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# EVALUATING MECHANISMS BY WHICH 17α-ESTRADIOL ELICTS HEALTH BENEFITS ACROSS SPECIES

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EVALUATING MECHANISMS BY WHICH 17α-ESTRADIOL ELICTS HEALTH BENEFITS ACROSS SPECIES

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| | |
|---|---|
| **17α-E2** | 17 alpha estradiol |
| **17β-E2** | 17 beta estradiol |
| **ERα** | Estrogen receptor alpha |
| **ERβ** | Estrogen receptor beta |
| **T2D** | Type 2 diabetes |
| **CR** | Caloric restriction |
| **HRT** | Hormone replacement therapy |
| **GTT** | Glucose tolerance test |
| **ITT** | Insulin tolerance test |
| **AUC** | Area under the curve |
| **OVX** | Ovariectomy |
| **WAT** | White adipose tissue |
| **EPI** | Epididymal WAT |
| **NOER** | Nuclear-only ERα |
| **MOER** | Membrane-only ERα |
| **POMC** | Proopiomelanocortin |

## ABSTRACT

There is an emerging need for novel pharmacological compounds that mitigate metabolic diseases that arise with obesity and advancing age. One such interventional compound is 17α-estradiol (17α-E2), a "non-feminizing" estrogen that prolongs lifespan in male mice. However, little is known regarding the mechanisms of action by which 17α-E2's elicits health benefits. Prior work in my laboratory has shown that 17α-E2 likely elicits benefits through estrogen receptor α (ERα) but it remains unclear if these benefits occur through a specific cell-type, or if these benefits are conserved in other species. I hypothesized that 17α-E2 elicits benefits in species other than mice. My studies provide evidence that 17α-E2 can elicit health benefits in both male and female rats, and male common marmosets. Collectively, my studies demonstrate that the benefits of 17α-E2 are translatable to other species. Future studies will be needed to determine the specific cell type responsible for 17α-E2 health benefits in males. This knowledge would provide greater opportunities for 17α-E2 to be translated into human clinical trials, while also enabling greater understanding of the mechanisms underlying sex differences in aging and chronic disease burden.

# CHAPTER I

# INTRODUCTION AND LITERATURE REVIEW

## Sex Differences and Aging

Striking sex differences in the physiology of aging have been discovered and these likely to underlie the sex differences in lifespan noted in many developed nations [1]. For instance, the prevalence of obesity, diabetes, and diabetes-related complications are greater in men than women [2,3]. However, postmenopausal women have obesity rates that increase in comparison with age-matched men [2], which put them at greater risk of chronic conditions. For instance, despite the incidence of osteoporosis being increased in both older women and men [2], the propensity to fracture is higher in older women rather than older men. Interestingly, prior to menopause women tend to have lower blood cholesterol levels compared to men, but after menopause the incidence of cardiovascular disease increases substantially [4,5]. Systemic inflammation arises during aging in both sexes [6, 7], but changes in immune function is significantly affected in women undergoing menopause, suggesting that hormonal changes are playing a role [8]. Moreover, the occurrence of stroke is higher in older women than older men [9], especially in women who entered menopause at 42 years of age or younger, which more than doubles the risk [10]. Collectively, these observations clearly demonstrate that menopause increases the risk of developing chronic diseases in women, which points to a positive role of estrogens in combating the mechanisms that promote certain diseases [11-13].

## The Relationship Between Estrogen Status and Age-Related Diseases

Circulating estrogen concentrations are correlated with the onset of age-associated diseases [12-14]. Estrogen receptor (ER) play key roles in modulating

estrogenic effects on metabolic, immune, and muscle/bone growth signaling pathways. Post-menopausal women [15] and mouse models of ovariectomy (OVX) [16] often display decreased longevity and increased susceptibility to age-associated disease.

There is considerable evidence indicating that estrogen deficiency promotes visceral adiposity, which is associated with inflammation and metabolic dysfunction (17-19). Increased visceral adiposity is an established risk factor for T2D, metabolic syndrome, and coronary artery disease (20). Experimental data demonstrates that people with estrogen deficiency are vulnerable to having worse outcomes, such as atherogenic changes, insulin resistance, pancreatic beta-cell dysfunction, glucose intolerance, and increased inflammation (21-22). Estrogen receptor alpha (ERα) and beta (ERβ) play critical roles in regulating glucose and lipid metabolism through actions in the liver, pancreas, adipose tissue, skeletal muscle, heart, and brain (23).

Estrogens also mitigate pro-inflammatory states through multiple ER-mediated signaling pathways (24). The most commonly studied estrogen, 17-beta estradiol (17β-E2), is protective against dysregulated metabolic pathways which helps to ward off the mechanisms that lead to T2D (25-26). 17β-E2 also has a suppressive effect on the NFκB pathway, a key regulator of pro-inflammatory gene expression and cellular senescence. 17β-E2 inhibits NFκB activation via direct interactions with its subunits, c-Rel and Rel A, thus suppressing inflammation (27-30).

17β-E2 also plays a unique role in regulating neuroinflammation (31). For instance, an estrogen-deficient state adversely affects DNA repair mechanisms, which are believed to regulate cognitive function and the risk for neurodegenerative diseases (32-34). There is now compelling evidence that 17β-E2 may attenuate the formation of beta-amyloid plaques and hyperphosphorylation of tau protein in Alzheimer's disease (AD) (35). Studies in post-menopausal women with AD indicate that those receiving estrogen patch therapy

showed significant improvement in attention and verbal and visual memory compared with those not receiving estrogen therapy (36). Animal studies indicate that estrogens improve cognitive function by facilitating both hippocampal and cortical long-term potentiation under ischaemic and oxidative stress conditions (37), but additional work is warranted to unravel how this may be occurring.

Bone health remains a critical concern in aging women due to diminished estrogen production and the essential roles of ERs in maintaining bone homeostasis [3, 4]. This results in sex-specific patterns of bone health, with women exhibiting higher rates of osteoporosis and associated fractures [2]. Hormone Replacement Therapy (HRT) has been shown to effectively alleviate these bone health issues [3, 4], and interestingly, 17α-Estradiol (17α-E2) is a constituent of many HRT formulations [38]. HRTs are known to prevent bone loss by inhibiting osteoclast activity, reducing bone turnover, and lowering the risk of other metabolic risk factors that may contribute to further bone loss [39-40]. Despite, the aforementioned benefits of HRTs, their potential health risks have led to numerous individuals to advocate against their use. This stems from controversial findings from the Women's Health Initiative study that reported an increased risk of adverse events such as stroke, pulmonary embolism, and breast cancer in women receiving HRT (41). This has led to the field pushing for alternatives to traditional HRTs.

## Synthesis of 17α-E2

17α-E2 is a naturally occurring enantiomer of 17β-E2 that can be found in all mammalian species that have been tested. The exact role that endogenous 17α-E2 plays in mammalian species is unknown, but as outlined above, exogenous administration of 17α-E2 elicits numerous health benefits, including lifespan extension in male mice. 17α-E2 has been studied for decades, yet enzymatic pathways leading to its production are still unknown (42). In humans, 17α-E2 and its conjugates circulate at very low

concentrations (43). It is possible that 17α-E2 can be produced from the testosterone enantiomer, namely 17α-testosterone (43-45). However, the exact biochemical pathways through which aromatase, or some other enzyme, may convert 17α-testosterone to 17α-E2 remain unknown. A few reports have linked aromatase to 17α-E2 biosynthesis (46), but these studies suggest that aromatase activity in the hippocampus, neocortex, and amygdala of the brain are the primary locations where 17α-E2 is synthesized (47). Another possible source of 17α-E2 production is the gonads and extragonadal tissues such as the placenta, skin, adipose tissue, and bone (48), although very little evidence exists to support this hypothesis, thus additional studies are needed.

## 17α-Estradiol Interactions with Estrogen Receptors

17α-E2 possesses only around 3-10 percent of the binding affinity for ERα and ERβ that 17β-E2 has. [44-45]. Prior work has suggested that 17α-E2 might elicits its effects through a novel receptor, termed ER-X [44, 49-50]. However, since these initial reports, very little new data has been reported to support the ER-X hypothesis.

## Benefits of Estrogens in Hepatic Steatosis

The liver heavily contributes to systemic control of carbohydrates, lipid, and protein metabolism, which wanes with advancing age (51-52). Aging promotes changes in lipid metabolism due to decreased activity of hepatic enzymes involved in cholesterol and drug oxidation (53). Aging also increases hepatic insulin resistance which further contributes to lipid accumulation in the liver. In the liver, most of the fatty acids synthesized are incorporated into triglyceride-rich very low-density lipoprotein (VLDL) particles and exported into the circulation. Estrogens have a significant impact on liver metabolism, especially during aging and obesity. Like the uterus, the liver is one of the tissues with the highest number of estrogen receptors and sexually dimorphic expression of many estrogen-responsive genes involved in hepatic lipid metabolism [54]. The actions of

estrogens on hepatic metabolism could be mediated by both direct actions through estrogen response elements (EREs) and indirect actions through other transcription factors [55-56].

Our group and others have shown that 17α-estradiol (17α-E2) heavily impacts hepatic lipid and glucose metabolism. 17α-E2 improves hepatic metabolic flexibility by suppressing de novo lipogenesis and gluconeogenesis, which limits the amount of substrate that is available locally for storage [57-59]. 17α-E2 also decreases hepatocyte DNA damage, which likely limits cellular senescence and aberrant proinflammatory stress [57]. Other groups have also shown that 17α-E2 decreases metabolites involved in betaine metabolism in females, while modulating urea metabolism in males [60]. Discrepancies in the few significant responses observed in females, and differences in male responses between intact and castrated UM-HET3 mice, suggest that male gonadal hormones are important, and likely play a role in mediating 17α-E2's effects. [61] 17α-E2's effects on proteo-stasis and aging are currently unknown, despite its regulation of the mTOR complexes [60-63]. These findings support the notion that 17αE2 can have similar effects to 17β-E2 via ER-mediated action, but also that it likely engages in additional pathways beyond classical ER signaling.

**17α-Estradiol and Its Role in Mitigating Systemic Inflammation**

In recent years, special attention has been paid to 17α-E2due to its potential to reduce systemic inflammation, a contributing factor in the appearance and progression of age-related disease. Also, known as 'inflammaging', chronic inflammation is commonly observed in both sexes. Excess fat tissue is one major driver, and visceral fat, found in and around the abdominal cavity, a key depot that contributes to systemic inflammation [64-67]. Menopause promotes visceral fat accumulation, with the potential to promote the development of metabolic syndrome. That's why estrogen replacement has been shown

to reverse this negative trend by increasing fat distribution in the healthier subcutaneous depot, and improving metabolic health as a result [68-69].

Nuclear factor kappa B (NFκB) by binding to the estrogen receptor (mainly via ERβ) and by direct binding to estrogens such as estrogen itself (17β-estradiol) and/or metabolites such as estrone sulfate, estriol, and 2-hydroxy-estrogen. The binding of subunits of NFκB, c-Rel and RelA (14, 28, 30, 70) increased the expression of IκB (inhibitor of κB) that traps NFκB in the cell's cytoplasm and prevents its binding and activation of proinflammatory genes (29, 71-72). This is important, because NFκB is a major regulator of proinflammatory mediators, including TNFα and IL6, and estrogen can prevent these mediators from being turned on and produced during inflammation. Indeed, NFκB is one of the many anti-inflammatory pathways activated by estrogen.

Although research into the mechanisms by which 17α-E2 may affect inflammation is in its infancy, a few reports suggest a connection. Prior reports showed that 17α-E2 reduces circulating levels of pro-inflammatory cytokines: TNFα, MCP-1, IL6, IL1α, and IL1β in male mice [57]. In vitro studies also suggest that 17α-E2 may differentially affect proinflammatory pathways in a sex-specific manner. For instance, studies using mouse embryonic fibroblasts (MEFs) revealed that 17α-E2 significantly reduced TNFα and IL6 levels in male cells, whereas its effect in female cells were on IL6 alone [73].

In short, 17α-E2 has the potential to reduce systemic inflammation through pathways that include the reduction of adiposity, inhibition of pro-inflammatory cytokines, and the upregulation of antioxidant pathways. Nevertheless, the hypothesis that there is a coordinated effect of 17α-E2 on inflammation, particularly under conditions of aging, still requires experimental investigation. Once our understanding of these mechanistic pathways increases, it may be possible to develop new therapeutic interventions that modulate inflammation to combat age-associated diseases.

## The Benefits of 17α-Estradiol in Aging and Metabolic Health

17α-estradiol (17α-E2) is the non-feminizing estrogen that showed a dose-dependent effect on extending the lifespan of male mice [74-75]. When the 17α-E2 treatment was started at mid-life in male UM-HET3 mice, it extended median lifespan by 19 percent [74-75]. In contrast to males, this lifespan-extension effect was not observed in female mice [74-75]. The reasons underlying these sex-specific effects on lifespan remain unresolved, but may be related to differences in endogenous hormone production. Other potential contributors may be related to how 17α-E2 alters physiological processes in a sex-specific manner. For instance,17α-E2 modulates feeding behavior, reduces adiposity, regulates metabolism, decreases oxidative stress, and lowers circulating proinflammatory mediators more dominantly in male mice [43, 57-59, 76]. Importantly, the doses shown to extend lifespan also elicit very little effect on feminizing characteristics [57]. This suggests that 17α-E2 may have therapeutic potential for humans. Interestingly, 17α-E2 is a minor constituent of some HRTs, such as Premarin, yet this form of estrogen has remained largely an understudied [38]. The promise demonstrated by 17α-E2 to attenuate age-related conditions, particularly those of metabolic origin indicates further exploration is needed to unravel the mechanisms by which 17α-E2 elicits these benefits.

## Estrogens and their receptors modulate Metabolism

In aging, a metabolic imbalance often arises, leading to insulin resistance, glucose intolerance, and altered nutrient-sensing capabilities, which contribute to excess adiposity, and muscle loss [20]. These disturbances contribute to several disease states in old people [77]. Insulin resistance (IR) is one of the most important features of metabolic syndrome and a major risk factor for type 2 diabetes [78]. Obesity and metabolic disorders can be managed through increased exercise and better dietary habits, but these interventions are

can be poorly tolerated due to anemia, muscle weakness, and/or poor thermoregulation [79-80].

It is well-established that various estrogens and their receptors can modulate metabolic processes to include lipogenesis and insulin signaling. When estrogens decrease due to menopause, subcutaneous fat is often reduced in parallel with an increase in intraabdominal visceral fat [81-82]. Interestingly, ER polymorphisms that affect receptor function have also been associated with excess adiposity [83].ERα knockout (KO) mice also demonstrate increased weight gain, insulin resistance, and glucose intolerance in both sexes, but particularly females [84]. ERαKO female mice become morbidly obese, whereas male ERαKO mice develop adipose tissue fibrosis and inflammation [85], which clearly shows that estrogen actions through ERα are important for physiological processes in both sexes. Similarly, GPER1 knockout mice are mildly obese, glucose intolerant, and have elevated inflammatory cytokines [86-87]. OVX mice, who lack endogenous estrogens, also suffer from the same metabolic deficiencies, but these are corrected by hormone replacement. Administering estrogen also reverses glucose intolerance, but this could conceivably be due to declines in adiposity [26]. These findings point towards a role for estrogen receptor-based pathways in addressing metabolic pathologies.

17α-E2, like 17β-E2, beneficially modulates metabolism. Our prior studies have shown that 17α-E2 improves the metabolic profiles of diet-induced obese mice by reduced weight gain, glucose intolerance, and lowering levels of proinflammatory markers [57]. 17α-E2 also reduces fasting glucose, insulin, HbA1C, and circulating free fatty acids [57]. Although very few studies have directly compared the metabolic effects of 17α-E2 and 17β-E2 in parallel, we have begun studies to account for differences in binding for ERs between the two estrogens, which should allow for direct comparisons. We speculate that 17α-E2 may be working in the brain to modulate systemic metabolism.

**Estrogen and their receptors modulate hypothalamic control of Metabolism**

As people age, one of the contributors to metabolic dysfunction is an impaired nutrient-sensing capacity [64]. We have shown that 17α-E2modulates hypothalamic feeding circuits, which is at least partially mediated through POMC neurons. [76]. We have previously speculated that the metabolic benefits of 17α-E2 may occur through direct actions on peripheral tissues that control systemic metabolism, such as liver. However, we and others have begun exploring the potential for 17α-E2-mediated effects in the hypothalamus [76]. For instance, 17α-E2 has been shown to suppress food intake in male mice by modulating Pomc neuron activity, although it remains unclear is these results from direct actions in these neurons [57, 76]. The ARC is the brain area and Pomc-expressing neurons represent important appetite-regulating neurons that critical for satiety signaling [76]. This appears to be similar to how 17β-E2 and estrogen receptor modulation regulates hypothalamic feeding mechanisms [88]. For example, both 17β-E2 and drug ERα agonists were demonstrated to undo the dysregulation of feeding neurocircuitry in the ARC of ovariectomized (OVX) mice [88]. These results and the corresponding food intake reduction with 17α-E2 are consistent with the notion that this compound could act through hypothalamic ERs. Indeed, 17α-E2 induces ERα expression in the hypothalamus, despite not appearing to increase ERα expression in other peripheral tissues [57, 76]. These observations suggest that 17α-E2 may predominantly elicit benefits through actions in the hypothalamus Similar to 17β-E2, we speculate that 17α-E2 binds to anorexigenic hypothalamic neurons that are modulated in response to peripheral endocrine factors such as insulin and leptin [57, 76]. In support of this, 17α-E2 increases the expression of leptin receptors (LEPR), melanocortin-4 receptors (MC4R), and Pomc in the hypothalamus Therefore, 17α-E2 likely exerts its benefits through actions in the hypothalamus.

## Overview

Aging is the primary risk factor for many chronic diseases, such as metabolic decline, chronic inflammation, and declines in muscle function. Interventions such as 17α-Estradiol (17α-E2) seem promisingly beneficial, but we don't know exactly how it acts. 17α-E2 likely acts through ERs in a cell-type-specific manner to elicit benefits, but more work is needed to clarify its effects. My studies reveal that 17α-E2 not only elicits benefits in mice, but also rats and common marmosets, which suggests its mechanisms of action are conserved across species.

## HYPOTHESIS AND AIMS

17α-E2 is a “non-feminizing” form of estrogen and several studies have shown that it elicits benefits in the context of aging and obesity. However, whether 17α-E2 metabolic benefits in male mice could be translated to other models such as rat and common marmosets are not yet fully known. Unraveling the effects of 17α-E2 in across the species will help us to move forward with 17α-E2 as a treatment option for aging and metabolic diseases. I hypothesized that 17α-E2 elicits its benefits in heterogeneous rat models, and potentially it can be used safely and effectively in male common marmosets. To prove this hypothesis, the following aims were tested:

1. Assess the metabolic benefits of 17α-E2 in a heterogeneous rat model (Chapter II)
2. Determine if male common marmosets tolerate 17α-E2 treatment (Chapter III)

# CHAPTER II

## A GENETICALLY HETEROGENEOUS RAT MODEL WITH DIVERGENT MITOCHONDRIAL GENOMES

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*All data in this chapter were generated by R.S., except for phenotypic characterization and mitochondrial function. [89]

## ABSTRACT

We generated a genetically heterogenous rat model by a 4-way cross strategy using 4 inbred strains (Brown Norway [BN], Fischer 344 [F344], Lewis [LEW], and Wistar Kyoto [KY]) to provide investigators with a highly genetically diverse rat model from commercially available inbred rats. We made reciprocal crosses between males and females from the 2 F1 hybrids to generate genetically heterogeneous rats with mitochondrial genomes from either the BN (OKC-HETB, a.k.a "B" genotype) or WKY (OKC-HETW a.k.a "W" genotype) parental strains. These two mitochondrial genomes differ at 94 nucleotides, more akin to human mitochondrial genome diversity than that available in classical laboratory mouse strains. Body weights of the B and W genotypes were similar. However, mitochondrial genotype antagonistically affected grip strength and treadmill endurance in females only. In addition, mitochondrial genotype significantly affected multiple responses to a high-fat diet (HFD) and treatment with 17α-estradiol. Contrary to findings in mice in which males only are affected by 17α-estradiol supplementation, female rats fed HFD beneficially responded to 17α-estradiol treatment as evidenced by declines in body mass, adiposity, and liver mass. Male rats, by contrast, differed in a mitochondrial genotype-specific manner, with only B males responding to 17α-estradiol treatment. Mitochondrial genotype and sex differences were also observed in

features of brain-specific antioxidant response to HFD and 17α-estradiol as shown by hippocampal levels of Sod2 acetylation, JNK, and FoxO3a. These results emphasize the importance of mitochondrial genotype in assessing responses to putative interventions in aging processes.

Keywords: Aging, Estrogen, Genetic diversity, Mitochondria, Physical function, Sex differences.

## INTRODUCTION

Largely because of its genetic tractability and short lifespan, the mouse has become the mainstay of mammalian biomedical research focused on elucidating mechanisms of aging and identifying putative anti-aging interventions. Because the translatability of effective pharmacological interventions from mice to humans has been weak (e.g., cancer [90], Alzheimer's disease [91], and amyotrophic lateral sclerosis [92]). It is critical to assess the generality of mouse findings with respect to disease-modifying interventions. One way to assess generality is to employ a second, experimentally tractable species to confirm or dispute the initial result, especially if the second species displays greater similarity to humans with a specific disease phenotype. Previously, we argued that the laboratory rat would be a useful animal model to test the translatability of observations in mice to humans [93]. Rats diverged from a common ancestor at about the same time that humans diverged from African monkeys and differ from mice in multiple physiological parameters important in aging (e.g., insulin sensitivity, muscle and bone biology, cognitive capacity, end-of-life pathology, and sex differences in longevity). In each of these cases, rats are more similar to humans than mice. Also, despite their larger size, which is advantageous for a variety of reasons, rats generally do not live longer than mice.

Because inbred rodents are genetically homozygous at every locus, they show strain-specific idiosyncrasies, some of which are known, others of which may not be. For

example, it is well- established that multiple inbred mouse genotypes have lost the ability to synthesize melatonin in their pineal glands [94]. Some strains also show idiosyncrasies in the primary pathologies of aging or cause of death [95]. A major improvement in mouse models to study aging was the development of the UM-HET3 model, which is a controlled, genetically heterogeneous stock in which each mouse is genetically unique but the population can be replicated at will [96].

We describe below the development of a similar genetically heterogenous rat model (OKC- $HET^{B/W}$) that allows investigators to test the translatability of data from mice to another species or to compare the translatability of results from a second rodent species to humans. Our rat model is generated using a four-way cross strategy, which was initially used by Roderick [97] in producing a population of genetically heterogenous mice to identify mouse strains that show different sensitivities to X-irradiation. Miller and colleagues used a similar strategy to develop UM-HET3 mice, described above, which are now widely used in aging research, including the NIA Intervention Testing Program [98]. The advantage of this four-way cross is that anyone can generate a similar population by obtaining the parental strains from commercial vendors. Although the UM-HET3 mice are genetically heterogenous with respect to nuclear encoded alleles, the breeding scheme was designed in a manner that leads to all pups having the same BALB/c mitochondrial genome.

It is important to note that the interaction between mitochondrial and nuclear genomes is an underappreciated intracellular dynamic. Mitochondrial function depends on more than 1200 proteins synthesized in the nucleus, but only thirteen produced from the mitochondria genome. These thirteen however are important in metabolism as they form critical components of four of the five electron transport chain complexes. Thus, coordination of transcription, translation, and intracellular transport of mito-nuclear proteins

must be tightly regulated under a range of cellular conditions that can alter the dynamics of mitochondrial biogenesis or mitophagy [99]. Illustratively, two mouse mitochondria that differ at only a handful of nucleotides can alter nuclear gene expression, bioenergetic and organismal metabolic efficiency, as well as cardiac function, and adiposity when paired with the same nuclear genome [100-101]. Another unique feature of mitochondrial nuclear dynamics is that because mitochondria are transmitted intergenerationally only through the female lineage, selection will be stronger for optimizing female mito-nuclear coordination relative to males, in which any male-specific deleterious mutations will have minimal evolutionary consequence. This sex-specific mito-nuclear inheritance dynamic has been adduced to explain sex differences in longevity and later life diseases [102] and in *Drosophila* has been shown to affect sex-specific aging phenotypes [103].

The OKC-HET$^{B/W}$ rat model we describe below was generated by crossing four commercially available inbred strains deliberately selected to maximize genetic diversity [104-105]. Traditional rat strains, because they represent multiple, disparate domestication events, are genetically far more diverse than traditional mouse strains used in research [93]. Specifically, we used Brown Norway (BN), Fischer 344 (F344), Lewis (LEW), and Wistar Kyoto (WKY) inbred rats to produce two F1 lines [(BN/F344) F1 and (WKY/LEW) F1]. By performing reciprocal crosses between males and females from these two F1 hybrids, two F2 stocks (OKC-HET$^{B}$ and OKC-HET$^{W}$) are created with all the advantages of typical four-way cross populations but also with half of the progeny having the BN (henceforth “B”) mitochondrial genotype and the other half the WKY (henceforth “W”) mitochondrial genotype. These mitochondrial genomes, again because of the domestication of rats from multiple wild populations, are far more diverse than mitochondrial genomes from traditional inbred mouse strains, which have a single mitochondrial ancestor and thus differ at only a handful of nucleotides [106]. Below, we

describe an initial characterization of the OKC-HET$^{B/W}$ rats. In addition to assessing their baseline phenotypes, we also challenged them metabolically with a high-fat diet (HFD) to investigate whether such metabolic stress might result in different responses depending on mitochondrial genotype. We also investigated the generality of sex differences previously observed in mice by supplementing the rats' diet with 17α-estradiol (17α-E2), an intervention demonstrated to have beneficial effects almost exclusively in male mice [57-58, 61-63, 74-76,107-110].

## METHODS

### ***Animals and Diets***

Brown Norway (BN), Fischer 344 (F344), Lewis (LEW) and Wistar Kyoto (WKY) rats were obtained from Charles River at approximately 2 months of age. BN females were bred to F344 males to produce BN/F344F1 hybrids, and WKY females were bred to LEW males to produce WKY/LEWF1 hybrids. The female BN/F344F1's was crossed with male WKY/LEWF1's to generate the OKC-HET$^{B}$ stock, and male BN/F344F1's was crossed with female WKY/LEWF1's rats to generate the OKC-HET$^{W}$ stock. We found that litter size differed between the two stocks: WKY/LEWF1 females produced a mean litter size of 17 pups (range: 14-20), whereas BN/F344F1 females produced a mean litter size of 11 pups (range: 9 to 12). The rats were bred and maintained in the animal facilities at the Oklahoma City VA Medical Center and fed *ad libitum* a commercial rodent chow diet (Picolab Rodent Diet 5053, LabDiet, St. Louis, MO). Animals were group housed with the number per cage dependent on the size of the rats. Once the rats reached 15- months of age males were housed 1 or 2 per cage and the females were housed 2 to 3 per cage. Their environment was enriched with nesting materials, plastic lodges, and wooden chew sticks. In the high-fat diet experiments, control rats were fed normal chow diet (58YP: 66.6% carbohydrates,

20.4% protein and 13% fat; TestDiet, IN) or a high-fat diet (58V8: 35.5% carbohydrate, 18.3% protein, 45.7% fat; TestDiet, IN) with, or without, 14.4ppm 17α-estradiol (Steraloids, Newport, RI). All experiments were performed in accordance with the National Institutes of Health's guidelines and approved by the Institutional Animal Care and Use Committee at the Oklahoma City VA Medical Center.

***Sequencing of mitochondrial genomes***

DNA isolation:

Total DNA was extracted from frozen quadriceps muscles using proteinase K digestion with SDS and EDTA, phenol/chloroform extraction, and ammonium acetate/ethanol precipitation as previously described [111].

Cas9 cleavage, library preparation, and nanopore sequencing:

We used a tiling approach for nanopore re-sequencing of rat mtDNA. Custom guide RNA sequences targeting rat mitochondrial DNA (mtDNA) are listed in the table below and purchased from IDT-DNA.

| Probe | Location of 5-prime end[a] | crRNA sequence | Sense |
|---|---|---|---|
| Probe-1 | 469 | ACGATAGCTAAGACCCAAAC | Positive |
| Probe-2 | 4444 | TATTCATCAATTGCCCACAT | Positive |
| Probe-3 | 8917 | GTTCCTACCCACGACCTAGG | Positive |
| Probe-4 | 14370 | ATCCGATACCTACACGCCAA | Positive |

[a] Based-on Brown-Norway mitochondrial DNA GenBank reference sequence AY172581.1

Pre-complexed Alt-R CRISPR-Cas9 single guide RNA (sgRNA) (IDT) was diluted to a concentration of 10 μM and combined with HiFi Cas9 Nuclease V3 (IDT, cat 1081060) in CutSmart Buffer (NEB, cat B7204). Cas9 cleavage, library preparation, and nanopore sequencing was performed in accordance with previously described methods [112]. Prepared libraries were run on a MinION flow cell (v9.4.1) using the MinION Mk1C sequencer. Sequencing and base-calling were done using the MinKNOW software

(v21.10.8).

Sequence analysis:

The analysis was done using python version 3.6.15 and R version 4.0.2. The reference mitochondrial genomes for the Brown-Norway strain was AY172581.1, and the WKY strain was DQ673907.1. Base calling was performed using MinKNOW (v4.5.4, release 4/20/21), and reads meeting a QC threshold of 5 were included in analysis. All fastqs passing quality control were aligned to the rotated mitochondrial genome using Minimap2, version 2.24. All nanopore sequencing data are available at BioProject PRJNA899912.

PCR-Restriction Length Polymorphism to genotype BN and WKY DNA samples:

To verify the mitochondrial DNA genotype in rat samples, we developed a PCR-restriction length polymorphism screen. A 614-bp portion of the rat mtDNA minor arc from 4902 bp to 5515 bp flanking a polymorphism at position 5269 (BN G to WKY C numbering according to BN rat reference mtDNA AY172581.1) was PCR amplified using forward primer AAGTACCCTTACCCTACCGC (bp 4902-4922) and reverse primer sequence TTACGAATGCATGGGCTGTGA (bp 5515-5494; IDT DNA). Restriction enzyme digestion of the PCR amplicon was used to discriminate between the BN and WKY rat strains as shown in **Figure 6: Supplementary Figure 1**. Five µL of each PCR reaction was prepared in three separate restriction digestion reactions with no restriction enzyme, BanII (Cat no.: R0119S to cut the BN sequence), or HaeIII (Cat no.: R0108S to cut the WKY sequence).

***Grip Strength and Treadmill Performance***

Rats from the Oklahoma VA Medical Center were sent to the University of Alabama at Birmingham at 2- to 3-months of age for performance-related outcomes. After habituation to their new environments, grip strength and treadmill performance was measured. Animals were also habituated and trained prior to the treadmill performance task using a Five Lane Touchscreen Convertible Treadmill (Panlab, Holliston, PA). On the first day of

habituation, the rats were placed on the treadmill for 10 minutes. On the second through fifth day, the treadmill was set to 8 cm/sec and then increased by 1 cm/sec until reaching 12 cm/sec (5 minutes) to train them to run a moderate speed. On the sixth day, the rats were placed on the treadmill apparatus at 12 cm/sec for 2 minutes, after which the speed increased by 2 cm/sec until 16 cm/sec max speed was achieved and thereafter, animals were allowed to run at this speed. When rats stopped moving, they were prodded with a stylus 3 times. If they did not respond by the third prodding, they were considered to have reached their endurance limit.

Grip strength was tested using a Chatillon Grip Strength apparatus (Columbus Instruments; Columbus, OH). Briefly, rats were held by the tail and allowed to place their forelimbs on the mesh grid. Once they gripped the mesh, they were firmly pulled away from the machine until their grip was broken. This was repeated for a total of three trials. Maximum grip strength measurement was then corrected for body weight. The same experimenter performed all grip strength testing for all rats and was blinded with respect to the mitochondrial genotype.

***Assays of Mitochondrial Function***

Preparation of skeletal muscle fiber permeabilization was performed as described previously with minor modifications [113]. A small piece (~3-5 mg) of red gastrocnemius muscle was dissected, and the separated fibers placed in ice-cold buffer X, containing (in mM): 7.23 $K_2$EGTA, 2.77 Ca$K_2$EGTA, 20 imidazole, 0.5 DTT, 20 taurine, 5.7 ATP, 14.3 PCr, 6.56 $MgCl_2$-$6H_2O$, 50 K-MES (pH 7.1). The muscle bundle was permeabilized in saponin-containing solution (30 ug/mL) for 30 minutes, followed by three 5-minute washes in ice-cold wash buffer Z containing (in mM): 105 K-MES, 30 KCl, 10 $K_2HPO_4$, 5 $MgCl_2$-$6H_2O$, 0.5 mg/ml bovine serum albumin, 0.1 EGTA (pH 7.1). Rates of mitochondrial respiration were determined using the Oxygraph-2k (O2k, OROBOROS Instruments,

Innsbruck, Austria). We sequentially added substrates and inhibitors to measure complex-specific respirations as follows: glutamate (10 mM), malate (2 mM), ADP (5 mM), succinate (10 mM), rotenone (1 μM), antimycin A (1 μM), and TMPD (0.5 mM) immediately followed by ascorbate (5 mM). All respiration measurements were normalized to antimycin A to account for non-mitochondrial oxygen consumption. Data for OCR were normalized by milligrams of muscle bundle wet weight.

***Western Blot Assays***

Brains were rapidly extracted from the skull following decapitation, hippocampi dissected out, and the tissue flash frozen in liquid nitrogen. Lysates from hippocampi were prepared in RIPA buffer containing protease inhibitors (Roche cOmplete™ Protease Inhibitor Cocktail) and protein concentration determined using the Pierce™ BCA assay (Thermo Fisher). Equal amounts of protein (10 μg/lane) were subjected to SDS/PAGE and subsequently transferred to a nitrocellulose membrane. The membrane was blocked in a solution containing 5% bovine serine albumin in TBST (20 mM Tris, 0.9% NaCl, 0.1% Tween-20, pH 7.4) and probed with primary antibodies for Sod2 (Abcam, Waltham, MA, cat# ab13533, 1:5000), Sod2-acetyl-K68 (Abcam, cat# ab137037, 1:5000), Sod1 (Abcam, cat# ab51254, 1:1000), GAPDH (Cell Signaling, cat# 97166, 1:2000), JNK (Cell Signaling, cat# 9252, 1:1000), p-JNK (Cell Signaling, cat# 4668, 1:1000), FoxO3a (Cell Signaling, cat# 2497, 1:1000), and p-FoxO3a (Cell Signaling, cat# 9466, 1:1000). Following secondary incubation with IRDye donkey anti-mouse and donkey anti-rabbit antibodies (LI-COR Biosciences, Lincoln, NE, 926-68072 and 926-32213, 1:5000), images were captured using an Odyssey CLX imaging system (LI-COR Biosciences, Lincoln, NE). Band densitometry was quantified using ImageStudio software (LI-COR Biosciences, Lincoln, NE) with all signals normalized to GAPDH as a loading control.

## RESULTS

### Generation of the OKC-HET$^{B/W}$ rats and mitochondrial DNA resequencing of both genotypes

To produce rats with the greatest genetic heterogeneity, we selected four commercially available, but phylogenetically diverse, inbred strains of rats as shown in **Figure 1A**: BN, F344, LEW, and WKY. Using a four-way cross of these strains, we generated two F2 populations of rats that have equal numbers of alleles from each of the four inbred strains but have mitochondrial genomes from either BN or WKY strains. We refer to these as OKC-HET$^{B}$ (abbreviated "B" genotype) and OKC-HET$^{W}$ (abbreviated "W" genotype) rats. The BN rat was the third complete mammalian genome, including the mitochondrial genome, to be sequenced after the human and mouse genomes [114] that we used as a reference sequence (GenBank Accession: AY172581.1). Similarly, the WKY rat mtDNA has been sequenced (GenBank Accession: DQ673907.1) [115]. Using nanopore Cas9-targeted sequencing [112], we resequenced the mtDNA from our founder female BN and WKY animals as well as the female F1 hybrids. With greater than 100-fold coverage across the BN and WKY mtDNA, we found no differences in the BN mtDNA sequence from the AY172581.1 BN reference genome and two bases that differed from the DQ673907.1 WKY mtDNA reference genome: one at position 11339 (A to C synonymous mutation in ND4) and one at 15351 (C to A in tRNA-Pro). Comparing our updated WKY and BN mtDNA sequences, we identified 94 nucleotide differences between the two mitochondrial genomes with 88 base substitutions and 6 base insertions/deletions (**Figure 1B**). These substitutions were distributed throughout 7 of the 13 protein coding genes, five tRNAs, both rDNA subunits, and the D-loop. Of these substitutions, 16 are nonsynonymous substitutions in the genes for ND2, COI, ATPase8, ATPase6, ND4, ND6, and Cytb.

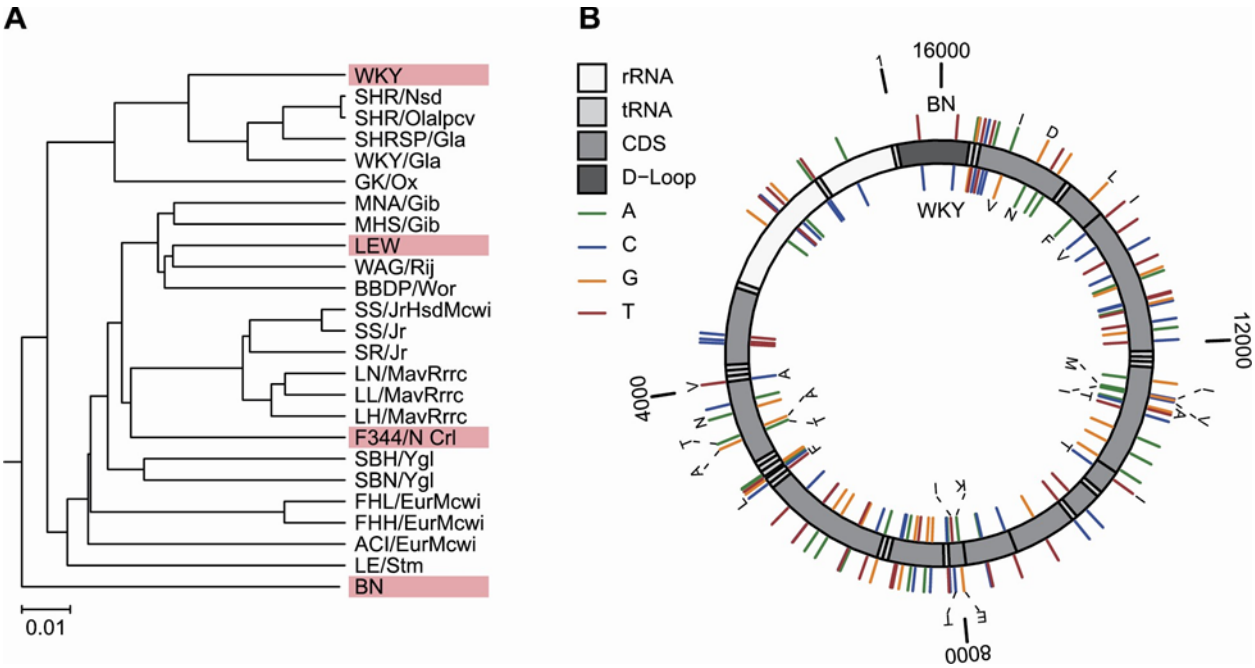


**Figure 1.** (**A) Phylogenetic tree of domesticated rats from Atanur et al. (16) for 28 inbred strains using 9.6 million single-nucleotide variants (SNVs).** The phylogenetic distribution of the 4 inbred lines of rats used to generate the OKC- HETB/W rat are shown in red outline. **(B) Nucleotide and amino acid differences between BN and WKY mitochondrial DNA**. The rat mitochondrial genome is denoted by the gray scale circular diagram. The 88 nucleotide differences between the two genomes are denoted in the color of the corresponding divergent nucleotide. Nonsynonymous amino acid changes are denoted using the single letter amino acid code. Differences in the BN genome is on the outer track and differences in the WKY genome on the inner track. Numbering is according to the BN reference genome (AY172581.1).

**General phenotypic characterization**

OKC-HET$^{B/W}$ rats come in three coat colors (**Figure 2A)**. Approximately 50% of the OKC- HET$^{B/W}$ rats are white albino, as are the F344, LEW, and WKY parental strains. The remaining 50% are equally divided between charcoal gray, similar to the BN parental strain, or a combination of white and charcoal gray. We observed no significant differences among the two mitochondrial genotypes in the body weights of either sex up to 20 months of age (**Figure 2B**). At 20 months of age, the average size of males was ~550 ± ~60(SD) grams compared to ~310 ± 40(SD) grams for females. Overall, the animals appeared normal with no major observable pathology up to 24 months of age at which point approximately 85% of animals were still alive. The mean lifespan of F344, BN, and F344BNF1 rats in the NIA Biomarkers colony was 24-29, 29-30, and 31-32 (male-female) months, respectively, with females being the longer-lived sex in all 3 genotypes [116].

Our small number of observations indicated antagonistic differences in grip strength and treadmill performance between females of the two mitochondrial genotypes at 4-months of age (**Figure 2 C and D**). Specifically, females with the B mitochondrial genotype showed significantly greater grip strength, whereas W females displayed greater treadmill performance. We observed no significant differences in grip strength or treadmill performance among males.

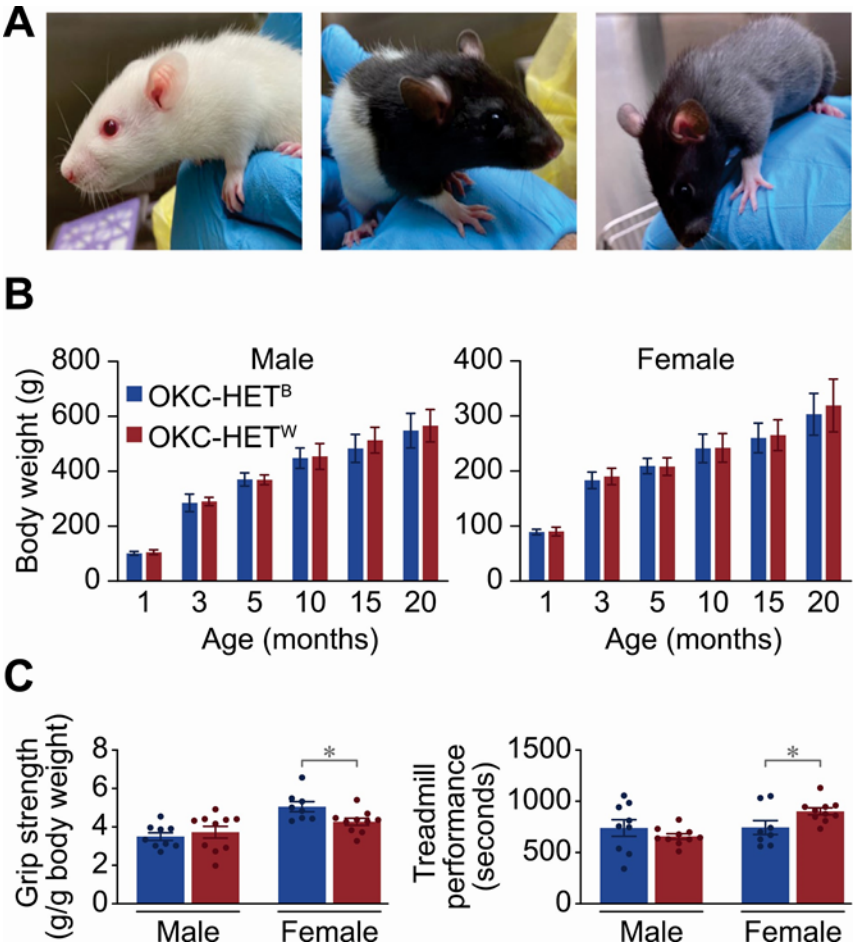


**Figure 2. Characterization of OKC-HET$^{B}$ and OKC-HET$^{W}$ rats. A: Coat Color** for OKC HET$^{B/W}$ rats. **B: Body weights** for male and female B (blue bars) and W (red bars) genotypes. Data are the mean ± SD for 40 to 50 rats per group. **C: Grip strength** (g/g body weight). **D: Treadmill performance** (seconds) for 4-month-old male and female rats as described in the Methods. The mean ± SEM are given for 8-10 rats per group, and the data were analyzed by Two-Way ANOVA with Tukey posthoc comparisons. * $p < 0.05$

**Mitochondrial function**

We measured mitochondrial function under basal conditions and under metabolically challenging high-fat diet (HFD) conditions. Specifically, under both these conditions we assessed mitochondrial respiration in permeabilized fibers from the gastrocnemius of 13- to 14-month-old animals of both sexes (**Figure 3A-D**). To establish adequate power for assessment of whether respiration differed as a function of mitochondrial genotype, we combined sexes for the analysis. Sex-specific effects were largely equivalent (**Figure 7: Supplement Figure 2**). Leak respiration, which occurs when electrons passed down the respiratory chain exit prior to the reduction of oxygen to water at cytochrome C oxidase, had little response to the HFD in the W mitochondrial genotype, but was robustly higher for the B mitochondrial genotype (**Figure 3A**). These relative responses persisted for Complexes I, I&II, and IV. In general, it appears that the B genotype had compensatory increases in respiration to the HFD, whereas the W genotype did not. Further, it appeared that the W genotype may have had some decline in respiratory rate to HFD as observed at Complex I (**Figure 3B**).

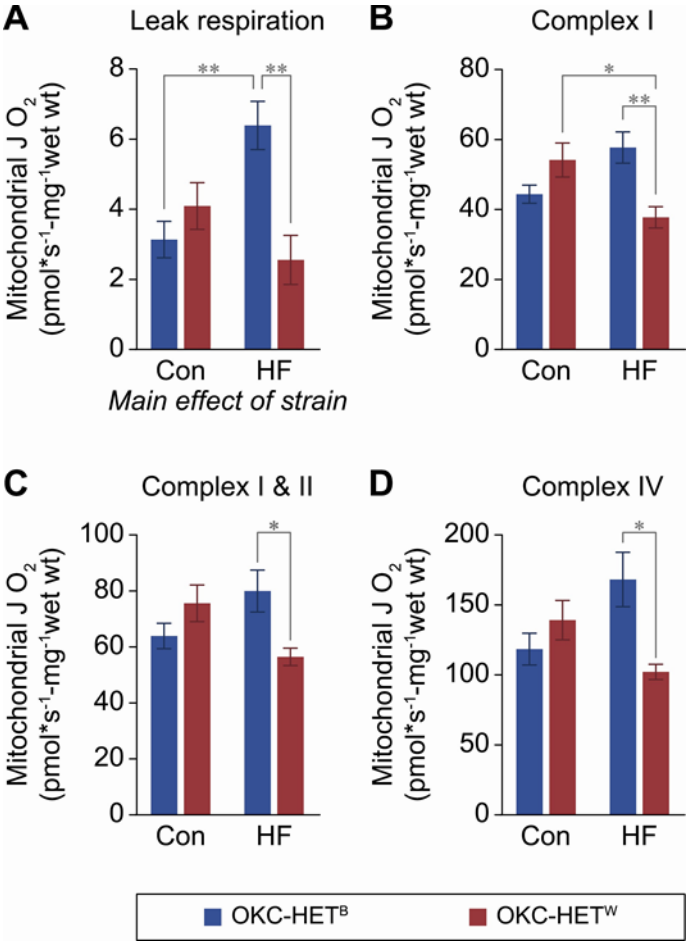


**Figure 3. Mitochondrial bioenergetics in the gastrocnemius from OKC- HET$^{B}$ and OKC-HET$^{W}$ rats.** Permeabilized fibers from red gastrocnemius of 13- to 14-month-old OKC-HET$^{B}$ (blue bars) and OKC-HET$^{W}$ (red bars) rats fed a normal chow (control) or high-fat diet (HFD) (45.7%) for 12 weeks were used to measure mitochondrial oxygen consumption rate. (A) Mitochondrial Leak respiration was measured after adding Complex I substrates in absence of ADP. Complex I (B), Complex I & II (C), and Complex IV (D) activities were measured by ADP-stimulated mitochondrial oxygen consumption rates with sequential additions of substrates and inhibitors. The mean ± SEM are given for 7–9 rats per group. Data were analyzed by two-way analysis of variance with Tukey posthoc comparisons. *$p < 0.05$.

**Response to high-fat feeding and 17α-estradiol supplementation**

The NIA Intervention Testing Program found that long-term administration of 17α-estradiol (17α-E2) extended median lifespan of male, but not female, mice in a dose-dependent manner (108, 165). Our group has been exploring potential mechanisms by which 17α-E2 may improve healthspan and extend lifespan in male mice specifically. We reported that 17α-E2 supplementation reduces calorie intake, body mass, and regional adiposity in combination with significant improvements in a multitude of systemic metabolic parameters in both middle-aged obese and old male mice without inducing deleterious effects (57-58, 63, 76, 109-110, 117). Given that 17α-E2 elicits sex-specific effects in mice, which could be at least partially mediated through altered mitochondrial activity, we sought to determine if 17α-E2 would elicit similar sex-specific effects in OKC-HET$^{B/W}$ rats. Interestingly, 17α-E2 prevented HFD-induced increases in body mass and organ mass [e.g., liver and white adipose tissue (WAT)] in a mitochondrial genotype- and sex- specific manner. In males, 17α-E2 prevented weight gains in body, liver, gonadal WAT, and inguinal WAT mass in the B mitochondrial genotype only (**Figure 4A and C**). This was an unexpected finding because prior work with male mice showed that 17α-E2 treatment elicited benefits to health parameters in both UM-HET3 as well as inbred (C57BL/6J or C57BL/6N) mice, suggesting a conserved species pattern. We observed similar effects of 17α-E2 on body mass of 13- to 14-month old OKC-HET$^{B/W}$ rats fed HFD ± 17α-E2 for 12 weeks (**Figure 8: Supplementary Figure 3**).

Female OKC-HET$^{B/W}$ rats receiving 17α-E2 also displayed unexpected results. Both mitochondrial genotypes beneficially responded to 17α-E2 treatment as evidenced by a prevention of HFD-induced gains in body, liver, gonadal WAT, and inguinal WAT mass (**Figure 4B and D**). These observations are contrary to previous studies in mice, in which females are generally unresponsive to 17α-E2 treatment unless ovariectomized [59, 62].

These observations suggest that unlike in mice, 17α-E2 may have significant biological activity in female rats.

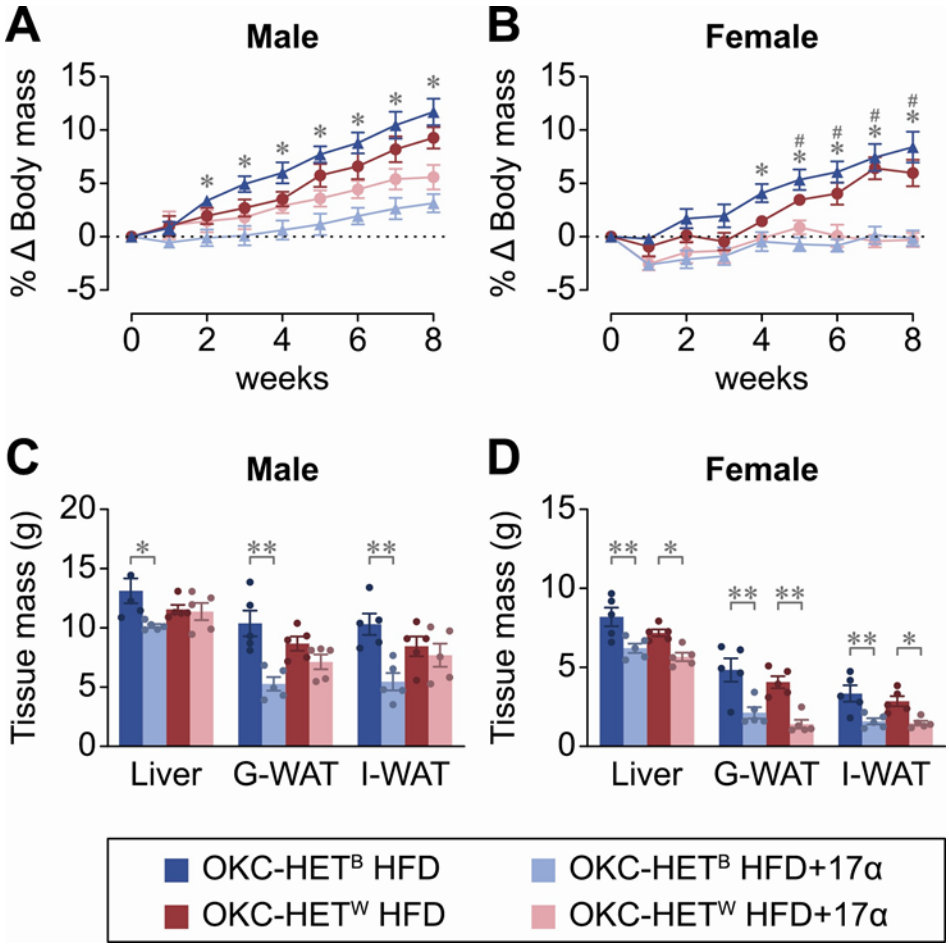


**Figure 4.** Young OKC-HET$^{B}$ and OKC-HET$^{W}$ rats respond differently to high- fat feeding and 17*α*-E2 treatment. Male and female B (blue) and W (red) genotypes at 4 months of age received a 45.7% high-fat diet (HFD) ± 17*α*-E2 (14.4 ppm) over an 8-week intervention. (A, B) Longitudinal percent change in body mass in male and female rats. The data are the mean ± SEM for 5 animals per group and were analyzed by 2-way repeated measures analysis of variance (ANOVA) with Tukey post-hoc comparisons. *$p$ < .05 between OKC-HET$^{B}$ HFD and OKC-HET$^{B}$ HFD+17*α*-E2; #$p$ < .05 between OKC-HET$^{W}$HFD and OKC-HET$^{W}$ HFD+17*α*-E2. (C, D) Liver, gonadal (G-WAT), and inguinal (I-WAT) white adipose tissue masses at necropsy. The data are mean ± SEM for 5 animals per group and were analyzed by 1-way ANOVA with Tukey posthoc comparisons within individual tissues and sexes. *$p$ < 0.05, **$p$ < 0.01.

17α-E2 has also been shown to have neuroprotective effects by mitigating the toxic effects of oxidative stress in humans and ovariectomized rats [118]. Therefore, we investigated whether markers of oxidative stress and response to HFD and 17α-E2 are impacted in our rat model by mtDNA genotype. We previously reported that mitochondrial function and antioxidant expression was altered in a mouse model of brain aging with reduced circulating levels of IGF-1 [119]. Additionally, a decline in levels of both Cu/Zn- and Mn-superoxide dismutase (Sod1 and 2, respectively) was associated with impaired hippocampal-dependent spatial learning [120-121]. We therefore measured the levels of Sod1, Sod2, and acetylated Sod2, in the hippocampus of W and B genotypes under normal and HFD feeding both with and without 17α-E2 supplementation. Acetylated Sod2 has been shown to lack its normal dismutation activity but a gain in peroxidase activity [122].

Brain weights (normalized to body weight) were comparable between mitochondrial genotypes within a sex, albeit female percent brain weights were higher than males (**Figure 5A**). No significant difference was found between the genotypes with respect to the levels of Sod1 (**Figure 5D**) or Sod2 (**Figure 5C**) in either sex. However, we did observe that levels of acetylated Sod2 (K68) were significantly lower in male W genotypes compared to B genotypes **(Figure 5B).** Conversely, when fed HFD, acetylated Sod2 levels were significantly increased in male W, but not B, genotypes. Acetylated Sod2 levels trended to be lower in female W, but not B genotype fed HFD with and without 17α-E2.

We also measured the levels of upstream regulators of the antioxidant pathway, Jun N-terminal kinase (JNK) and FoxO3a. It is well established that oxidative stress can activate the JNK pathway [123], and FoxO transcription factors, which are activated by the JNK pathway, regulate the transcription of genes coding for antioxidant proteins [124]. Levels of JNK were significantly lower in W genotype male rats fed the HFD with or without 17α-E2, while JNK levels were significantly elevated in the OKC-HET$^{B}$ male rats fed the HFD with

17α-E2 only (**Figure 5E**). No significant differences were found in JNK levels in the two genotypes in female rats. FoxO3a levels were comparable in the hippocampus of the two mitochondrial genotypes for male rats and feeding HFD with or without 17αE2 had no detectable effect on FoxO3a levels (**Figure 5F**). However, female FoxO3a levels were significantly reduced in the W genotype when fed HFD with 17α- E2 compared to rats fed HFD alone. These data suggest that adaptive changes in cellular response to stressful stimulus such as HFD, and responsiveness to 17α-E2 treatment, may be mediated through mtDNA variants in a sex-specific manner, potentially contributing to cognitive health.

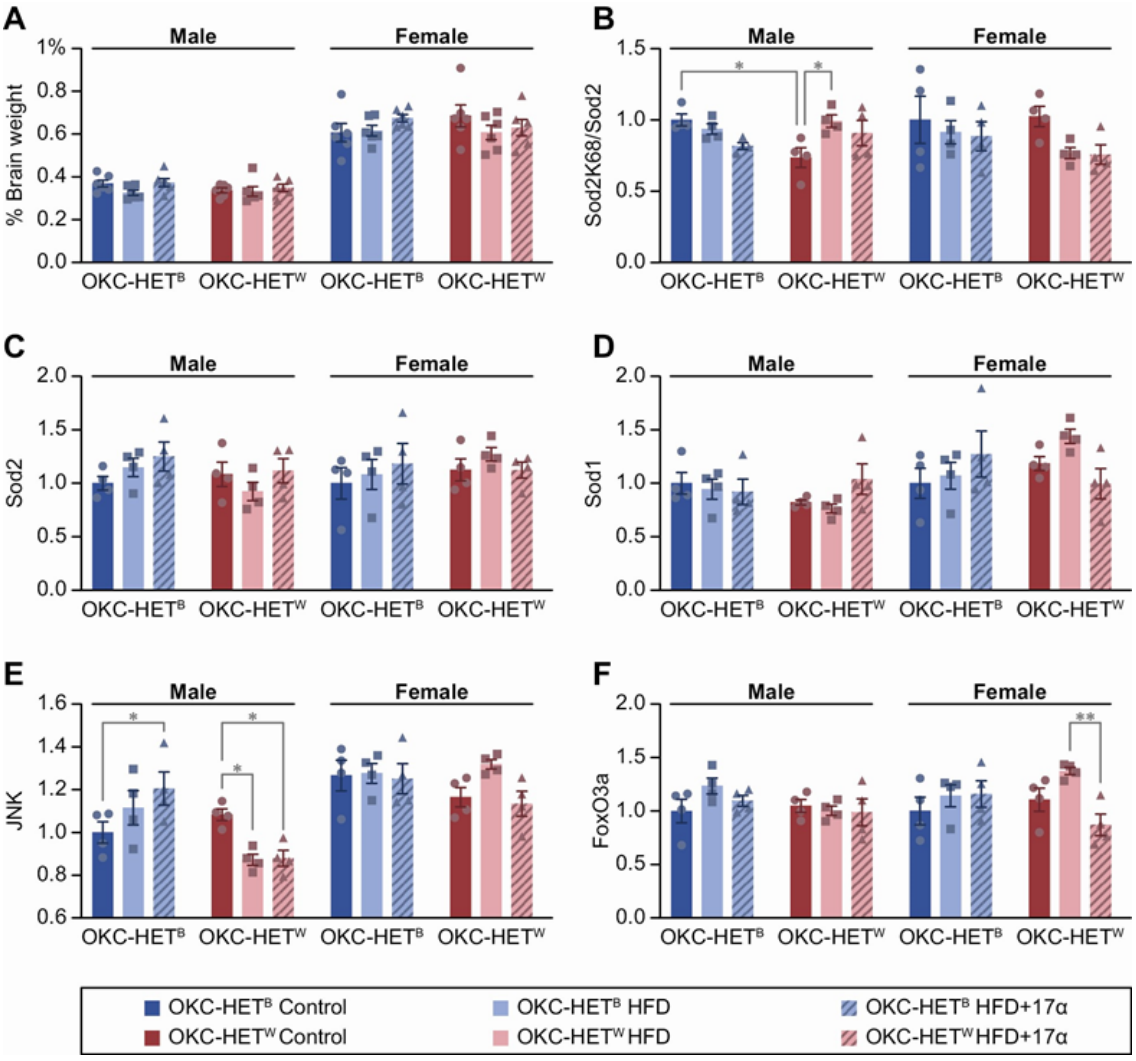

**Figure 5. Expression of antioxidant and oxidative stress pathway proteins in hippocampus of OKC-HET$^{B}$ and OKC-HET$^{W}$ rats.** (A) Percent brain weight (normalized to body weight). (B) Levels of acetylated SOD2(K68), (C) Sod2, (D) Sod1, (E) JNK, (F) FoxO3a from hippocampi of 13–14-month-old male and female rats fed either a control diet (dark blue or dark red bars), a 45.7% high- fat diet (HFD) (light blue or light red bars), or HFD + 17*α*-E2 (stripped blue or red bars) for 12 weeks, and levels were determined by Western blots, which are shown in **Figure 9: Supplementary Figure 4**. The data are the mean ± SEM for 4 animals and were analyzed using a 2-way analysis of variance with Tukey posthoc comparisons. *$p < 0.05$.

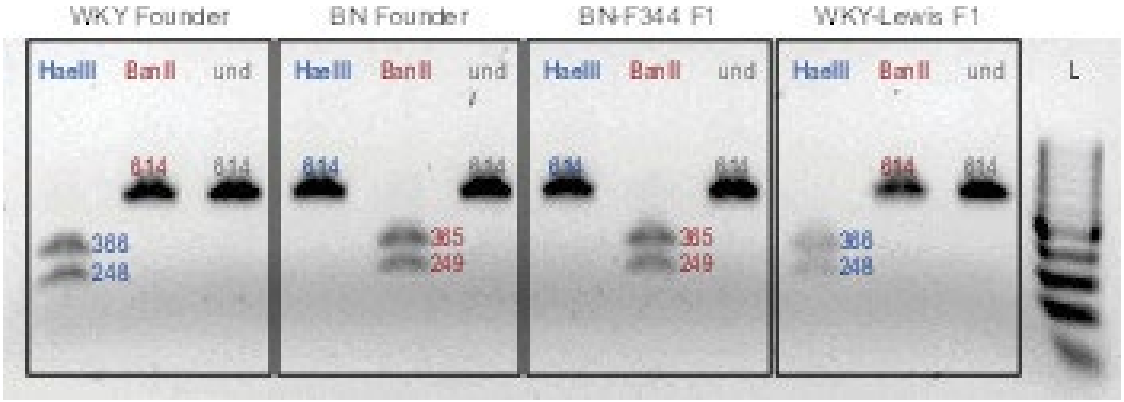


**Figure 6: Supplementary Figure 1. PCR-RFLP analysis of two female founder rats and two female F1 hybrids.** The PCR amplicon flanking a known single nucleotide polymorphism from each DNA sample was digested with HaeIII, BanII, or left undigested. HaeIII only digests the WKY amplicon, and BanII only digests the BN amplicon. L denotes the DNA ladder.

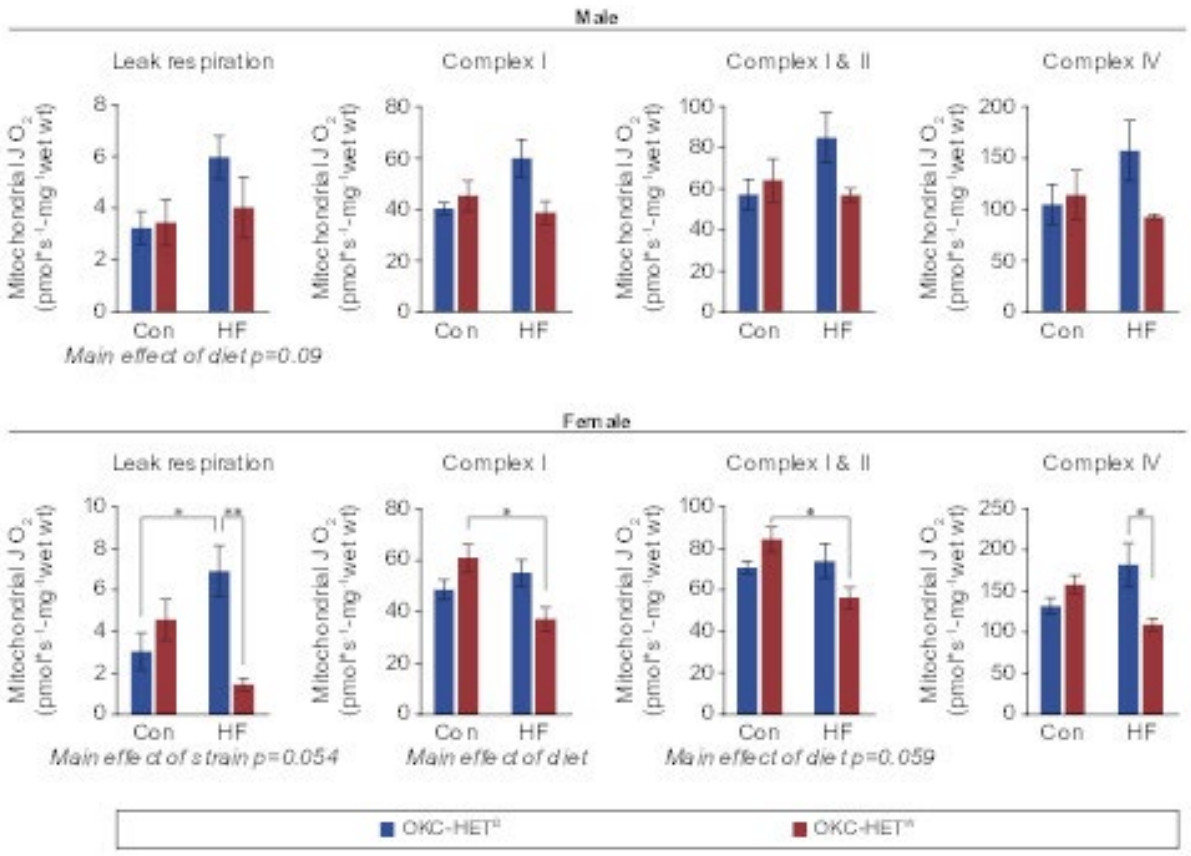


**Figure 7: Supplementary Figure 2. Mitochondrial bioenergetics in the gastrocnemius from OKC-HET$^{B}$ and OKC-HET$^{W}$ rats by sex.** Permeabilized fibers from red gastrocnemius of 13- to 14-month-old B (blue bars) and W (red bars) genotypes fed a normal chow (control) or HFD for 12 weeks were used to measure mitochondrial oxygen consumption rate. The mean ± SEM are given for 3-5 rats per group and were analyzed by Two-Way ANOVA with Tukey posthoc comparisons. * $p < 0.05$

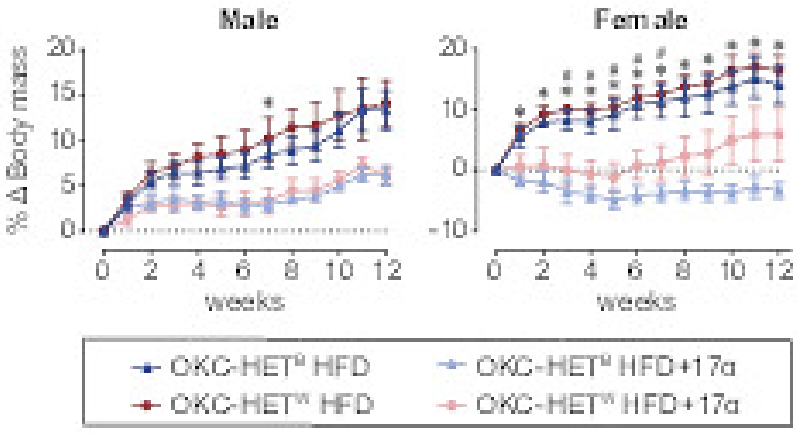


**Figure 8: Supplementary Figure 3. Adult OKC-HET$^{B}$ and OKC-HET$^{W}$ rats respond differently to high-fat feeding and 17α-E2 treatment.** Adult (13- to 14-month old) male and female B (blue) and W (red) genotypes 45.7% HFD ± 17α-E2 (14.4ppm) over a 12-week intervention. **A & B: Longitudinal percent change in body mass** in male and female rats. The data are the mean ± SEM for 6 animals per group and were analyzed by two-way repeated measures ANOVA with Tukey post-hoc comparisons. *p<0.05 between OKC-HET$^{B}$ HFD and OKC-HET$^{B}$ HFD+17α-E2; #p<0.05 between OKC-HET$^{W}$ HFD and OKC-HET$^{W}$ HFD+17α-E2.

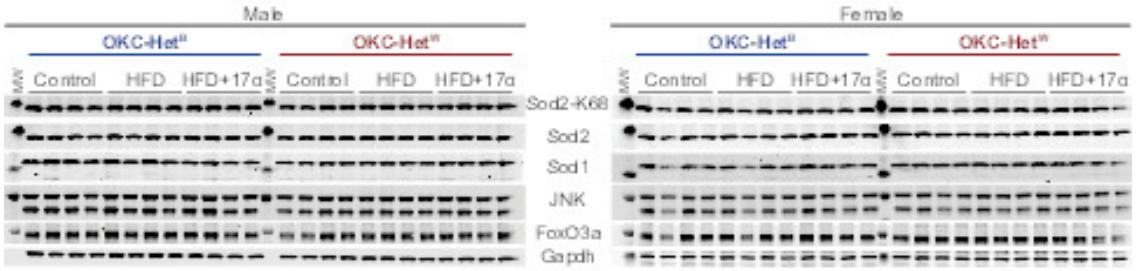


**Figure 9: Supplementary Figure 4. Western blots for acetylated Sod2(K68), Sod2, Sod1, JNK, and FoxO3a from the hippocampi** of 13- to 14-month-old male and female B and W genotypes fed either a control diet, a HFD, or HFD + 17α-E2.

## DISCUSSION

We have developed a new genetically heterogeneous four-way cross rat model with two genetically divergent mitochondrial backgrounds. We have furthermore shown that mitochondrial genotype, often interacting with sex, affects multiple physiological parameters important to the biology of aging. Specifically, we observed that one mitochondrial genotype was associated with greater grip strength, whereas the other associated with greater exercise endurance in female rats only. The two mitochondrial genotypes also differentially affected gastrocnemius respiratory response to HFD. We also discovered that mitochondrial genotype interacting with sex had some dramatic effects on cellular and physiological response to HFD and supplementation with 17α-E2 and that these effects clearly differed from previous research done in mice. These findings emphasize the importance of this new model for understanding the role of mitochondrial genotype in multiple aspects of aging biology and also for distinguishing phenomena that may or may not be generalizable across species.

An important difference between laboratory mice and rats is the genetic diversity represented in commonly used domesticated stocks and strains. Virtually all traditional inbred strains of laboratory mice originated from a small number of “fancy mice”, so their diversity is highly constrained [125]. Phylogenetic mtDNA studies show that 50 of 52 common inbred strains of mice descended from a single female *Mus musculus domesticus* mouse [106]. In contrast, laboratory rats have come from multiple domestications and in fact were often bred back to wild-caught animals; hence, both nuclear and mitochondrial diversity are considerably greater in laboratory rats compared with laboratory mice.

A unique feature of the OKC-HET$^{B/W}$ rat that we have generated is that it gives investigators a rodent model where substantial differences in the mitochondrial genome can be studied in rats with the same genetically diverse nuclear backgrounds. Our BN-

derived mitochondria differ from our WKY-derived mitochondria in 94 nucleotides with 88 base substitutions and 6 base insertions/deletions (**Figure 2A**). The importance of even considerably less divergent mitochondrial genomes on a variety of physiological outcomes was shown by Ballinger's group when they generated mitochondrial nuclear exchange mouse models (MNX) in which the mtDNA from the C3H mouse was merged with the C57BL/6 nuclear background and vice versa [100]. They showed that the mitochondrial genetic background modulated a variety of functions and pathways, e.g., bioenergetics and susceptibility to acute cardiac volume overload [100]; macrosteatosis, inflammation, fibrosis, and mitochondrial function in liver induced by an atherogenic diet [126]; metabolic efficiency, body composition, and gene expression in adipose tissue [101]; and insulin sensitivity [127]. Importantly, the mitochondrial genomes from C57BL/6 and C3H mice differ by only 5 nucleotides [106].

The normal response to relatively short-term HFD as seen in mice is a compensatory increase in mitochondrial biogenesis and respiration [128]. Interestingly, our B genotype rats appeared to make this compensation with trends or significant increases as expected. However, the W genotype rats did not and, in fact, had significantly lower Complex I activity with HFD compared to control. Unfortunately, we did not assess ROS generation in this preliminary work to determine if the failure to respond was associated with increased ROS production, although we suspect it would [129]. Finally, we found similar trends in the changes in respiration between sexes. It is worth noting that our preliminary data indicate the failure to adapt to HFD is most pronounced in females of the W genotype. This finding is interesting because rats (compared to mice) and female rats (compared to males) are generally more resistant to increases in fat deposition and declines in insulin sensitivity to HFD [130].

Because 17α-E2 has been shown to modulate metabolic- and age-related

outcomes in different strains of male, but not female mice, we studied the effects of 17α-E2 in both sexes and mitochondrial genotypes of rats undergoing HFD feeding. This is the first study to test the effect of feeding 17α-E2 to rats. Contrary to findings in mice [58, 61-62, 74-75, 107-109], female rats of both genotypes beneficially responded to 17α-E2 treatment as evidenced by the prevention of HFD-induced gains in body mass and adiposity. Liver mass was also reduced by 17α-E2 treatment in female rats of both mitochondrial genotypes. Male rats in our study responded in a mitochondrial genotype-specific manner with only B genotype males showing a decline in body mass, adiposity, and liver mass, similar to previous studies in mice [57-58, 61-63, 76, 109-110]. Our findings in both sexes were unexpected and interesting because we anticipated that both male genotypes would beneficially respond to treatment and that female responsiveness would be mild or absent. The mechanisms underlying these observations remain unknown. It should be noted that the dose provided in these studies is equivalent to what mice are provided. As such, the dosing regimen has not yet been allometrically scaled downward to account for differences in size and surface area be- tween mice and rats. Thus, in the current studies female rats are consuming a higher dose than female mice relative to size and surface area, which could contribute to female responsiveness in our studies.

Future studies will be required to determine if lower dosing regimens will effectively mimic 17α-E2 blood levels observed in mice. We surmise that genotype-specific differences in male responsiveness to 17α-E2 arises from differences in mitochondrial activity, which almost certainly affects substrate utilization, and thus, attenuates effects on adiposity. Additional studies will be needed to confirm this possibility.

Sex and genotype-specific changes were also evident in the stress response in the hippocampus with HFD feeding, potentially ameliorated by 17α-E2. While Sod2 detoxifies superoxide in the mitochondrial matrix, acetylated Sod2 (K68) can generate hydrogen

peroxide and have detrimental effects on tissue function [122]. Thus, the increase in Sod2 acetylation we observed in OKC-HETW males could impact hippocampal mitochondrial and cognitive dysfunction with HFD feeding. Additional functional studies on brain function in these models would be pertinent to unraveling mitochondrial contri- bution to cognitive decline in aging and neurodegenerative disorders.

These observations on 17α-E2 provide support for our concept that studying anti-aging compounds in both mice and rats will provide additional insights, particularly related to the mechanisms that underlie sex-specific aging. Although our findings should be considered preliminary, they suggest that the sex differences in the impact of 17α-E2 on life span might be different in rats and that mitochondrial genotype may play a role in male-responsiveness to 17α-E2. However, more studies are needed to unravel the interaction between mitochondrial genotypes and sex-specific aging and disease burden. Notably though, this short interventional study provides proof-of-concept that mice and rats may age differently in a sex- specific fashion and these differences, depending on mitochondrial genotype could impact the effect of aging-interventions. These findings raise the prospect that the OKC-HETB/W rat may be applied to study dietary interventions that purport to improve cognitive and performance phenotypes associated with improvements in mitochondrial function.

We note that tissues and OKC-HETB/W rats are available to the scientific community and can be obtained by contacting Arlan Richardson (Arlan-Richardson@ouhsc.edu), Michael Stout (Michael-Stout@omrf.org), or Steven Austad (austad@uab.edu)

# CHAPTER III

## A PILOT STUDY EVALUATING DOSING TOLERABILITY OF 17α-ESTRADIOL IN MALE COMMON MARMOSETS (*CALLITHRIX JACCHUS*)

*Sathiaseelan R, Isola JVV, Santín-Márquez R, et al. *A pilot study evaluating dosing tolerability of 17α-estradiol in male common marmosets (Callithrix jacchus).* 2024. Geroscience.

*All data in this chapter were generated by R.S., animal experiment was conducted by D.A. and A.B.S [131]

## ABSTRACT

17α-estradiol extends health span and lifespan in male mice without significant feminization or deleterious effects on reproductive function, making it a candidate for human translation. However, studies in animal models that more accurately replicate human physiology are necessary to establish 17α-estradiol dosing standards for clinical trials. This study evaluated the tolerability of 17α-estradiol treatment in the common marmoset over a short treatment duration. We found that male marmosets tolerated two dosing regimens (0.37-0.47 or 0.62-0.72 mg/kg/day) as evidenced by the absence of gastrointestinal distress, changes in vital signs, or overall health conditions. 17α-estradiol treatment mildly decreased body mass, adiposity, and glycosylated hemoglobin, although these changes were not statistically significant in most instances. However, neither dose of 17α-estradiol elicited feminization in our study, thereby suggesting that optimized dosing regimens may provide health benefits without feminization in primates. Additional studies are needed to determine if longer duration treatments would also be non-feminizing and elicit significant health benefits, which would aid in developing dosing regimens targeting healthy aging in humans.

**KEYWORDS:** androgen, endocrinology, estrogen, follicle stimulating hormone, luteinizing hormone, nonhuman primate

## INTRODUCTION

In recent years, 17α-estradiol (17α-E2) has been shown to elicit beneficial health outcomes in mice. The National Institute on Aging NIA Interventions Testing Program (ITP) has reported that 17α-E2 significantly extends lifespan in male mice when administered during mid-life [74-75] or late-life [132]. 17α-E2-mediated lifespan extension in male mice is similar to that of calorie restriction [133] or rapamycin administration [75], which suggests 17α-E2 may hold therapeutic potential for translation into humans. Our prior work has focused on identifying pathways through which 17α-E2 enhances health span and prolongs lifespan in a sex-specific manner. Our previous reports indicate that 17α-E2 administration decreases calorie consumption in male rodents, which is mirrored by significant declines in adiposity and improvements in a variety of metabolic parameters including glucose tolerance, insulin sensitivity, and ectopic lipid deposition [57-58, 63, 76, 89, 109-110, 134-135]. Others have also reported that 17α-E2 treatment elicits benefits on glucose tolerance, mTORC2 signaling, hepatic urea cycling, neuroinflammation markers, and sarcopenia [61-62, 107-108, 136-137]. We have also shown that the reduction in food intake caused by 17α-E2 is not essential for its metabolic benefits [76], indicating that 17α-E2 potentially influences metabolism and aging through mechanisms that are independent of hypothalamic anorexigenic signaling. It remains unresolved if the benefits of 17α-E2 are contingent upon changes in reproductive endocrinology, although we recently demonstrated that 17α-E2 treatment does not adversely affect sperm parameters or fertility in breeding age male mice [117].

In addition to the aforementioned rodent reports, a few studies have evaluated 17α-E2 administration in humans. However, these studies primarily evaluated pharmacokinetic parameters of 17α-E2 or were short-term Phase 1 tolerability trials [43]. The human studies found no evidence that exogenous 17α-E2 administration elicited adverse health outcomes or feminization in men regardless of dose and duration [43]. Despite these prior studies, no 17α-E2 dosing regimens have been established for treating age-related morbidity in humans. In contrast, we recently evaluated the tolerability of 17α-E2 in male rhesus macaques. We chose this species of monkeys because they closely recapitulate human aging and chronic disease onset [138]. In the rhesus macaque study, 17α-E2 elicited only mild benefits on body mass, adiposity, and glycosylated hemoglobin, with no other changes in health status. However, our dosing regimens were significantly feminizing as evidenced by decreased testis size, suppressed circulating androgen concentrations, and elevated circulating estrogen levels [139]. The significant feminizing effects of 17α-E2 observed in rhesus monkeys were unexpected and point to a need for additional studies that employ alternative dosing regimens, delivery methods, and/or nonhuman primate models to potential direct clinical studies.

Pre-clinical translational studies in non-human primates share many challenges with clinical studies, such as the long lifespan of primates. Common marmosets (Callithrix jacchus) offer some advantages due to their relatively short natural lifespan, small size, and similarity to other primates, including humans. They also develop a variety of age-related diseases similar to those in humans [140-141]. Marmosets display age-related changes in pathologies associated with diabetes, cardiac disease, cancer, and renal disease similar to those seen in humans [142-143]. We and others have also recently shown naturally occurring changes in oral health and osteoarthritis with age that recapitulate the normal course of aging in humans [144-145]. Moreover, we have recently

developed tools to address the functional, cellular, and molecular changes associated with aging in marmosets [146-148]. Marmosets are proving to be valuable animal models to determine potential for pharmaceutical aging interventions [149-153], and this study addressed the question of tolerability of 17α-E2 in marmosets as a first step towards addressing translation to humans. Herein, we present data from a short-term 17α-E2 dosing study in male common marmosets in which two different dosing regimens were evaluated.

## METHODS

### ***Subjects***

Nine 3-year-old male common marmosets (Callithrix jacchus) were used in our study. Captive marmosets are considered to have a 1:8 equivalent age ratio to humans, therefore the marmosets used in the current study are representative of 24-year-old men [154]. All marmosets were housed as pairs (with non-breeding females) in standard nonhuman primate caging under established conditions (temperature, 22-28 °C; humidity, 50-60%; 12:12 light-dark) at the University of Texas Health San Antonio. Throughout the studies, all marmosets received a base diet that was a mix of Purina Mazuri® and Harlan-Teklad™ marmoset purified diets and had ad libitum access to water. Food enrichment items were offered daily and included things such as Zupreem® marmoset diet, dried cranberries, raisins, or Teklad™ primate enrichment mix. Animals were monitored at least 3-times daily by trained care staff. This research was reviewed and approved by the UTHSA IACUC and experiments were conducted in compliance with the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals and adhered to the American Society of Primatologists principles for the ethical treatment of non-human primates. Animals chosen

for enrollment in this study were selected based on age as well as a record of relatively good health over the preceding 6 months as assessed by veterinary examination.

***17α-E2 Dosing and Delivery***

Our previous studies in mice have established that the average dietary intake of 17α-E2 ranges from 30-45 μg/day [57-58, 76]. Normalizing these intake levels by body mass revealed that male mice generally consumed 0.92-1.15 mg/kg/day during our studies. These data were used to calculate dosing regimens in marmosets through allometric scaling. Allometric scaling is used to calculate pharmacological dosing paradigms because it accounts for differences in metabolic rate and surface area between species [155-156]. These calculations determined that 17α-E2 doses ranging from 0.40-0.70 mg/kg/day would be roughly equivalent to what mice ingest on a daily basis. Therefore, we decided to evaluate two dosing ranges in the current study, a low dose (0.37-0.47 mg/kg/day) and a high dose (0.62-0.72 mg/kg/day). Four male subjects were assigned to the low dose group and five subjects were assigned to the high dose group for a 28-days intervention. Initial body masses ranged from 378.4 to 528.6 grams with an average of 491.57 ± 46.52 grams. 17α-E2 was purchased from Steraloids (Newport, RI) and reconstituted in EtOH at a concentration of 10 mg/ml. This stock solution was created fresh each week and then aliquoted into prelabeled cryovials to account for daily individualized dosing. Delivery of 17α-E2 was achieved by adding the appropriate volume of stock solution into yogurt (Hill Country Fare low-fat strawberry-banana yogurt) and provided to marmosets each day in their home cage [157]. Subjects were monitored several times each day for signs of vomiting, diarrhea, and/or constipation, which are initial indicators of poor tolerability of an orally delivered pharmacotherapy in nonhuman primates.

***Food Intake***

Food intake was monitored for a single day during baseline testing, following 14 days of treatment, and 28 days of treatment. Each animal received half of its daily calorie allotment twice daily, which was based on body mass and estimated from the National Research Council Guidelines [158]. Marmosets were part of an established colony and weight stable prior to the onset of the study. During these 24-hour periods, the animals were single housed in their home cage, but were physically separated using cage dividers, so that food intake could be evaluated. Any chow remaining prior to the next feeding period was subtracted from the amount provided to determine daily consumption.

***Blood Collection and Analyses***

Blood was collected from fasted animals following an overnight fast during baseline testing, following 14 days of treatment, and 28 days of treatment. Blood draws occurred during the morning hours (08:00–11:00) in non-anesthetized animals that were restrained in a custom assembly. Femoral vein blood collection (1.0–2.0 mL) was performed on each animal and blood was placed in EDTA-coated blood collection tubes. Whole blood was frozen and stored at − 80 °C. Fasting glucose was determined during blood collection via hand-held glucometer. Fasting insulin was evaluated using an ELISA from Novus Biologicals (Centennial, CO). Glycosylated hemoglobin (HbA1C) was measured in whole blood using a Siemens DCA Vantage Analyzer (Siemens Medical Solutions, Malvern, PA). Plasma was isolated and sent to Mayo Clinic (Rochester, MN) for assessment of circulating 17α-E2, 17β-estradiol (17β-E2), estrone and testosterone as previously described [57]. Additional plasma was sent to the Endocrine Technologies Core at the Oregon National Primate Research Center (Beaverton, OR) for assessment of circulating luteinizing hormone (LH) by radioimmunoassay as described elsewhere [159].

***Body Composition Measures***

Animals were weighed weekly in the morning prior to feeding. Echo-MRI scanning for fat and lean mass was performed during baseline testing, following 14 days of treatment, and 28 days of treatment as previous described [153]. Chest and abdominal circumferences were measured during baseline testing and following 28 days of treatment as previously described [160].

***Gonadal Ultrasounds***

Testicle size was determined in non-anesthetized animals that were restrained in the same custom assembly used for blood draws. Size was measured during baseline testing and following 28 days of treatment using a GE Logiq Book XP portable ultrasound system (GE Healthcare, Chicago IL) by a trained technician blinded to group designation. Two dimensional measures were recorded for both the left and right testis and testis volumes were determined using the ellipsoid equation.

***Statistical Analyses***

Results are presented as mean ± SEM with p values less than 0.05 considered to be significantly different. Analyses of differences between groups were performed by two-way ANOVA using Graphpad Prism 9.0 Software. **Supplemental Table 1** includes confidence intervals and p-values for each statistical comparison we performed.

## RESULTS

### Oral dosing of 17α-E2 appears tolerable and does not affect calorie consumption in male marmosets

We initially sought to determine if daily oral administration of 17α-E2 at two different doses (0.37-0.47 or 0.62-0.72 mg/kg/day) would be tolerated by male common marmosets. We found no evidence of adverse events (vomiting, diarrhea, constipation) with either dose of 17α-E2, which is suggestive of tolerable dosing regimens. Calorie

consumption remained relatively constant in all animals regardless of dosing regimen (**Figure 10**).

**Oral dosing of 17α-E2 mildly reduces body mass and adiposity in male marmosets**

17α-E2 treatment elicited mild reductions in body mass and adiposity. Specific reductions included declines in the percent change in body mass ($p=0.057$) and fat mass ($p=0.097$) from baseline to day 28 in animals receiving the high dose of 17α-E2 (**Figure 11C, F**). We also found that the average fat mass ($p=0.065$) was mildly reduced from baseline to day 28 in animals receiving the low dose of 17α-E2 (**Figure 11E**). These observations aligned with a subsequent finding of a mild, but trending, reduction in the average abdominal circumference ($p=0.108$) from baseline to day 28 in animals receiving the high dose of 17α-E2 (**Figure 12B**), although the percent change in abdominal circumference did not display a similar trend ($p=0.266$; Figure 12C). Interestingly, low dose treatment with 17α-E2 increased chest circumference ($p=0.048$; **Figure 12E**), which is commonly used as an indicator of axillary fat pad size around the nipple area in both male and female marmosets [160]. At this juncture the significance of this finding is unclear, particularly in the context of only mild, nonsignificant reductions in body mass and whole-body adiposity. The increase in chest circumference in the low dose treatment group could be interpreted as an initial indication of gynecomastia, but the lack of this observation in the high dose treatment group lessens this possibility. Lean mass was unaffected by either treatment in our study (**Figure 11G-I**). Despite the mild changes outlined above, it should be noted that none of these observations rose to the level of statistical significance, except the increase in chest circumference.

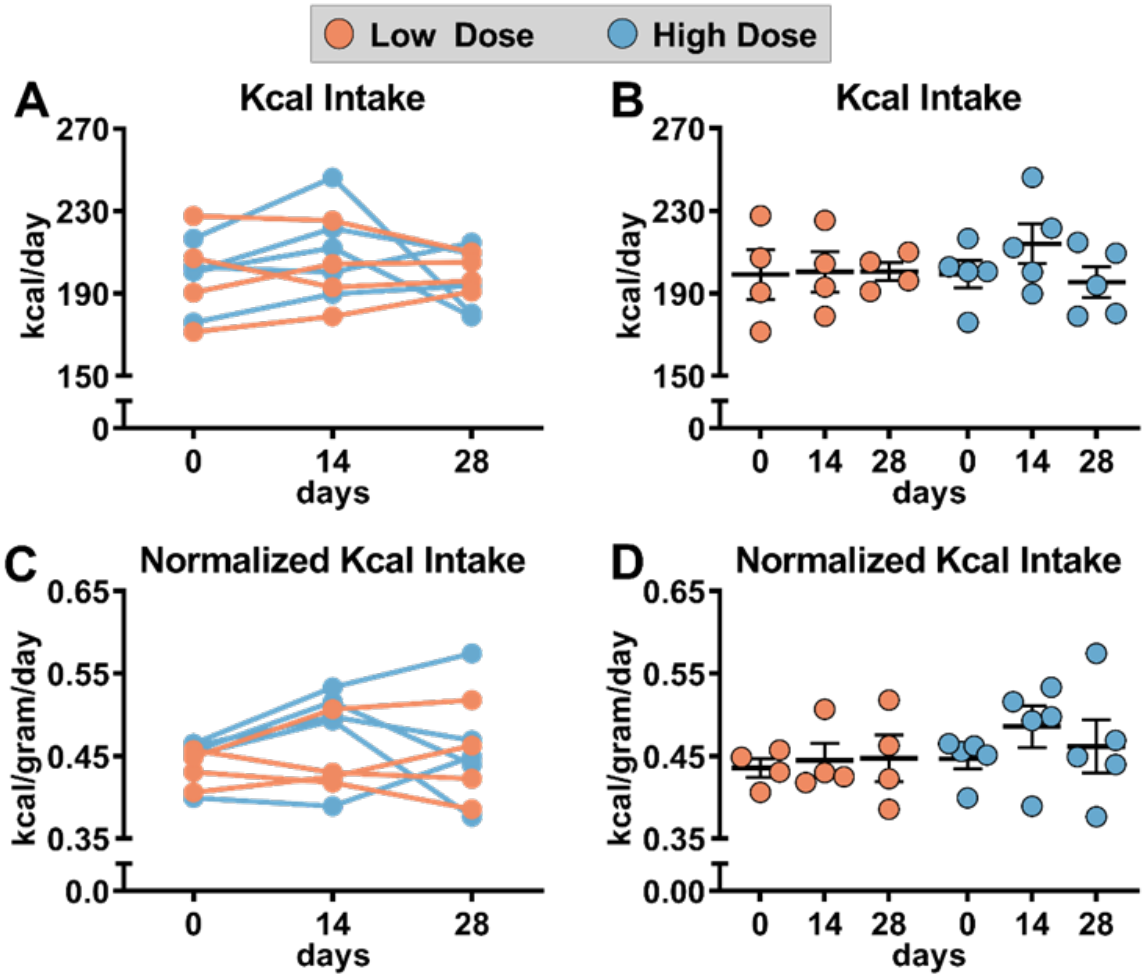


**Figure 10. Food intake is unaffected by 17α-E2 treatment in male common marmosets.** (A) Daily calorie consumption in individual animals, (B) Average calorie consumption, (C) Daily calorie consumption normalized to body mass in individual animals, and (D) Average calorie consumption normalized to body mass at baseline (day 0), day 14, and day 28 in male marmosets receiving either a low dose (0.37-0.47 mg/kg/day) or high dose (0.62-0.72 mg/kg/day) of 17α-E2. All data are presented as mean ± SEM and were analyzed by two-way repeated measures ANOVA with Sidak post-hoc comparisons. Low dose: n=4; High dose: n=5.

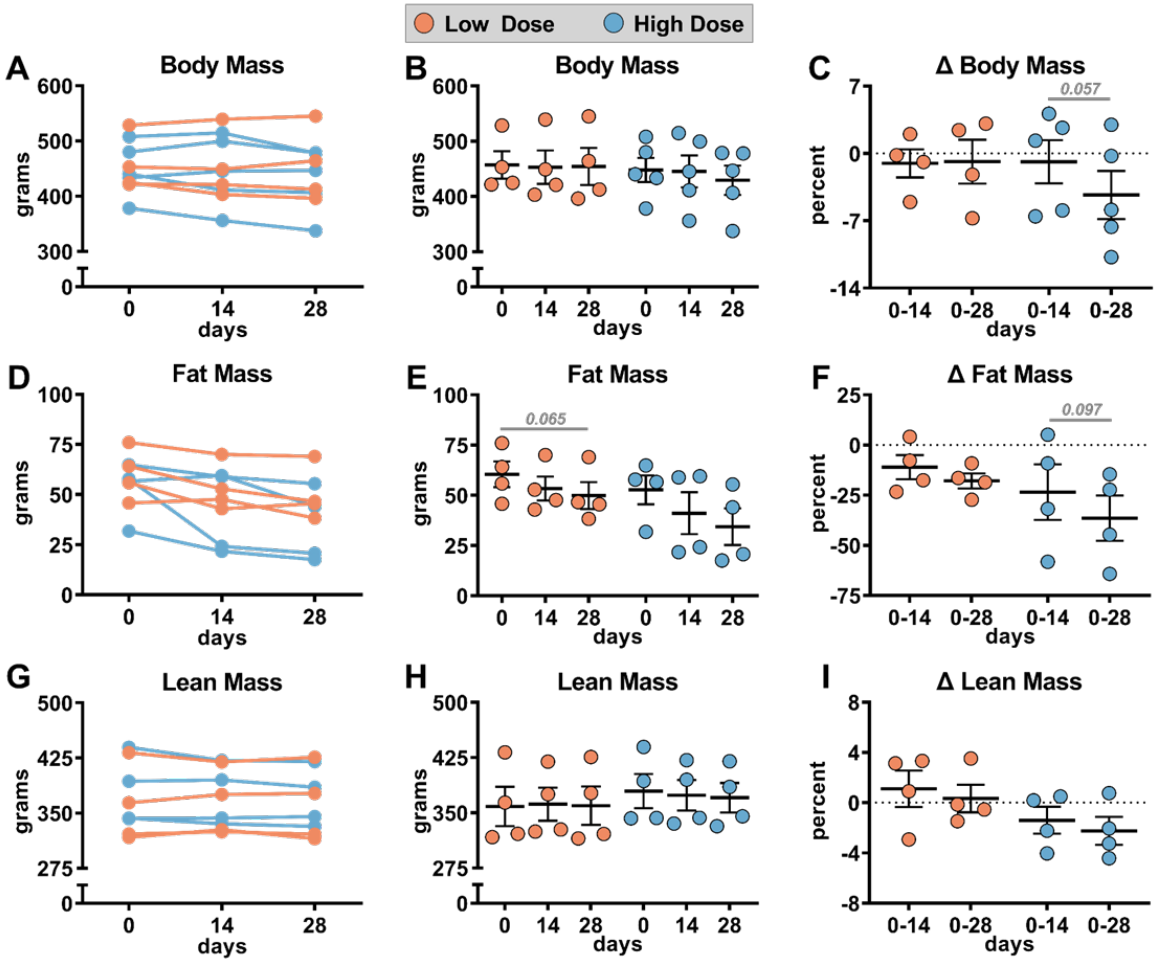


**Figure 11. High dose of 17α-E2 mildly reduces body mass and adiposity in male common marmosets.** (A) Body mass of individual animals, (B) Average body mass, (C) Average percent change in body mass, (D) Fat mass of individual animals, (E) Average fat mass, (F) Average percent change in fat mass, (G) Lean mass of individual animals, (H) Average lean mass, and (I) Average percent change in lean mass at baseline (day 0), day 14, and day 28 in male marmosets receiving either a low dose (0.37-0.47 mg/kg/day) or high dose (0.62-0.72 mg/kg/day) of 17α-E2. All data are presented as mean ± SEM and were analyzed by two-way repeated measures ANOVA with Sidak post-hoc comparisons. Low dose: n=4; High dose: n=4-5. Notably, a single animal in the high dose treatment group had difficulty acclimatizing to entry into the plastic tube for body composition analyses, therefore their fat and lean mass data were inconsistent and excluded. No statistical differences were observed but trending outcomes were labeled with respective p values.

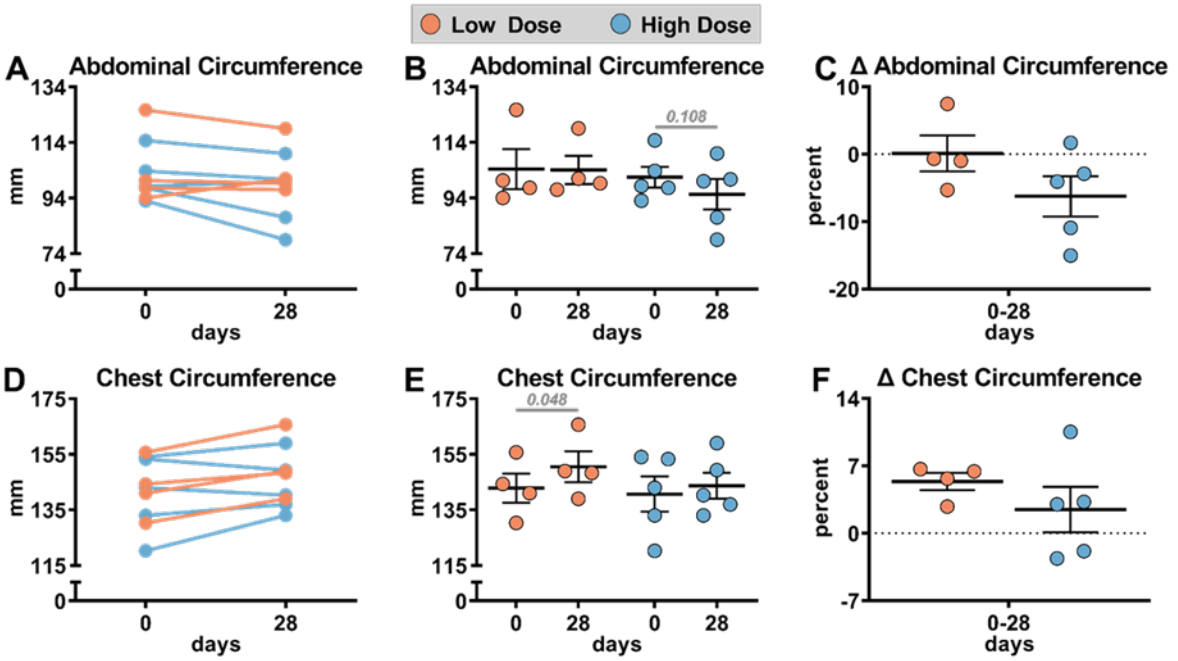


**Figure 12. 17α-E2 mildly alters abdominal and chest circumference outcomes in male common marmosets.** (A) Abdominal circumference of individual animals, (B) Average abdominal circumference, (C) Average percent change in abdominal circumference, (D) Chest circumference of individual animals, (E) Average chest circumference, and (F) Average percent change in chest circumference at baseline (day 0) and day 28 in male marmosets receiving either a low dose (0.37-0.47 mg/kg/day) or high dose (0.62-0.72 mg/kg/day) of 17α-E2. All data are presented as mean ± SEM and were analyzed by two-way repeated measures ANOVA with Sidak post-hoc comparisons. Low dose: n=4; High dose: n=5. Significantly different and trending outcomes were labeled with respective p values.

**Oral dosing of 17α-E2 inconsistently affects glucose homeostasis in male marmosets**

17α-E2 treatment has been consistently shown to improve glucose homeostasis in male mice [57-58, 63, 76, 109], although essentially no data has been reported in higher order mammals other than our prior study in rhesus monkeys [139]. In the current study, 17α-E2 treatment inconsistently affected fasting glucose and insulin outcomes, although our treatment duration was quite short and our animals were young and healthy. For instance, the low dose of 17α-E2 actually appeared to increase fasting glucose levels ($p=0.057$), while the high dose of 17α-E2 elicited no change (**Figure 13A-B**). These observations occurred despite no changes in fasting insulin levels within both treatment groups (**Figure 13C-D**), thereby making interpretations difficult. We also evaluated HbA1c levels, which is reflective of long-term glucose management, and found that the low dose of 17α-E2 reduced HbA1c ($p=0.046$), while the high dose of 17α-E2 elicited no change (**Figure 13E-F**). This observation is in direct conflict with the aforementioned increase in fasting glucose following low dose treatment with 17α-E2 and supports the idea that this observation may represent some form of an environmental stress response during the blood collection period.

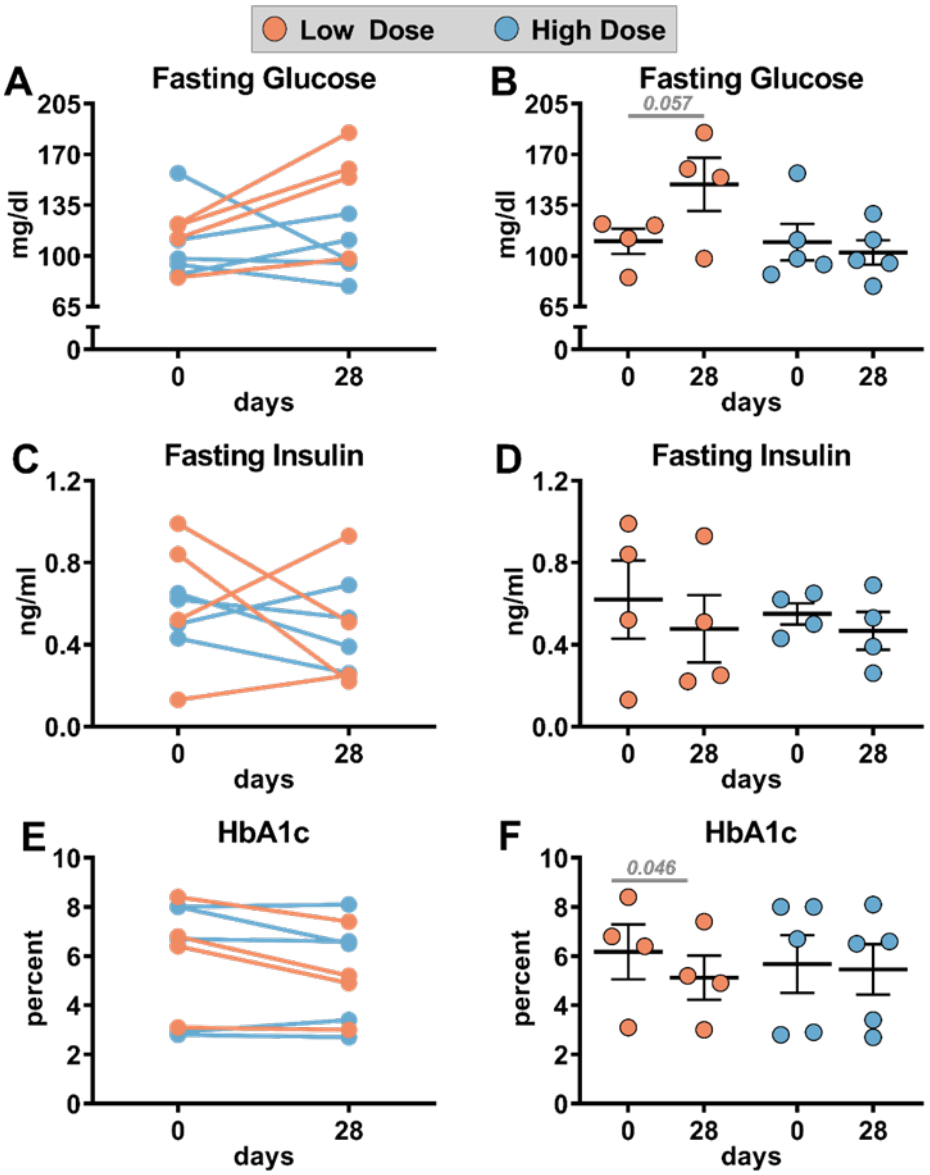


**Figure 13. 17α-E2 inconsistently affects glucose homeostasis in male marmosets.** (A) Fasting glucose of individual animals, (B) Average fasting glucose, (C) Fasting insulin, (D) Average fasting insulin, (E) HbA1c of individual animals, and (F) Average HbA1C at baseline (day 0) and day 28 in male marmosets receiving either a low dose (0.37-0.47 mg/kg/day) or high dose (0.62-0.72 mg/kg/day) of 17α-E2. All data are presented as mean ± SEM and were analyzed by two-way repeated measures ANOVA with Sidak post-hoc comparisons. Low dose: n=4; High dose: n=4-5. Notably, measurements of insulin from a single animal in the high dose treatment group were inconsistent and excluded. Significantly different and trending outcomes were labeled with respective p values.

**Oral dosing of 17α-E2 does not induce feminization in male marmosets**

In mice, 17α-E2 treatment does not elicit feminization or adversely affect reproduction in males [117]. However, our previous study in rhesus macaques showed a dose-dependent feminization suggesting potential differences in outcomes between rodents and nonhuman primates [139]. Here we found that neither low or high dose of 17α-E2 dosing elicited testis atrophy in marmosets (**Figure 14**). Furthermore, our dosing regimens did not significantly alter the circulating levels of the sex hormones 17β-E2, estrone, testosterone, or LH (**Figure 15C-J**). With regards to circulating 17α-E2, an increase up to 100-200-fold was observed in the treatment groups, though this did not reach statistical significance likely due to small sample sizes (**Figure 15A-B**), but which also mirrored the findings from our previous investigation in rhesus macaques [139]. However, we did not observe characteristics of feminization in marmosets and it remains unclear if this discrepancy is due to species-specific differences in 17α-E2 metabolism or the study design. Nevertheless, our findings suggest that exposure to 17α-E2 does not feminize male marmosets.

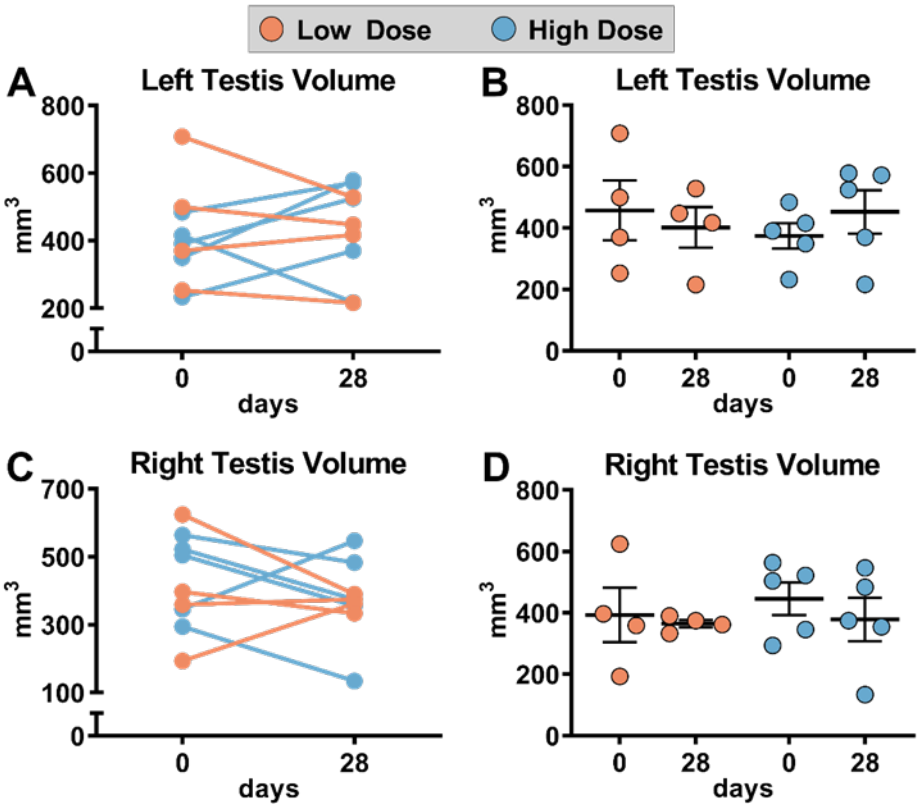


**Figure 14. 17α-E2 does not induce testis atrophy in male marmosets.** (A) Left testis volume of individual animals, (B) Average left testis volume, (C) Right testis volume, and (D) Average right testis volume at baseline (day 0) and day 28 in male marmosets receiving either a low dose (0.37-0.47 mg/kg/day) or high dose (0.62-0.72 mg/kg/day) of 17α-E2. All data are presented as mean ± SEM and were analyzed by two-way repeated measures ANOVA with Sidak post-hoc comparisons. Low dose: n=4; High dose: n=5.

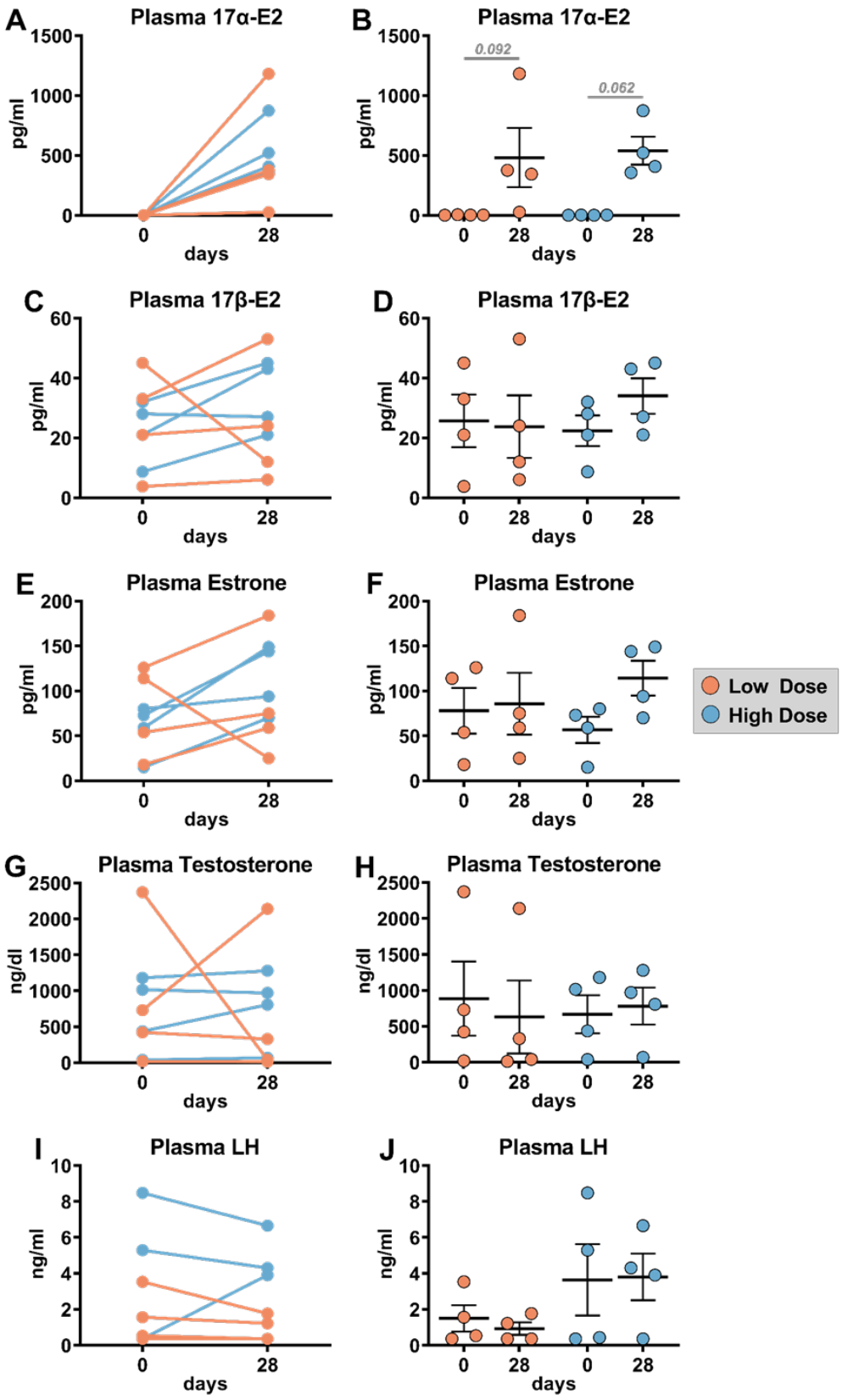
A
Plasma 17α-E2
pg/ml
1500
1000
500
0
0
28
days
B
Plasma 17α-E2
0.092
0.062
C
Plasma 17β-E2
60
40
20
D
Plasma 17β-E2
E
Plasma Estrone
200
150
100
50
F
Plasma Estrone
Low Dose
High Dose
G
Plasma Testosterone
ng/dl
2500
2000
H
Plasma Testosterone
I
Plasma LH
ng/ml
10
8
6
4
2
J
Plasma LH

**Figure 15. 17α-E2 does not elicit significant changes to the circulating sex hormone milieu in male common marmosets.** (A) Plasma 17α-E2 in individual animals, (B) Average plasma 17α-E2, (C) Plasma 17β-E2 in individual animals, (D) Average plasma 17β-E2, (E) Plasma estrone in individual animals, (F) Average plasma estrone, (G) Plasma testosterone in individual animals, (H) Average plasma testosterone, (I) Plasma LH in individual animals, and (J) Average plasma LH at baseline (day 0) and day 28 in male marmosets receiving either a low dose (0.37-0.47 mg/kg/day) or high dose (0.62-0.72 mg/kg/day) of 17α-E2. All data are presented as mean ± SEM and were analyzed by two-way repeated measures ANOVA with Sidak post-hoc comparisons. Low dose: n=4; High dose: n=4. Notably, insufficient blood was collected during baseline blood draws for hormone analyses from a single animal in the high dose treatment group, therefore no longitudinal comparisons could be made in this animal so it was excluded. No statistical differences were observed but trending outcomes were labeled with respective p values.

**SUPPLEMENTAL TABLE 1**

| | Low Dose | | |
|---|---|---|---|
| | **0 vs 14** | **0 vs 28** | **14 vs 28** |
| **Figure 1B** | -30.72 to 28.16 (p=0.9964) | -45.88 to 43.06 (p=0.9986) | -27.53 to 27.26 (p>0.999) |
| **Figure 1D** | -0.1006 to 0.0821 (p=0.9600) | -0.1558 to 0.1327 (p=0.9792) | -0.0739 to 0.0693 (p=0.9986) |
| | | | |
| | **0 vs 14** | **0 vs 28** | **14 vs 28** |
| **Figure 2B** | -28.06 to 35.86 (p=0.9347) | -46.97 to 52.22 (p=0.9937) | -28.30 to 25.75 (p=0.9955) |
| **Figure 2E** | -9.090 to 23.20 (p=0.3313) | -1.079 to 22.24 (p=0.0646) | -9.383 to 16.44 (p=0.6247) |
| **Figure 2H** | -29.91 to 23.81 (p=0.9458) | -20.93 to 18.82 (p=0.9936) | -17.01 to 21.00 (p=0.9560) |
| | | | |
| | **0-14 vs 0-28** | | |
| **Figure 2C** | -4.166 to 3.817 (p=0.9910) | | |
| **Figure 2F** | -8.893 to 22.42 (p=0.4350) | | |
| **Figure 2I** | -1.994 to 3.538 (p=0.6870) | | |
| | | | |
| | **0 vs 28** | | |
| **Figure 3B** | -8.128 to 8.828 (p=0.9919) | | |
| **Figure 3E** | -15.26 to -0.0930 (p=0.0476) | | |
| | | | |
| | **0 vs 28** | | |
| **Figure 4B** | -79.79 to 1.287 (p=0.0568) | | |
| **Figure 4D** | -0.4076 to 0.6926 (p=0.7217) | | |
| **Figure 4F** | 0.0207 to 2.079 (p=0.0461) | | |
| | | | |
| | **0 vs 28** | | |
| **Figure 5B** | -139.5 to 250.6 (p=0.6938) | | |
| **Figure 5D** | -197.8 to 254.3 (p=0.9291) | | |
| | | | |
| | **0 vs 28** | | |
| **Figure 6B** | -1049 to 90.16 (p=0.0920) | | |
| **Figure 6D** | -23.40 to 27.25 (p=0.9709) | | |
| **Figure 6F** | -84.87 to 69.37 (p=0.9499) | | |
| **Figure 6H** | -1372 to 1884 (p=0.8834) | | |
| **Figure 6J** | -2.043 to 3.190 (p=0.7890) | | |

Note: Confidence intervals and p-values for each statistical comparison performed for low dose analyses.

**SUPPLEMENTAL TABLE 1 (continued)**

| | High Dose | | |
|---|---|---|---|
| | **0 vs 14** | **0 vs 28** | **14 vs 28** |
| **Figure 1B** | -35.83 to 6.302 (p=0.1447) | -38.03 to 45.76 (p=0.9813) | -37.89 to 75.15 (p=0.6010) |
| **Figure 1D** | -0.0929 to 0.0149 (p=0.1322) | -0.1370 to 0.1069 (p=0.9581) | -0.1082 to 0.1562 (p=0.8851) |
| | | | |
| | **0 vs 14** | **0 vs 28** | **14 vs 28** |
| **Figure 2B** | -35.51 to 40.71 (p=0.9922) | -22.19 to 59.23 (p=0.3805) | -10.23 to 42.07 (p=0.2071) |
| **Figure 2E** | -25.99 to 49.36 (p=0.5457) | -12.27 to 48.97 (p=0.1771) | -7.586 to 20.91 (p=0.2932) |
| **Figure 2H** | -16.24 to 27.63 (p=0.6551) | -12.96 to 30.98 (p=0.3686) | -8.755 to 15.38 (p=0.6222) |
| | | | |
| | **0-14 vs 0-28** | | |
| **Figure 2C** | -0.1188 to 7.022 (p=0.0572) | | |
| **Figure 2F** | -2.707 to 28.61 (p=0.0974) | | |
| **Figure 2I** | -1.921 to 3.612 (p=0.6408) | | |
| | | | |
| | **0 vs 28** | | |
| **Figure 3B** | -1.443 to 13.72 (p=0.1080) | | |
| **Figure 3E** | -9.782 to 3.782 (p=0.4382) | | |
| | | | |
| | **0 vs 28** | | |
| **Figure 4B** | -29.06 to 43.46 (p=0.8330) | | |
| **Figure 4D** | -0.4676 to 0.6326 (p=0.8929) | | |
| **Figure 4F** | -0.7007 to 1.141 (p=0.7698) | | |
| | | | |
| | **0 vs 28** | | |
| **Figure 5B** | -252.6 to 96.30 (p=0.4300) | | |
| **Figure 5D** | -134.9 to 269.4 (p=0.6126) | | |
| | | | |
| | **0 vs 28** | | |
| **Figure 6B** | -1108 to 31.62 (p=0.0618) | | |
| **Figure 6D** | -36.90 to 13.75 (p=0.3993) | | |
| **Figure 6F** | -134.6 to 19.62 (p=0.1342) | | |
| **Figure 6H** | -1741 to 1515 (p=0.9758) | | |
| **Figure 6J** | -2.781 to 2.453 (p=0.9801) | | |

Note: Confidence intervals and p-values for each statistical comparison performed for high dose analyses.

## DISCUSSION

Prior research has established that 17α-E2 treatment prolongs lifespan and mitigates various mechanisms associated with aging and chronic disease burden in male rodents [57-58, 61-63 74-76, 89, 107-110, 132, 134-137]. Interestingly, these male-specific benefits occur without notable feminization of sex hormone profiles [57] or impairment of reproductive function [117]. In contrast, the effects of 17α-E2 treatment in intact female mice are minor compared to those seen in males [61-62, 107-108, 136, 161-162]. The removal of ovaries increases the responsiveness of female mice to 17α-E2 treatment [59]. However, long-term administration in ovariectomized females has limited effectiveness in mitigating pro-aging mechanisms to the same extent observed in male mice [61-62, 107-108, 136]. Considering the aforementioned findings and the limited information available on the translatability of 17α-E2 treatment, this study aimed to establish dosing regimens of 17α-E2 in male common marmosets. Additionally, we assessed metabolic and endocrine parameters to evaluate efficacy and potential feminizing effects. Our findings offer valuable insights for future studies concerning the translation potential of 17α-E2.

Upon initiation of treatment, our primary goal was to determine if oral dosing of 17α-E2 elicited acute signs of illness or distress in common male marmosets. We found no evidence of illness or distress with either dose of 17α-E2. We had anticipated this outcome for several reasons. First, 17α-E2 occurs naturally in both male and female mammals [45, 163-164] and constitutes a minor component of various FDA-approved hormone replacement therapies for menopause (2.5-9.5%) [43]. Therefore, thousands of women have been exposed to exogenous 17α-E2 over extended periods without clinically relevant adverse effects being commonly reported [165-169]. Second, 17α-E2 is

prescribed to men as a topical treatment for androgenic alopecia in parts of Europe and South America [170] with no significant side effects reported. Third, a few Phase 1 trials have evaluated 17α-E2 treatment in humans and no tolerability issues were reported [43, 163]. For instance, men receiving 2 mg/day of 17α-E2 presented no instances of gynecomastia or breast tenderness, with only one patient reporting a decrease in libido.

We subsequently evaluated whether 17α-E2 would elicit metabolic benefits in male common marmosets despite the short treatment duration. We found that both dosing regimens were associated with mild reductions in body mass and/or adiposity, although these changes were subtle when compared to those observed in male mice undergoing similar treatment durations [76]. Similar results were observed in rhesus monkeys receiving 17α-E2 for a longer duration [139]. A potential contributor to the discrepancies observed between mice and primates could be attributed to the intrinsic variability in the data resulting from the genetic diversity among primates [171] as well as the limited sample sizes, common to studies using nonhuman primates. However, these conditions fail to completely elucidate the limited impact of 17α-E2 therapy on these parameters in male marmosets, especially given that the subjects do not seem to segregate into distinct categories of responders and non-responders. It is conceivable that substantial decreases in body mass and adiposity were impractical within non-obese, healthy marmosets used for these studies. Nevertheless, the trend towards reduction in body and fat mass appeared to be more pronounced in animals receiving the higher dosing regimen, indicating a necessity for additional studies with higher dosing regimens and/or longer durations of treatment.

Similar to the effects on body mass and adiposity, we observed only minor, yet inconsistent, effects of 17α-E2 treatment on fasting glucose and insulin. No changes were observed in fasting insulin levels in both treatment groups, although the low dose of 17α-

E2 appeared to increase fasting glucose levels, but this observation was contradicted by a statistically significant reduction in HbA1c within this group. Considering the age and health status of the animals involved, we speculate that the increase in fasting glucose among those receiving the lower dose of 17α-E2 may represent responsiveness to some form of transient environmental stress, rather than a metabolic homeostatic alteration, particularly since fasting insulin levels remained unchanged. In rhesus macaques, 17α-E2 treatment significantly reduced HbA1c levels, despite the animals displaying healthy HbA1c levels when the study began [139]. This observation was paralleled in the low-dose marmoset group, which followed a dosing regimen comparable to that used in the rhesus monkey study (marmoset low dose: 0.37-0.47 mg/kg/day; rhesus monkey dose: 0.30 mg/kg/day). These findings suggest that 17α-E2 at doses close to 0.30 mg/kg/day may reduce circulating HbA1C levels in nonhuman primates. Previous studies from our group have shown that 17α-E2 improves glucose metabolism through estrogen receptor α (ERα) in male mice [58, 172]. Activation of ERα has been associated with reduced HbA1C levels in male mice [173]. This leads us to speculate that 17α-E2 may act similarly in nonhuman primates. Also, we speculate that future studies might yield clearer outcomes by assessing obese, pre-diabetic, and/or older marmosets.

It is noteworthy that the study performed in rhesus macaques was longer (12 weeks), compared to the short 28 days treatment provided in the present study. This is relevant for the interpretation of our results given that no differences in weight and adiposity were found after 6 weeks of treatment in rhesus monkeys, similar to the four-week dosing regimen utilized in the current study. Conversely, differences in body composition were evident in male rhesus monkeys after 12 weeks of treatment. This leads us to hypothesize that longer treatment durations may be necessary to observe significant changes in non-human primates.

In the current study we observed no effects of 17α-E2 treatment on circulating levels of 17β-E2, estrone, testosterone, and LH, which is suggestive of no significant hormonal feminization. Nevertheless, circulating levels of 17α-E2 were significantly increased after 17α-E2 treatment. The increase in 17α-E2 levels were as high as 100-200-fold greater than baseline levels. This magnitude of increase was also observed in rhesus macaques [139]. Chronic 17α-E2 treatment in mice also elicits increase in 17α-E2, but to a much lesser degree, around 5- to 20-fold. This discrepancy might be due to the fact that dietary administration in mice results in slower ingestion rates due to their intermittent nibbling behavior [174], whereas both marmosets and rhesus macaques received their entire daily dose of 17α-E2 during a single feeding. This 'bolus' effect likely contributes to the higher serum levels of 17α-E2 seen in the primate studies compared to the mouse studies. Future studies in primates and humans would likely benefit from the development and implementation of 17α-E2 transdermal patches, which are commonly used to administer hormone therapies [175].

In male mice, 17α-E2 treatment does not elicit overt feminization or adverse effects on reproduction [57, 117]. In humans, a prior report also indicates no significant effects on serum 17β-E2, estrone, testosterone, or sex hormone binding globulin (SHBG) following 12 weeks of daily 17α-E2 administration at 2 mg/day in middle-aged men [43, 163]. Interestingly, male rhesus monkeys receiving 17α-E2 displayed severe feminization as evidenced by testis atrophy, suppressed circulating androgens and gonadotropins, and increased circulating estrogens [139]. Since both marmosets and rhesus macaques showed similar circulating 17α-E2 levels, the discrepancy related to the levels of feminization observed between the two species remain unresolved. It is important to consider the differences in the duration of 17α-E2 administration between rhesus macaques and marmosets. 17α-E2 was administered to rhesus macaques for 12 weeks,

whereas in marmosets, we administered 17α-E2 for only 28 days, constituting a much shorter intervention. It is plausible that long-term administration of 17α-E2 may induce feminizing effects in male primates, while shorter times of administration do not exert such effects. Another factor that might be associated with differences in feminization levels observed between species might be differences in SHBG. The abundance and function of SHBG is known to vary between species [176], which may differentially regulate 17α-E2 availability to target tissues.

There are important limitations to consider regarding the current study. First, our study is likely underpowered due to the limited number of animals available for enrollment. This almost certainly affected our ability to detect statistically significant differences across timepoints. Second, our study lacked an untreated control group for the reasons outlined above. As such, each animal served as their own control which limited our ability to detect potential environmental disturbances that could affect phenotypes within the colony. Fortunately, all the outcomes reported herein are subtle, which is suggestive that environmental disturbances either did not occur or were transient in nature during the intervention period. Third, our study was short in duration, which enabled us to evaluate tolerability, but prevented us from comprehensively assessing variables related to metabolic homeostasis. Forth, our food intake analyses were only performed at a single timepoint at baseline, day 14, and day 28, which is less sensitive than the common practice of evaluating several consecutive days. Lastly, oral administration of hormone therapies is an antiquated approach for clinical administration; thus, alternative delivery approaches should be considered during future studies. For instance, transdermal patches offer a slow-release option that could potentially address some of the issues associated with bolus delivery. Despite these limitations, the primary objectives of our trials were to evaluate the tolerability of 17α-E2 and to assess metabolic and endocrine responses over a relatively

short treatment period, which we successfully achieved with the animals available for study.

In summary, the data presented herein is the first to show 17α-E2 treatment effects in male common marmosets. Our study demonstrates that doses in the ranges of 0.37-0.47 and 0.62-0.72 mg/kg/day are well-tolerated by male common marmosets. 17α-E2 treatment did elicit mild reductions of body mass and/or adiposity without causing feminization, although these changes did not rise to a level of statistical significance. Hence, we conclude that future studies that use larger sample sizes and alternative delivery methods, such as transdermal patches, will be needed in an effort to establish definitive 17α-E2 dosing paradigms that maximize beneficial metabolic effects and minimize feminization in higher order mammals.

# CHAPTER IV

## DISCUSSION AND FUTURE DIRECTIONS

17α-E2 has shown promise as a potential therapeutic for alleviating age- and metabolic- related detriments due to its ability to extend health-span and lifespan in male mice in a non- feminizing manner [42-43]. However, translatability of 17α-E2 is limited by our current lack of understanding of the modes of action underlying its numerous health benefits. In the current report, I aimed to determine if the beneficial effects of 17α-E2 is translatable to rat model, and if the doses of 17α-E2 may be tolerable in higher order mammals such as male common marmosets. My studies found that 17α-E2 benefits on body mass and adiposity is translated into a heterogenous rat model, and it also tolerated in male common marmosets. These findings greatly contribute to our understanding of the beneficial effects of 17α-E2 in multiple different species. Future studies with 17α-E2 will both advance the compound towards clinical use as an adjunct therapeutic as well as enhance our understanding of sexually divergent mechanisms of action on health and lifespan.

Starting with Chapter II, the OKC-HETB/W rat model illustrates the dynamic relationship between mitochondrial genotype, sex, and physiological sensitivity to HFD and 17α-E2. Interestingly, one of the female rats' mitochondrial genotypes was associated with increased grip strength, while the other mitochondrial genotype as associated with enhanced exercise endurance. These results show that mitochondrial genotypes (which frequently interacts with sex) have an enormous influence on metabolic responses, contrary to what was previously reported in mice [89]. The B genotype rats experienced compensatory increases in mitochondrial biogenesis and respiratory rates during HFD,

whereas the W genotype rats had reduced Complex I and lower respiratory rates (mainly in females) [89]. This observation is particularly remarkable as female rats tend to be more resistant to fat accumulation and loss of insulin sensitivity [130].

In contrast to previous experiments in mice [58, 61-62, 74-75, 107-109], female rats of both mitochondrial genotypes were responsive to 17α-E2 administration as evidenced by an attenuation of HFD-induced gains in body mass and liver mass. Only the male B-genotype were responsive to 17α-E2, which indicates that genotype-specific responses occur in rats, which conflicts with mice [57-58, 61-63, 76, 109-110]. What drives these genotype-specific responses are not yet fully understood. Further, hippocampus alterations in stress responses and Sod2 acetylation are evidence of a mitochondrial role in cognitive deterioration that should be investigated more closely [122] in the future studies. The, data presented in Chapter II emphasizes the importance of further investigating how mitochondrial dysfunction might serve a direct role in aging and responsiveness to different treatment options.

Chapter III looks at the effect of 17α-E2 administration on male common marmosets, which determined that the compound is well tolerated as evidenced by no major adverse effects. This is consistent with other studies on the safety of 17α-E2 in humans and other animals [43, 163-164, 170]. We also noticed subtle losses in mass and adiposity, although the magnitude of these effects was not as robust as those seen in male mice [76]. The incongruent effects on fasting glucose and insulin in this study points to a transient environmental stressor since hyperglycemia is often accompanied by hyperinsulinemia that is compensatory in nature. Unexpectedly, despite allometrically scaling the dose of 17α-E2 in mice up to marmosets, the circulating level of 17α-E2 was several folds higher than that observed in mice, but no negative hormonal feminization was observed. This finding is contrary to male rhesus monkeys who did show feminization

following prolonged treatment [139]. This leads to questions of species differences and treatment duration effects. Because the sample size was small and there wasn't a stand-alone control group, additional studies will be needed to disentangle the mechanisms underlying these outcomes. Future trials should employ different forms of 17α-E2 administration, such as transdermal patches. Overall, 17α-E2 treatment in male common marmosets has potential to beneficially affect metabolic pathways without feminization and should be pursued for proper dosing guidelines.

Through these studies I have shown that 17α-E2 is effective in rat model and it could be used safely in common marmosets. 17α-E2 is particularly beneficial in male mice. Women tend to live longer and develop metabolic diseases later in their life [177-178]. The loss of endogenous estrogen at menopause is also linked to an increased risk of metabolic disease [179-180]. We have to figure out if health benefits of 17α-E2 in male mice occurs via nuclear transcription or membrane-bound ERα signaling. These mechanisms will need to be explained in future research through nuclear-only (NOER) and membrane-only (MOER) mouse models. Overall, my work shows 17α-E2's clinical potential as a therapy for metabolic and age-related conditions. I demonstrate that 17α-E2 promotes systemic metabolic benefits in both male and female heterogenous rats, and the dose of 17α-E2 is well tolerated by male common marmosets – all important precursors for its clinical potential as an adjunct to hormonal therapy and as a key tool for exploring sex differences in aging and the prevention of metabolic disease.

# CHAPTER V

## SUMMARY

Collectively, the studies in my dissertation made it so much easier to understand whether 17α-E2 cause metabolic benefits in heterogenous rat model and if the dose of 17α-E2 is well tolerated by male common marmosets.

The main findings of my studies were these:

1. In our rats, we found mitochondrial genotype-dependent responses with only B genotype males showing decreases in body mass, fat, and liver mass, while female rats of both genotypes had a beneficial effect under 17α-E2 treatment because the HFD-induced increases in body mass and fat were inhibited. (Chapter II).

2. We have also shown that male common marmosets tolerate doses of 0.37-0.47 and 0.62-0.72 mg/kg/day with high tolerance. The 17α-E2 treatment caused slight alterations in body mass and/or fat (with no feminization), although not enough to be statistically significant (Chapter III).

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