC were starved for 24hrs; 9AM to 9AM. Water was provided *ad libitum*. Body was weighed, food intake was measured, serum was collected, and glucose levels were monitored before and after the fast.

HFD/LFD Study

At 4 months of age, mice were randomly fed, a high-fat diet (60%, Research Diets) or a low-fat diet (10%, Research Diets), and water *ad libitum*. Only body weight and food intake were measured. After 10 weeks on diet, mice were perfused at 7 months of age.

Statistics

Statistics done by Student's two-tailed t-test via Microsoft Office Excel 2007 and Graphpad Prism 5. Statistical difference defined as *p-value<0.05, **p-value<0.01, ***p-value<0.001.

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