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# 17α-estradiol does not adversely affect sperm parameters or fertility in male mice: implications for reproduction-longevity trade-offs

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**Abstract** 17α-estradiol (17α-E2) is referred to as a nonfeminizing estrogen that was recently found to extend healthspan and lifespan in male, but not female, mice. Despite an abundance of data indicating that 17α-E2 attenuates several hallmarks of aging in male rodents, very little is known with regard to its effects on feminization and fertility. In these studies, we evaluated the effects of 17α-E2 on several markers of male reproductive health in two independent cohorts of mice. In alignment with our previous reports, chronic 17α-E2 treatment prevented gains in body mass, but did not adversely affect testes mass or seminiferous tubule morphology. We subsequently determined that chronic 17α-E2 treatment also did not alter plasma 17β-estradiol or estrone concentrations, while mildly increasing plasma testosterone levels. We also determined that chronic 17α-E2 treatment did not alter plasma follicle-stimulating hormone or luteinizing hormone concentrations, which suggests 17α-E2 treatment does not alter gonadotropin-releasing hormone neuronal function. Sperm quantity, morphology, membrane integrity,

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and various motility measures were also unaffected by chronic 17α-E2 treatment in our studies. Lastly, two different approaches were used to evaluate male fertility in these studies. We found that chronic 17α-E2 treatment did not diminish the ability of male mice to impregnate female mice, or to generate successfully implanted embryos in the uterus. We conclude that chronic treatment with 17α-E2 at the dose most commonly employed in aging research does not adversely affect reproductive fitness in male mice, which suggests 17α-E2 does not extend lifespan or curtail disease parameters through tradeoff effects with reproduction.

**Keywords** Aging · Androgen · Estrogen · Hypothalamic-pituitary–gonadal axis · Reproduction

## Introduction

Despite significant increases in human lifespan over the past several decades, human healthspan has failed to increase in a similar fashion. In fact, the period of morbidity in mid-to-late life, and prevalence of multimorbidity, has increased dramatically in recent decades [1]. Although it is well established that dietary interventions including chronic calorie restriction and various forms of fasting can delay and/or reverse basic mechanisms of aging, many of these strategies are poorly tolerated [2]. Compliance issues remain a paramount hurdle with dietary interventions due to adverse effects on mood, thermoregulation, and/or musculoskeletal mass [3]. These adverse health outcomes demonstrate the need for pharmacological approaches aimed at curtailing aging and disease.

17α-estradiol (17α-E2) is one of the more recently studied compounds to demonstrate efficacy for beneficially modulating age- and disease-related outcomes. The NIA Interventions Testing Program found that 17α-E2 administration extends median lifespan of male mice in a dose-dependent manner [4–6]. Our group has reported that 17α-E2 reduces calorie intake and adiposity while concomitantly improving several markers of metabolic homeostasis including glucose tolerance, insulin sensitivity, and ectopic lipid deposition in obese and aged male mice [7–11]. Others have reported similar findings including improvements in glucose tolerance, mTORC2 signaling, hepatic amino acid composition and markers of urea

cycling, markers of neuroinflammation, and sarcopenia [12–16]. Although the mechanisms of action for 17α-E2 remain debated, we recently reported that the ablation of estrogen receptor α (ERα) completely attenuates all beneficial metabolic effects of 17α-E2 in male mice [7], thereby indicating that 17α-E2 signals through ERα to elicit benefits, but also that ERα could be a "druggable" target for mitigating aging and disease in males. Despite an abundance of data demonstrating that 17α-E2 improves a multitude of parameters related to metabolism and aging in males, very little has been done to determine if 17α-E2 promotes significant feminization, and more importantly, if any of these outcomes deleteriously affects male fertility.

Interestingly, several interventions that extend lifespan can also impart some impact on reproduction. For instance, females subjected to calorie restriction (CR), rapamycin, or metformin display a suppression of ovarian primordial follicle activation, thereby extending the reproductive window [17, 18] and improving fertility [19] once treatment has subsided. The effects of lifespan-extending interventions on male reproduction are conflicting and exceedingly unclear. CR is known to reduce libido in men [20] which could be related to a suppression of the reproductive axis [21]. However, other reports indicate that CR does not alter circulating testosterone or semen quality in rhesus macaques [22, 23]. In rodents, moderate CR (20–30%) has been reported to decrease testes and seminal vesicle mass [24], increase sperm defects [24, 25], and suppress serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone [24]. Conversely, other studies have found that CR has no effects on testes mass [26, 27], sperm production [26], serum testosterone [27], or fertility [27]. The effects of rapamycin on male fertility are more consistent and clearly indicate deleterious effects related to testes mass, spermatogenesis, and testosterone production in both rodents and humans [28–30]. To date, no study has thoroughly evaluated the effects of 17α-E2 on sperm parameters or fertility in male model systems.

The work outlined in this report sought to determine if chronic 17α-E2 treatment adversely affected testes morphology, sperm parameters, reproductive fitness, and/or the serum hormonal milieu in middle-aged male mice. Two independent cohorts of mice were studied and found to respond nearly

identically to 17α-E2 treatment. We determined that chronic treatment with 17α-E2 at the dose most commonly employed in aging research does not adversely affect reproductive fitness in male mice. It remains unknown if higher dosing regimens of 17α-E2 induce reproductive abnormalities in rodents, or if allometric scaling to higher-order mammals for the purpose of translational studies can be effectively undertaken.

## Materials and methods

### Control and experimental diets

TestDiet, a division of Purina Mills (Richmond, IN), prepared all the diets. LabDiet 58YP (66.4% CHO, 20.5% PRO, 13.1% FAT) was used as the control (Con) diet, and LabDiet 58YP was supplemented with 17α-E2 (14.4 ppm Steraloids, Newport, RI) for the treatment diet.

### Animals

All mice (C57BL/6) used in these studies were obtained from the UFPel Central Vivarium. Unless otherwise noted, all mice were group housed at $24 \pm 2$ °C on a 12:12-h light–dark cycle with ad libitum access to food and water. At 3 months of age, mice were randomly assigned to Con or 17α-E2 treatment groups. Two cohorts of mice were evaluated independently for comparison purposes and to determine outcome repeatability. In Cohort 1, Con ($n = 8$) and 17α-E2 ($n = 10$) treatment groups were evaluated for 5 months. In Cohort 2, Con ($n = 30$) and 17α-E2 ($n = 30$) treatment groups were evaluated for 4 months. Body mass was monitored monthly. During the final week of treatment, male mice from both cohorts were housed with 3-month-old female mice (1 male with 2 females) that had undergone estrus synchronizations so male fertility could be assessed. Female mice were synchronized with IP injections of equine chorionic gonadotrophin (eCG; 5UI) 2 days prior to exposure to males and human chorionic gonadotrophin (hCG; 5UI) when housed with males. Male mice were humanly euthanized with isofluorane 1 week after being paired with females and plasma, testes, and semen were collected. Females that were bred with males from Cohort 1 were humanely euthanized with isoflurane 3 days after being paired with

males so that embryos could be collected as previous described [31]. Any female found to have at least one embryo was deemed to have been successfully fertilized by the corresponding male. Females that were bred with males from Cohort 2 were humanely euthanized with isoflurane 8 days after being paired with males so that the number of implantation sites could be counted. All procedures were approved by the Ethics Committee for Animal Experimentation from the Universidade Federal de Pelotas.

### Semen collection and analysis

Following euthanasia of males, the epididymis was removed and placed into a microtube containing 300 μL of preheated (36.5 °C) extender solution (3.634 g TRIS, 0.50 g glucose, 1.99 g citric acid, 6.0 g BSA, 100 ml of $H_2O$). The epididymis was then gently fractionated with scissors and agitated for 5 min in order to release the sperm. For sperm concentration analyses, 25 μL of the semen solution was aliquoted and diluted with 25 μL of formaldehyde-saline (8.5 g NaCl, 100 ml of 40% formaldehyde, 900 ml of $H_2O$). Concentration was determined by counting sperm cells using a hemocytometer. Sperm motility was evaluated using a Zeiss Axio Scope A1 microscope (Jena, Germany) coupled to a computer-assisted semen analysis system (CASA, SpermVision, Minitube, Tiefenbach, Germany). For this assay, 6 μL of semen solution was placed on a glass slide under a coverslip and observed at $200 \times$ magnification. The CASA system determined velocity average path (VAP), velocity curved line (VCL), velocity straight line (VSL), beat cross frequency (BCF), amplitude of lateral head displacement (ALH), total motility (TMO), and progressive motility (PMO). The automated system evaluated a minimum of 500 cells for each sample with an average of 822.6 and a range of 530 to 974 cells/sample. The membrane integrity of sperm cells was assessed by a hypoosmotic swelling test (HOS) [32], which is done by determining the % of sperm tail swelling following the exposure to a hypoosmotic solution. For this analysis, 10 μL of the semen solution was aliquoted and diluted with 90 μL of HOS solution (2.7 g of fructose, 1.47 g of sodium citrate, and 100 ml of H2O) and incubated at 36.5 °C for 30 min. After incubation, 200 cells were counted as non-swollen or swollen tails using an Olympus BX 51 epifluorescence microscope (América INC,

São Paulo, SP) at 400× magnification. As a negative control, 6 µL of semen solution was smeared on a glass slide before incubation with HOS solution and 200 cells were counted for swollen tails. The percentage of membrane integrity was calculated by the number of sperm with swollen tails before and after incubation as previously described [33]. For the acrosome integrity analysis, 3 µL of semen solution was aliquoted and diluted with 3 µL of a buffer containing Lectin from *Arachis hypogaea* FITC conjugate (Sigma-Aldrich, Saint-Louis, MO, USA) and incubated at room temperature in the dark for 15 min. In this analysis, by observing the emission of green fluorescence, the number of intact acrosome cells was counted in a total of 100 cells. This analysis was performed on an Olympus BX 51 epifluorescence microscope at 400× magnification (América INC, São Paulo, SP, Brazil) using a WU filter (450–490 nm excitation and 516–617 emission) as previously described [34]. Lastly, sperm were also evaluated for morphological defects. For this, a drop of semen was smeared on a glass slide and dried. Slides were then stained using a fast-panoptic staining method [35]. One hundred cells were observed on a Nikon Eclipse E200 microscope (Tokyo, Japan) at 40× magnification, and the number of normal cells or those with defects (head, midpiece, tail) were recorded [35].

### Testes histology

One of the testes was fixed in formalin, dehydrated in alcohol, cleared in xylol, and embedded in paraplast (Sigma-Aldrich, Saint-Louis, MO, USA). Paraplast blocks were transversally cut into 5 µm sections starting in the middle of the testicle. The sections were then dewaxed in xylene, rehydrated in descending series of alcohol, and stained with H&E. H&E stained sections were photographed by a camera coupled to a microscope using the software TC Capture (Tucsen Photomics Co.) at 40× magnification on a microscope (Nikon Eclipse E200, Nikon, Tokyo, Japan). In the images, twenty round or near round seminiferous tubules were randomly chosen for each animal. Perimeter, area, and diameter of the seminiferous tubules and its lumen were measured using the Motic 2.0 software (Motic®, Hong Kong, China). For statistical comparison, the mean measurements from 20 seminiferous tubules were used.

### Circulating hormone analyses

Plasma testosterone, 17β-E2, 17α-E2, and estrone were evaluated by LC/MS/MS as previously described [11]. Plasma FSH and LH levels were evaluated by ELISA (FSH: KA2330, Abnova, Taipei, Taiwan; LH: ABIN6574077, Antibodies-online, Limerick, PA, USA).

### Statistical analyses

Statistical analysis was performed using the GraphPad Prism 6 or SPSS version 28. The Shapiro–Wilk test was performed to test normality. Body weight was compared by repeated measures ANOVA, and percentage of fertilized females and viable embryos was compared by chi-square, whereas the other variables were compared by Student's *t*-test. For analysis of testes mass relative to body mass, we used a general linear model, including testes mass as the dependent variable, treatment as a fixed factor, and body weight as a continuous covariate. Non-parametric variables from CASA were compared by Mann–Whitney test. *P*-values lower or equal to 0.05 were considered significant.

## Results

### 17α-E2 treatment does not affect testes mass or seminiferous tubule morphology

Similar to our previous reports [7, 10, 11], 17α-E2 treatment reduced male body mass in both cohorts we evaluated (Fig. 1A, B, E and F). We did not evaluate adiposity in these studies but we have previously established that reductions in adipose accounts for the vast majority of declines in mass during 17α-E2 treatment [7, 10, 11]. We next sought to determine if chronic 17α-E2 treatment altered testes mass or seminiferous tubule morphology. We found that testes mass was unaffected by 17α-E2 treatment in both cohorts of animals tested (Fig. 1C, G). Evaluation of testes mass relative to body mass revealed no association between the two variables (Fig. 1D, H), which is aligned with a previous report indicating that testes mass is only mildly correlated with body mass in mice [36]. Subsequent analysis of seminiferous tubules within the testes revealed that 17α-E2
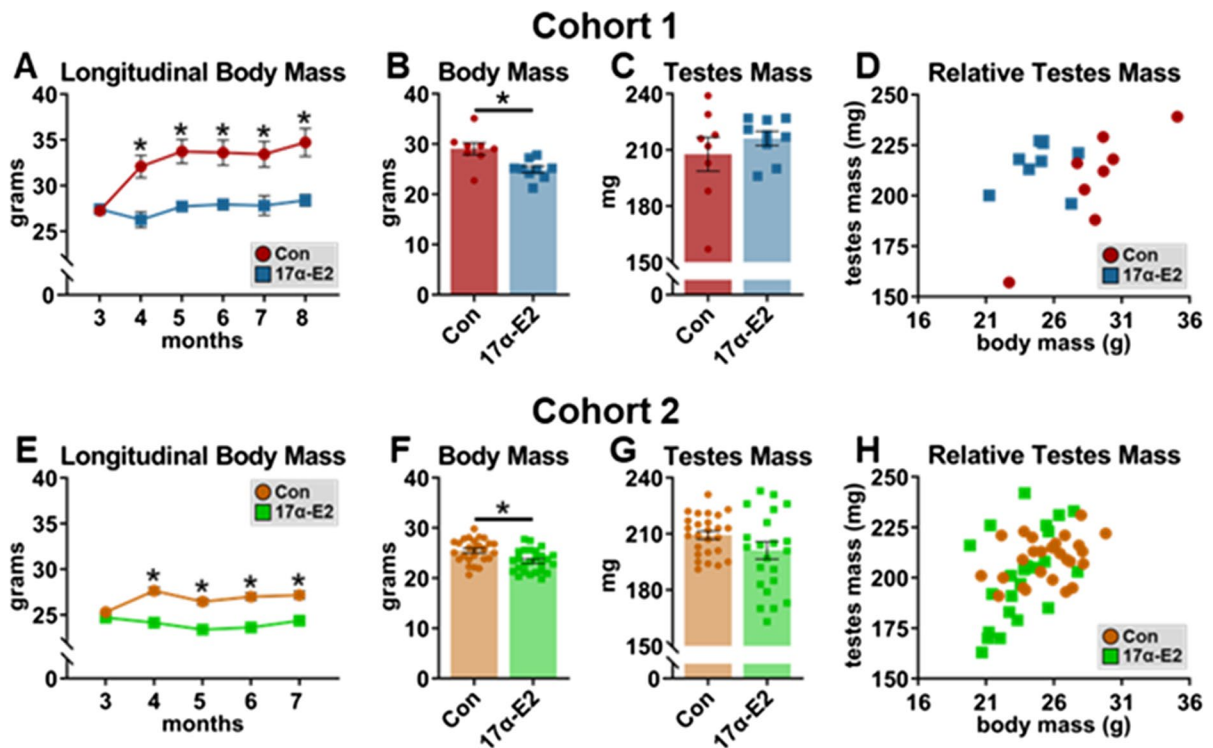
**Fig. 1** Chronic 17α-E2 treatment decreases body mass, but not testes mass, in male mice. **A** Longitudinal changes in body mass, **B** body mass at necropsy, **C** testes mass at necropsy, and **D** relative testes mass at necropsy in control (Con) and 17α-E2 treated male mice over a 5-month period (Cohort 1). **E** Longitudinal changes in body mass, **F** body mass at necropsy, **G** testes mass at necropsy, and **H** relative testes mass at nec-ropsy in Con and 17α-E2 treated male mice over a 4-month period (Cohort 2). Mice received LabDiet 58YP ± 17α-E2 (14.4 ppm) throughout the intervention periods. All data are presented as mean ± SEM and were analyzed by repeated measures ANOVA, Student's $t$-test, or ANCOVA where appropriate. $n = 8$–10/group for cohort 1 and 30/group for cohort 2. *$p < 0.05$

treatment did not adversely affect the tubule or lumen morphology (Fig. 2A–B) or size (Fig. 2C–H). This latter observation suggests that 17α-E2 treatment does not alter sperm parameters because changes in seminiferous tubule size, particularly lumen size, are associated with declines in sperm quality [37].

17α-E2 treatment does not alter circulating sex hormones or gonadotropins

Given that 17α-E2 is known to be a mild 5α-reductase inhibitor [38], coupled with several reports suggesting that 17α-E2 treatment elicits health benefits by modulating androgen metabolism [12, 13, 15], we next sought to determine if the endogenous sex hormone milieu was altered in the current study. As expected, plasma 17α-E2 was robustly increased with 17α-E2 treatment (Fig. 3A). Surprisingly, the plasma

levels of 17α-E2 observed in the current study were several times higher than we found in much older mice receiving an identical dose in a very similar diet [11]. Despite the higher level of plasma 17α-E2 in the current study, plasma 17β-E2 was essentially unchanged by 17α-E2 treatment (Fig. 3B). Plasma estrone (Fig. 3C) was undetectable (< 3.3 pg/ml) in most samples, and thus, nearly identical between Con and 17α-E2 treated animals. Plasma testosterone (Fig. 3D) was not statistically different between treatment groups, but was found to be trending higher in mice treated with 17α-E2. This observation may result from the aforementioned 5α-reductase inhibition properties of 17α-E2, which may increase circulating testosterone levels due to a reduced capacity to convert testosterone into dihydrotestosterone (DHT) [39]. Due to limited plasma volumes, we were unable to directly evaluate DHT in these studies. However,
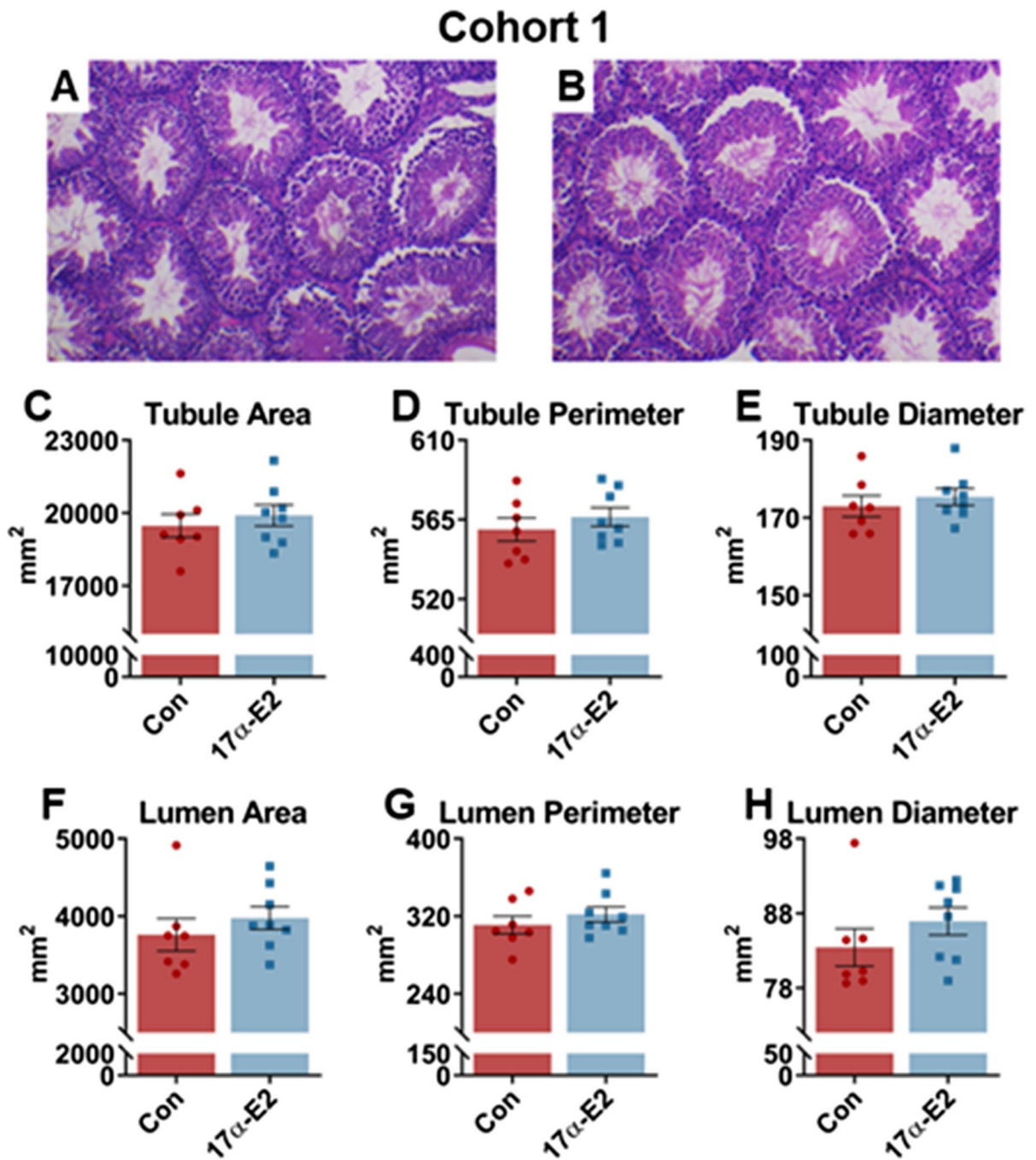
# Cohort 1



**Fig. 2** Chronic 17α-E2 treatment does not affect seminiferous tubule morphometry in male mice. Representative H&E stained sections of seminiferous tubules at 40×magnification from **A** control (Con) and **B** 17α-E2 treated male mice following a 5-month intervention period. Seminiferous **C** tubule area, **D** tubule perimeter, **E** tubule diameter, **F** tubule lumen area, **G** tubule lumen perimeter, and **H** tubule lumen diameter from Con and 17α-E2 treated male mice following a 5-month intervention period. Mice received LabDiet 58YP±17α-E2 (14.4 ppm) throughout the intervention timeframe. All data are presented as mean±SEM and were analyzed by Student's *t*-test. *n*=7–8/group
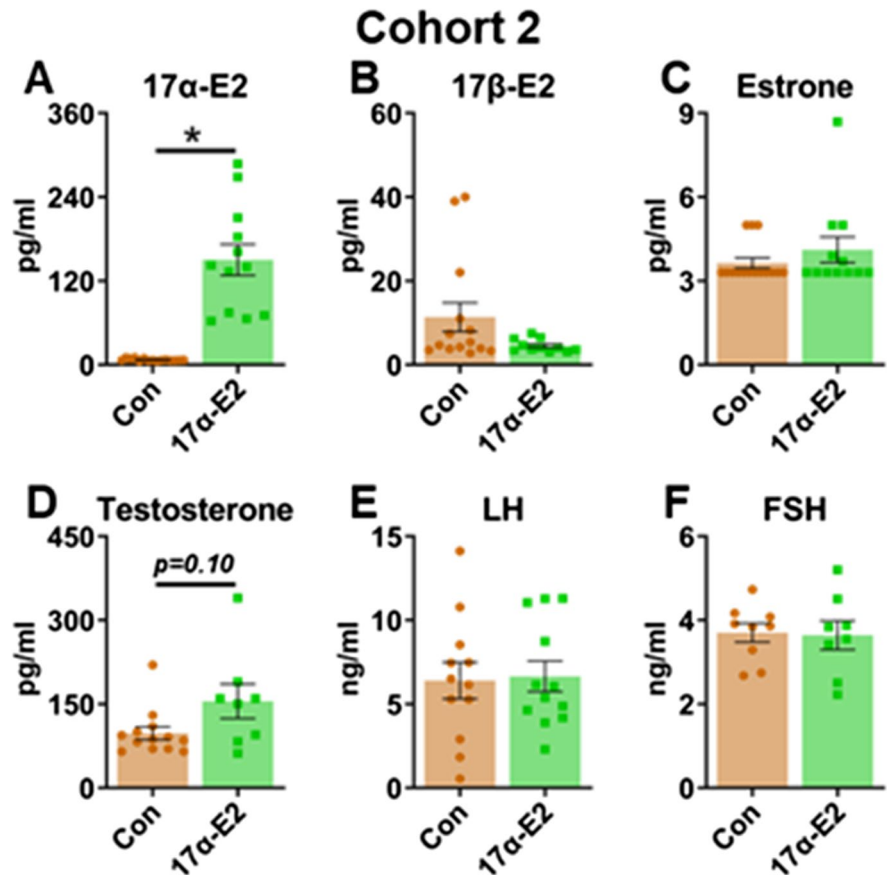
we did evaluate the effects of 17α-E2 treatment on circulating gonadotropins because estrogens are known to signal in the hypothalamus and pituitary as part of a negative feedback loop to control gonadotropin production and secretion [40]. We found that plasma LH (Fig. 3E) and FSH (Fig. 3F) were not significantly altered by 17α-E2 treatment, suggesting that exogenously administered 17α-E2 does not influence androgen-mediated hypothalamic-pituitary–gonadal (HPG) signaling in male mice.

**17α-E2 treatment does not alter sperm morphology, quantity, or motility**

Males with higher-than-normal plasma 17β-E2 often display low sperm counts and/or declines in sperm quality to include motility [41], which contributes to infertility [42]. Therefore, we sought to determine if chronic 17α-E2 treatment would adversely affect sperm parameters. Morphological assessment of sperm from both cohorts of mice revealed that 17α-E2 treatment did not diminish the quantity of normal sperm, or increase sperm with apparent defects, including decapitation (Fig. 4). Subsequent evaluation of sperm concentrations and various measures of motility, including straight and curvilinear velocities, amplitude of lateral head displacement, and beat cross frequency, were not different between Con and 17α-E2 treatment groups in both cohorts of mice tested (Table 1). Although these variables do not directly predict fertility outcomes, they are useful indicators of sperm quality because they represent sperm kinetics [43]. Both sperm cell membrane integrity and acrosomal integrity, which are important factors for determining fertilizing capacity [44], were found to be unaffected by 17α-E2 treatment in cohort 1.

**17α-E2 treatment does not adversely affect male fertility**

In addition to the variables outlined above, we also wanted to determine if chronic 17α-E2 treatment would adversely affect male fertility. To do this, we
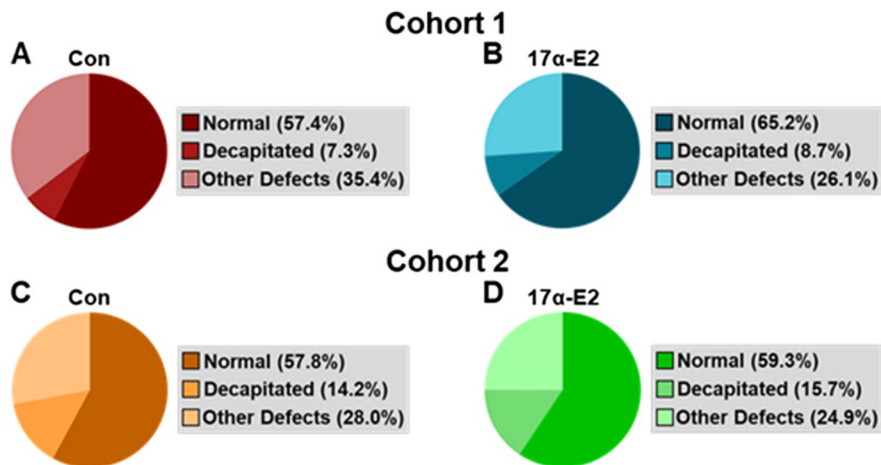
**Fig. 4** Chronic 17α-E2 treatment does not affect sperm morphology in male mice. Percentage of normal, decapitated, or otherwise defective sperm from **A** control (Con) and **B** 17α-E2 treated male mice following a 5-month intervention period (Cohort 1). Percentage of normal, decapitated, or otherwise defective sperm from **C** Con and **B** 17α-E2 treated male mice following a 4-month intervention period (Cohort 2). Mice received LabDiet 58YP ± 17α-E2 (14.4 ppm) throughout the intervention periods. $n = 8–10$/group for cohort 1 and 25/group for cohort 2

subjected male mice from both treatment groups to female mice that had undergone estrus synchronization. We then determined the percentage of successful fertilizations in cohort 1, and the pregnancy rates and number of implanted embryos in cohort 2. We found that Con and 17α-E2 treated males were equally effective at fertilizing females in cohort 1 and at successfully fertilizing oocytes that result in implanted embryos in the cohort 2 (Table 1). These findings clearly indicate that the 17α-E2 dosing regimen used in these studies does not adversely affect the propensity for male mice to breed, or their ability to fertilize oocytes that become implanted embryos.

## Discussion

17α-E2 treatment was recently found to extend healthspan and lifespan in male mice [4, 5, 7–16]. Despite several lines of evidence indicating that 17α-E2 delays aging hallmarks in male rodents, very little is known with regard to its effects on feminization, sperm quality, and fertility. We previously reported that 17α-E2 administration only mildly affects circulating testosterone levels, gonadal mass, or seminal vesicle mass in aged male mice [11]. Although informative, the mice evaluated in those studies were over 18 months of age, and thus, well-beyond their reproductive window. Therefore, it remains unclear if

17α-E2 administration adversely affects reproductive fitness in breeding age males. In the current studies, we evaluated the effects of chronic 17α-E2 treatment on several parameters indicative of male reproductive health, in addition to directly assessing fecundity in young, breeding age male mice.

In alignment with our previous reports, 17α-E2 administration prevented gains in body mass in male mice in these studies. We previously established that 17α-E2-mediated changes in body mass are due to an almost exclusive loss of adiposity, which we later linked to the modulation of hypothalamic anorexigenic signaling pathways [7, 10, 11]. Given the close association between hypothalamic regulation of metabolism and reproduction [45], coupled with the close proximity of hypothalamic neurons that control satiety, metabolism, and reproduction [45], we speculated that 17α-E2 treatment would also modulate the hypothalamic-pituitary–gonadal (HPG) axis in a manner that would attenuate reproductive vigor. Surprisingly, we found no evidence that chronic 17α-E2 treatment altered reproductive health in male mice. Both testes mass and seminiferous tubule morphology were unchanged following several months of 17α-E2 treatment in our studies. These observations provided the first indication that 17α-E2 treatment does not alter reproductive health in male mice, at least at the dose known to extend lifespan [4].

**Table 1** Sperm parameters and fertility in control and 17α-estradiol treated male mice

**Cohort 1**

| Sperm parameters | Con ($n=8$) | 17α-E2 ($n=10$) | $p$ value |
|---|---|---|---|
| Epididymal concentration ($\times 10^4$ cells/uL) | $2.3 \pm 0.8$ | $1.8 \pm 0.9$ | 0.28 |
| Motility (%) | $65.6 \pm 3.9$ | $62.7 \pm 4.8$ | 0.66 |
| Progressive motility (%) | $52.4 \pm 4.9$ | $50.0 \pm 5.5$ | 0.64 |
| Average path velocity (VAP) | $120.4 \pm 5.6$ | $127.6 \pm 4.9$ | 0.67 |
| Curvilinear velocity (VCL) | $229.3 \pm 10.1$ | $233.9 \pm 11.0$ | 0.76 |
| Straight line velocity (VSL) | $83.7 \pm 4.5$ | $85.9 \pm 2.7$ | 0.67 |
| Amplitude of lateral head (ALH) | $9.8 \pm 0.3$ | $10.1 \pm 0.4$ | 0.61 |
| Beat cross frequency (BCF) | $17.9 \pm 0.2$ | $17.6 \pm 0.2$ | 0.20 |
| Hypoosmotic swelling test ($\Delta$%) | $28.3 \pm 3.2$ | $21.0 \pm 2.9$ | 0.18 |
| Acrosomal integrity (%) | $34.5 \pm 4.0$ | $36.9 \pm 3.3$ | 0.65 |
| Fertility parameters | Con ($n=16$) | 17α-E2 ($n=18$) | $p$ value |
| Fertilized females (%) | 75.0 | 94.4 | 0.11 |

**Cohort 2**

| Sperm parameters | Control ($n=22$) | 17α-E2 ($n=21$) | $p$ value |
|---|---|---|---|
| Epididymal concentration ($\times 10^4$ cells/uL) | $1.6 \pm 0.6$ | $1.6 \pm 0.6$ | 0.95 |
| Motility (%) | $57.9 \pm 3.9$ | $59.0 \pm 4.4$ | 0.85 |
| Progressive motility (%) | $46.8 \pm 4.0$ | $46.8 \pm 4.6$ | 0.60 |
| Average path velocity (VAP) | $72.2 \pm 2.4$ | $71.1 \pm 2.4$ | 0.76 |
| Curvilinear velocity (VCL) | $134.6 \pm 4.9$ | $131.3 \pm 4.9$ | 0.39 |
| Straight line velocity (VSL) | $45.5 \pm 1.5$ | $45.0 \pm 1.7$ | 0.83 |
| Amplitude of lateral head (ALH) | $4.1 \pm 0.1$ | $4.0 \pm 0.1$ | 0.33 |
| Beat cross frequency (BCF) | $30.0 \pm 0.2$ | $30.3 \pm 0.2$ | 0.46 |
| Fertility parameters | Con ($n=40$) | 17α-E2 ($n=40$) | $p$ value |
| Pregnancy rate (%) | 20.0 | 22.5 | 0.78 |
| Implanted embryos | $13.6 \pm 1.3$ | $14.6 \pm 2.7$ | 0.77 |

Data presented as mean ± SEM with the exception of % fertilized females and % pregnancy rate

Our subsequent analyses provided additional evidence indicating that chronic 17α-E2 treatment fails to adversely affect reproductive health in male mice. First, 17α-E2 treatment did not alter the endogenous sex hormone milieu as evidenced by only mild changes in plasma 17β-E2, estrone, or testosterone. Interestingly, circulating testosterone trended slightly higher in the 17α-E2 treatment group, which we speculate is due to 17α-E2 being a mild 5α-reductase inhibitor [38], thus limiting the conversion of testosterone into DHT. Since we did not evaluate DHT in these studies, future experiments will be needed to determine if 17α-E2 treatment alters circulating DHT in male mice, and more importantly, if this potential change underlies benefits attributed to 17α-E2 treatment. It

should be noted that Garratt et al. previously reported that responsiveness to 17α-E2 was significantly attenuated in castrated male mice [13], which could be at least partially due to a lack of endogenous DHT and its effects on adiposity [46]. Regardless of the potential effects of 17α-E2 treatment on circulating DHT, our findings clearly demonstrate that chronic 17α-E2 treatment does not feminize the endogenous sex hormone milieu, which suggests 17α-E2 does not directly inhibit Leydig cell steroidogenesis as has been shown with 17β-E2 [47]. Furthermore, we also determined that chronic 17α-E2 treatment does not suppress plasma gonadotropins, LH, or FSH. This observation suggests that the dose of 17α-E2 employed in our studies does not interfere with gonadotropin-releasing

hormone (GnRH) neuronal function, which stimulates the pituitary to produce and secrete LH and FSH [45]. However, it should be noted that GnRH-mediated production of LH and FSH in the pituitary relies heavily on GnRH pulse frequency and amplitude as opposed to operating in a binary fashion [48]. Since GnRH pulse frequency and amplitude have recently been linked to male aging [48], it would be prudent for future studies to evaluate the direct effects of 17α-E2 on GnRH neuronal function and/or neuronal populations shown to modulate GnRH neuronal activity, including agouti-related peptide (AgRP), neuropeptide Y (NPY), proopiomelanocortin (POMC), and kisspeptin (KISS) [48].

Based on the lack of adverse effects of chronic 17α-E2 treatment on testes mass, seminiferous tubule morphology, and the sex hormone milieu, we surmised that sperm parameters and fertility would also be unaffected by 17α-E2 administration. Indeed, we found no evidence suggesting that chronic 17α-E2 treatment perturbed sperm quantity or quality. This suggests that our dose of 17α-E2 does not elicit ERβ responsiveness in Sertoli cells due to its role in regulating spermatogenesis [49]. If true, this would support our previous work suggesting that 17α-E2 elicits the vast majority of its benefits through ERα [7]. Given that sperm parameters were unchanged by 17α-E2 treatment, it came as little surprise that fertility was also unaffected in animals receiving 17α-E2. Two different approaches were used to evaluate male fertility. In the first cohort, we assessed the percentage of females that had fertilized oocytes in their oviducts following exposure to males from control or 17α-E2 treatment groups. We found that control and 17α-E2 treated mice successfully fertilized a similar percentage of females, with 17α-E2 treated mice actually fertilizing a greater percentage of females they were paired with. In the second cohort, we evaluated the ability of control and 17α-E2 treated males to produce embryos that successfully implant in the uterus. We found that pregnancy rates and the number of implanted embryos were essentially identical in female pairs with control and 17α-E2 treated males. Since oocyte fertilization and embryo implantation represents the gold-standard for evaluating fertility, these observations strongly indicate that the dose of 17α-E2 employed in our studies does not adversely affect male fertility.

There are a few caveats to our studies that should be noted. First, we were unable to evaluate plasma DHT in our studies due to limited sample availability. Investigating how 17α-E2 potentially modulates plasma DHT could provide additional insight into how 17α-E2 beneficially alters male metabolism and aging; therefore, future studies would be helpful in answering this question. We also did not measure seminal vesicle mass, an organ that is highly sensitive to androgen exposure, particularly DHT [50]. A previous study reporting that plasma testosterone levels were unaffected by 17α-E2 also reported that seminal vesicle mass was slightly, but consistently, reduced following three months of treatment in old male mice [16]. Given that seminal vesicles produce the majority of the proteins found in seminal plasma, which are important for promoting embryo implantation and placental development [51], it is possible that 17α-E2 treatment in males could elicit subtle effects on offspring development in utero. Lastly, our evaluation of circulating LH and FSH in these studies is a nice surrogate marker of central GnRH activity, but they provide only limited insight into GnRH pulse frequency and amplitude, which is reported to play a key role in male aging [48]. Future studies evaluating GnRH pulsatility will provide tremendous insight into a potential mechanism by which 17α-E2 may modulate male aging.

In summary, the data presented herein are the first to show that chronic 17α-E2 treatment does not adversely affect male reproductive health, including testes mass, seminiferous tubule morphology, plasma sex hormone milieu, sperm parameters, and fertility. These observations suggest that 17α-E2 does not extend lifespan or curtail disease parameters through tradeoff effects with reproduction. Future studies are still needed to determine if 17α-E2 is mildly altering GnRH pulsatility through actions in AgRP, NPY, POMC, or KISS neurons, which may underlie its lifespan extending effects.

**Declarations**

**Conflict of interest** The authors declare no competing interests.

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