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Testosterone use in female mice does not impair fertilizability of eggs: Implications for the fertility care of transgender males.

Running title: Egg fertilizability in testosterone treated females

C.B. Bartels[1,2], T.F. Uliasz[1], L. Lestz[1], and L.M. Mehlmann[1*]

[1]Department of Cell Biology, UConn Health, Farmington, CT 06030, USA
[2]Center for Advanced Reproductive Services, Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, UConn Health, Farmington, CT 06030, USA

[*]Correspondence address. Department of Cell Biology, UConn Health, 263 Farmington Ave., Farmington, CT 06030. Phone 001 860-679-2703. E-mail lmehlman@uchc.edu

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1 STUDY QUESTION: Does testosterone use in females affect reproductive potential, particularly
2 with regard to the production of fertilizable gametes?
3 SUMMARY ANSWER: Testosterone cypionate injections given to post-pubertal female mice
4 caused virilization and ovaries were smaller than control ovaries, but ovaries were still
5 responsive to hormonal stimulation and produced fertilizable eggs when superovulated.
6 WHAT IS KNOWN ALREADY: Studies to examine the effects of testosterone on reproductive
7 potential in transgender males are lacking. Recently, a model was developed that simulates
8 many aspects of testosterone use in transgender males in order to look at reproductive effects
9 of testosterone in female mice. This study found masculinizing effects on the mice but did not
10 find significant deficits on the number of ovarian follicles; however, effects of testosterone use
11 on ovarian stimulation and fertilizability of oocytes were not investigated.
12 STUDY DESIGN, SIZE, DURATION: A total of 66, 6-week-old Hsd:NSA(CF-1) female mice and
13 6 Hsd:ICR (CD-1) mice were used for this study. Mice were injected subcutaneously with 400
14 μg testosterone cypionate or sesame oil once a week for 6 weeks and were either sacrificed a
15 week after the 6th injection (active exposure group), or were sacrificed 6-7 weeks after the final
16 testosterone injection (washout group).
17 PARTICIPANTS/MATERIALS, SETTING, METHODS: Both active exposure and washout
18 groups were further subdivided into 3 groups: unstimulated, eCG-stimulated, or eCG/hCG-
19 stimulated. eCG-stimulated mice were sacrificed 44-48 hrs after eCG injection. eCG/hCG-
20 stimulated mice were injected with eCG, followed 48 hrs later with hCG. Mice were sacrificed
21 ~13-18 hrs after the hCG injection. Data collected included daily vaginal cytology, terminal
22 hormone levels and ovary weights, ovarian histology, number of oocytes/eggs collected in each
23 group, and cleavage to the 2-cell stage following in vitro fertilization.
24 MAIN RESULTS AND THE ROLE OF CHANCE: Testosterone cypionate-treated mice had
25 testosterone levels elevated to the level of male mice and ceased cycling. Ovaries were
26 significantly smaller in testosterone-treated mice, but they contained normal cohorts of follicles

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27 and responded to gonadotropin stimulation by ovulating similar numbers of eggs that fertilized
28 and cleaved in vitro.
29 LIMITATIONS, REASONS FOR CAUTION: Our model treated female mice for only 6 weeks,
30 whereas many transgender men use testosterone for many years before considering biological
31 children. Importantly, a mouse system may not perfectly simulate human reproductive
32 physiology.
33 WIDER IMPLICATIONS OF THE FINDINGS: The current standard of care for transgender men
34 who desire biological children is to cease testosterone therapy prior to ovarian stimulation, but
35 the necessity for stopping testosterone is not known. Our model demonstrates that it is possible
36 for testosterone-suppressed ovaries to respond to gonadotropic stimulation by producing and
37 ovulating fertilizable eggs, thereby obviating the need for testosterone cessation prior to ovarian
38 stimulation. In time, these results may provide insights for future clinical trials of fertility
39 treatment options for transgender men.
40

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41 Introduction

42 Transgender males are individuals who were assigned female at birth but identify as males.
43 Many, but not all, transgender males opt to undergo gender-affirming treatment, which can
44 consist of surgery and/or hormone therapy (HT) by long-term administration of testosterone
45 (Quinn et al., 2017). HT improves gender dysphoria through testosterone-driven development
46 and maintenance of desired male secondary sex characteristics; however, a potential adverse
47 effect of testosterone exposure is a decrease in fertility.

48 A recent study by the Williams Institute estimated that about 1.4 million individuals identify as
49 transgender in the United States (Flores, 2016), and there are reports that approximately half of
50 transgender adults desire biological children (Moravek, 2019; Wierckx et al., 2012). The
51 reproductive consequences of HT are still unclear, and both the World Professional Association
52 for Transgender Health (WPATH) and the Endocrine Society recommend that all transgender
53 males be counseled regarding options for fertility preservation before initiating testosterone
54 therapy (Hembree et al., 2017; Meyer, 2009). Transgender males may not consider fertility
55 preservation to be important at the start of testosterone therapy, which can be initiated as early
56 as 14 years old. In addition, assisted reproductive technology centers have little experience in
57 stimulation of peripubertal ovaries, nor in performing transvaginal oocyte harvest in children.
58 Due to a variety of physiological and psychological barriers, ovarian stimulation and oocyte
59 harvest is best avoided in children, if it can be safely postponed to adulthood. A recent study
60 showed that only 2 of 72 (2.8%) young transgender individuals chose to utilize fertility
61 preservation after counseling (Nahata et al., 2019), which reflects a priority for HT initiation to
62 attain features of their affirmed gender while avoiding the delay, invasiveness, or costs of fertility
63 preservation (Armuand et al., 2017; Insogna et al., 2020). The desire for biological children may
64 arise later in life, after months or years of HT exposure.

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65 The approach to fertility options in transgender males already taking HT therefore warrants
66 more investigation. To date, studies to evaluate the impact of HT on reproductive potential for
67 transgender males are lacking (ASRM Committee Opinion, 2015). The options for transgender
68 males presenting for fertility preservation after HT is either surgical oophorectomy to collect
69 ovarian tissue or surgical oocyte retrieval following ovarian stimulation (De Roo et al., 2016;
70 Neblett and Hipp, 2019). Methods for maturation and fertilization of oocytes collected directly
71 from isolated ovarian tissue without hormonal stimulation, while improving, are still considered
72 to be experimental (Yang and Chian, 2018) and to date, there have been no studies to examine
73 this method for fertility preservation in transgender males. Accordingly, if a transgender male
74 presents for fertility treatment now or in the near future and plans to have the pregnancy carried
75 by a cis-female partner or gestational carrier, the best option is in vitro fertilization (IVF) after
76 ovarian stimulation and oocyte retrieval. Due to the unknown effects of high-level testosterone
77 on ovarian response and oocyte quality, the current recommended practice before IVF is
78 discontinuation of testosterone to allow the resumption of menses (Adeleye et al., 2019;
79 Broughton and Omurtag, 2017; Leung et al., 2018). While this treatment regimen can be
80 effective, HT cessation for the purpose of fertility treatment has been reported to cause
81 significant psychological distress in the form of gender dysphoria attributed to the gender-
82 incongruous effects of testosterone withdrawal, estrogen exposure, and menses (Armuand et
83 al., 2017). These negative consequences could lead to treatment avoidance even when fertility
84 is desired.

85 Ovarian tissue taken from HT-exposed transgender males has demonstrated changes including
86 a thickened cortex, stromal hyperplasia, an increased number of atretic follicles, and increased
87 cortical stiffness (De Roo et al., 2019; Ikeda et al., 2013). However, the ovarian tissue follicular
88 pool is not diminished (De Roo et al., 2016; Van Den Broecke et al., 2001) Markers of ovarian
89 reserve, including anti-Müllerian hormone and inhibin, are unchanged (Rodriguez-Wallberg et

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90 al., 2014), and successful pregnancies have been reported after testosterone use (Light et al.,
91 2014). Case reports have been published of subjects successfully undergoing IVF after
92 temporarily discontinuing testosterone therapy for 1-12 months, and healthy live births were
93 reported (Adeleye et al., 2019; Broughton and Omurtag, 2017; Leung et al., 2018). These data
94 suggest that the follicular pool and oocyte quality are preserved.

95 A primary mouse model for HT in transgender males was recently published and found that
96 ovaries from testosterone-treated mice were generally normal, with the exception of some cyst-
97 like late antral follicles (Kinnear et al., 2019). The fertility potential in terms of ovarian response
98 to gonadotropins, oocyte integrity, or fertilizability was not examined. There is very limited
99 information about the necessity for cessation of testosterone therapy prior to ovarian
100 stimulation, and no mouse models have addressed this problem. The aim of the present study
101 was to establish a mouse model in which reproductive potential could be evaluated following
102 prolonged HT in female mice with and without a period of testosterone cessation.

103

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104 Materials and Methods

105 *Ethical approval*

106 Animal studies were performed in accordance with the Guide for the Care and Use of
107 Laboratory Animals (National Academy of Sciences 1996) and were approved by the
108 Institutional Animal Care & Use Committee at UConn Health (protocol number 101977-0122).
109

110 *Media and Reagents*

111 All chemicals were purchased from Millipore Sigma (St. Louis, MO, USA) unless otherwise
112 indicated. Testosterone cypionate was from Steraloids (Newport, RI, USA) and was prepared
113 as an 8 mg/ml solution in sesame oil. Equine chorionic gonadotropin (eCG) was from
114 Calbiochem. The medium for oocyte collection was HEPES-buffered MEMα (Gibco 12000022,
115 Thermo Fisher, Waltham, MA, USA) containing penicillin, streptomycin, and polyvinyl alcohol
116 (PVA), and 10 μM milrinone to prevent spontaneous meiotic maturation (Mehlmann et al.,
117 2019). For overnight oocyte maturation, oocytes were washed into bicarbonate-buffered
118 MEMα (Mehlmann et al., 2019) containing 5% fetal bovine serum (Invitrogen, Carlsbad, CA,
119 USA) without milrinone. For in vitro fertilization, cumulus masses were collected in human tubal
120 fluid medium (HTF; Cook Medical Inc. IVF medium (#K-RVFE; Fisher) containing reduced
121 glutathione. Sperm were capacitated in IVF medium (Mehlmann and Kline, 1994) containing 15
122 mg/ml Fraction V bovine serum albumin.
123

124 *Experimental design*

125 Six-week-old female CF-1 (Envigo, Indianapolis, IN, USA) and >8-week-old male CD-1 mice
126 (Envigo) were used for all experiments. Three female mice were housed per cage in a
127 temperature and light-controlled room on a 14L:10D light cycle. Male mice were housed
128 individually.

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129

130 Six-week-old female mice were lightly sedated with isoflurane and injected weekly,
131 subcutaneously, with 400 μg testosterone cypionate or vehicle using 27-gauge needles. In the
132 first set of experiments, mice were sacrificed within 8 days after the 6th testosterone injection,
133 when testosterone levels were high. These mice are referred to as the "active exposure" group.
134 In the second set of experiments, mice were sacrificed 6-7 weeks after the 6th testosterone
135 injection, when testosterone returned to basal levels. These mice are referred to as the
136 "washout" group. Both groups were subdivided into 3 more groups: mice that were not
137 stimulated with gonadotropins; mice that were stimulated with eCG only; and mice that were
138 stimulated with eCG followed by hCG to induce ovulation.

139

140 For both the active exposure and washout groups, we performed vaginal smears to examine
141 cyclicity. We analyzed the following: serum testosterone levels; serum estrogen levels; ovary
142 weights and histology; oocyte number prior to and after priming with eCG or eCG + hCG;
143 structure of the meiotic spindle; and egg fertilizability. Control mice were injected with sesame
144 oil and were treated in parallel with the testosterone-injected groups. All mice were euthanized
145 by isoflurane overdose followed by cervical dislocation.

146

147 *Vaginal cytology*

148 For the active exposure group, daily vaginal smears were performed using standard methods
149 (Goldman et al., 2007) starting in the 5th week of testosterone treatments. For the washout
150 group, daily vaginal smears were done starting one week after the final testosterone injection.
151 Staging of the estrous cycle was determined by the presence and distribution of leukocytes,
152 cornified epithelial cells, and nucleated epithelial cells. Proestrus was identified by nucleated
153 epithelium, estrus was identified by large cornified epithelial cells, metestrus was identified by

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154 leukocytes and large cornified epithelial cells, and diestrus was identified by the predominance
155 of leukocytes in the presence of nucleated and cornified cells (Gaytan et al., 2017). Clitoral size
156 was visually assessed at the time of cytology.

157 *Blood collection and hormone analysis*

158 After the mice were sacrificed, they were weighed and terminal blood was collected by cardiac
159 puncture using a heparinized 18-gauge needle and syringe. Blood samples were kept on ice.
160 Within 30 minutes of collection, samples were centrifuged at 4°C for 15 minutes (1000 x *g*) and
161 supernatants were stored at -80°C. Diethyl ether extraction was performed as directed by
162 Cayman Chemical, with extracted samples stored at -20°C in Cayman ELISA buffer.
163 Testosterone and estradiol-17β analyses were performed using ELISA kits (Cayman Chemical,
164 Ann Arbor, MI, USA) according to the manufacturer's instructions. For comparison, we also
165 collected blood from mature male mice that were used for in vitro fertilization (see below).
166 These males had been acclimated to the lab for at least one week prior to the experiment, were
167 housed individually, and were not exposed to females prior to blood collection.

168 *Ovarian histology*

169 Ovaries were collected and most of the fat and oviducts were removed by dissection under a
170 stereoscope and were then weighed. Ovaries were fixed in 10% formalin for 24-48 hours,
171 washed into PBS, then were dehydrated in ethanol, embedded in paraffin, and 5 μm serial
172 sections were cut and processed by the Histology Core at UConn Health. Sections were
173 stained with hematoxylin and eosin.

174 Total numbers of antral follicles and corpora lutea were counted. Antral follicles were defined as
175 being ~250-320 μm in diameter with a clearly visible antral cavity and oocyte with two or more
176 layers of granulosa cells. Preovulatory follicles were defined as being >320 μm in diameter.
177 Each antral follicle was counted only when the oocyte was present and while scanning between

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178 adjacent sections to prevent duplicate counting. Corpora lutea were defined as discrete
179 eosinophilic round structures. Corpora lutea were numbered as the sections were serially
180 assessed through the entire ovary to prevent duplicate counting. Two of the investigators
181 independently counted follicles.

182 *Oocyte collection and immunofluorescence staining*

183 Ovaries from unstimulated and eCG-primed mice were weighed and one ovary from each
184 mouse was fixed for histological analysis while the contralateral ovary was used for oocyte
185 collection. The ovary for oocyte collection was placed in HEPES-buffered MEMα containing
186 milrinone and punctured using a 30-gauge needle. Oocytes were collected with a mouth pipet
187 and counted. For in vitro maturation, oocytes were washed into bicarbonate-buffered MEMα
188 without milrinone and were incubated overnight at 37°C in a humidified incubator containing 5%
189 CO_2/95% air. In vitro maturation was confirmed by the disappearance of the nuclear envelope
190 and the formation of first polar bodies using a stereoscope. Oocytes were fixed for 30-60 min at
191 37°C in 2% formaldehyde, 100 mM HEPES, 50 mM EGTA, 10 mM $MgSO_4$, and 0.2% Triton X-
192 100, then were permeabilized in PBS containing 0.1% Triton X-100, and blocked for at least 15
193 minutes in PBS containing 3% BSA and 0.01% Triton X-100. Oocytes were incubated overnight
194 at 4°C in primary antibody against tubulin (YL1/2; Serotec Inc., Raleigh, NC) diluted to 10 μg/ml
195 in blocking buffer. After washing in PBS-PVA, oocytes were incubated in Alexa488-conjugated
196 secondary antibody for 1 hr at room temperature in the dark. Oocytes were washed in PBS-
197 PVA containing 5 μM SYTOX Orange (ThermoFisher) to label chromosomes. Imaging for
198 spindle integrity was performed using a Zeiss Pascal confocal microscope with a 40X, 1.2 NA
199 water immersion objective (C-Apochromat; Carl Zeiss MicroImaging, Inc., Thornwood, NY,
200 USA).

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202 *In vitro fertilization*

203 Female mice were superovulated with 5 IU eCG, followed 48 hrs later with 5 IU hCG.
204 Approximately 13-15 hrs later, ovaries and oviducts were removed and cumulus masses
205 obtained by puncturing the swollen ampullae. Cumulus masses were incubated in 200 μl drops
206 of HTF containing reduced glutathione for ~30 min prior to adding sperm. Sperm were collected
207 from the epididymides of male mice by gently snipping with fine scissors into a 100 μl drop of
208 capacitation medium and were capacitated for 1-2 hrs before adding 3-5 μl of the sperm
209 suspension to the drops containing the eggs. The sperm and eggs were incubated together for
210 4 hrs, then were washed into 200 μl drops of HTF without glutathione. Fertilized eggs were
211 incubated overnight in a humidified incubator containing 5% CO_2/95% air. The next day, 2-cell
212 embryos were counted.

213 *Statistical analysis*

214 Statistical analyses were performed using Prism 6.0 software for Windows, GraphPad Software,
215 La Jolla, California (www.graphpad.com). Specific statistical tests for each experiment are
216 indicated in the figure legends. $P<0.05$ was considered to be significant.

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222 Results

223

224 *Testosterone cypionate elevates serum testosterone levels and induces virilization in female*
225 *mice.*

226 In a recent study investigating the effects of testosterone on female mice, Kinnear et al. (2019)
227 injected testosterone enanthate twice weekly to maintain elevated testosterone levels. In the
228 current study, we injected a similar form of testosterone, testosterone cypionate (referred to
229 hereafter as “T”), which is commonly used by transgender men to elevate T levels (Luthy et al.,
230 2017; Moravek et al., 2020), weekly. T-injected mice showed signs of virilization, including
231 distinct clitoromegaly and cessation of estrous cycles (Fig. 1A). All control-injected mice clearly
232 cycled throughout the entire experiment, whereas all T-injected mice appeared to be in diestrus
233 (Fig.1B), which is consistent with what was observed previously (Kinnear et al., 2019). One
234 week after the 6th T injection (referred to herein as the “active exposure” group), mice were
235 sacrificed, trunk blood was collected, and T levels were measured. T-injected mice had
236 significantly higher T levels than controls, and the amount of T was comparable to the levels in
237 adult males (Fig. 2). In one set of experiments, we did not sacrifice females after the 6-week
238 injection period; rather, the mice were kept for several weeks after cessation of injections
239 (referred to herein as the “washout” group). T levels declined to baseline levels within 5 weeks
240 following the last injection (Fig. 2) and these “washout mice” resumed cycling, as assessed by
241 daily vaginal smears. Interestingly, clitoromegaly was no longer apparent in these washout
242 mice 5 weeks after the last injection (Fig. 1A). The weights of the mice did not differ between T-
243 treated and controls. T-treated and control mice in the active exposure group weighed 30.5 ±
244 0.8 g (SEM) vs 31 ±1 g, respectively, whereas in the washout group T-treated and control mice
245 weighed 33.9 ± 1 and 33.7 ± 0.8 g, respectively.

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247 *Ovaries from T-treated mice are smaller than control ovaries but contain normal complements of*
248 *follicles and respond to stimulation by gonadotropins.*
249
250 Currently, little is known about the effects of T treatment on the ability of ovaries to respond to
251 gonadotropic stimulation. To investigate this, both the active exposure and washout groups of
252 mice were divided into 3 sub-groups: 1) not stimulated by gonadotropins; 2) stimulated with
253 eCG only; and 3) stimulated with eCG and hCG to induce ovulation. For groups 1 and 2,
254 ovaries were collected after euthanasia, most of the fat and oviducts were removed under a
255 stereoscope, one ovary was fixed and processed for histological analysis, and oocytes were
256 collected from the other ovary. For group 3, ovulated eggs were collected from both oviducts
257 prior to weighing ovaries.
258
259 Ovaries from T-treated mice in the active exposure group weighed significantly less than control
260 ovaries whether or not they were stimulated with gonadotropins (Fig. 3A). Notably, the lower
261 ovarian weights were still apparent in the washout group, which more closely mimics the
262 standard of care for T-treated transgender males who wish to obtain functional eggs (Fig. 3B).
263
264 Histological analysis of unstimulated, eCG-stimulated, and ovulated ovaries from the active
265 exposure groups (approximately 12 weeks in age) showed similar follicle morphology and
266 comparable numbers of follicles from T-treated mice with their respective controls, despite the
267 overall smaller size of ovaries in T-treated mice (Fig. 4). Because the numbers of preantral and
268 primary follicles have already been reported to be the same for control and T-treated ovaries
269 (Kinnear et al., 2019), we focused on counting antral follicles of various sizes. We analyzed
270 follicles in detail from 3 mice per group, one ovary from each mouse. In the unstimulated group,
271 T-treated and control ovaries contained similar numbers of antral follicles, as well as similar
272 numbers of atretic follicles. Most of the atretic follicles were in the 250-320 μm size range, while

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273 there were almost no atretic follicles in the preovulatory size range. The major difference
274 between T-treated, unstimulated ovaries and control ovaries was a significantly lower number of
275 corpora lutea (CLs) in the T-treated group compared with controls (Fig. 4). The eCG-stimulated
276 ovaries contained significantly more preovulatory follicles than the unstimulated ovaries in both
277 control and T-treated mice and contained 0-1 atretic preovulatory follicles. Similar to
278 unstimulated ovaries, ovaries from T-treated mice contained fewer CLs than controls. Most of
279 the CLs present in the T-treated ovaries were likely to be from cycles that occurred prior to T
280 treatment, as they were eosinophilic rather than basophilic (Gaytan et al., 2017) and, in general,
281 located deep within the ovary rather than at the periphery (Fig. 4). The one exception was a T-
282 treated mouse that ovulated in response to the eCG injection; this mouse contained mostly
283 basophilic CLs (Fig. 4A). We did not evaluate ovulated ovaries in detail with histology.

284 Measurements of estradiol-17β (E2) levels from trunk blood obtained at the time of sacrifice
285 provided further evidence that T-stimulated ovaries responded to gonadotropins: E2 was low in
286 the unstimulated groups, increased in response to eCG injection, then fell back to basal levels
287 after ovulation (Fig. 5). E2 levels in T-treated mice in both the active exposure group and the
288 washout group were lower than respective controls in response to eCG stimulation, and E2
289 levels in the eCG-stimulated mice from the washout group were ~3X higher than in the active
290 exposure group (Fig. 5), though the basal levels in the unstimulated and ovulated groups were
291 similar to those in the active exposure group.

292

293 *T-treated mice contain comparable numbers of meiotically competent oocytes and ovulate*
294 *similar numbers of fertilizable, mature eggs as controls.*

295

296 To examine if the follicles from T-treated mice contain normal oocytes, we first collected
297 immature oocytes from ovaries of unstimulated and eCG-stimulated mice, and then collected

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298 ovulated eggs from the groups that were injected with both eCG and hCG. In the active
299 exposure group, we recovered significantly more immature oocytes from the T-exposed ovaries
300 than from their respective controls (Fig. 6A). In the washout group, we obtained similar
301 numbers of immature oocytes from T-treated and control ovaries, though statistical analysis was
302 not possible in this group due to the small sample size (n=2; Fig. 6B). Both active exposure and
303 washout groups contained similar numbers of ovulated eggs for T-treated and control mice (Fig.
304 6A,B). Immature oocytes from T-treated ovaries (active exposure and washout groups)
305 underwent germinal vesicle breakdown (GVBD), extruded first polar bodies, and formed
306 morphologically normal meiotic spindles in culture (Fig. 6C,D). The proportion of oocytes with
307 intact spindle structure vs poor spindle structure - characterized by degeneration or misaligned
308 chromosomes - was similar between the groups (Fig. 6D). The diameters of in vitro matured
309 eggs from the active exposure T group were the same as controls (Fig. 6E).

310 We tested the fertilizability of ovulated eggs using in vitro fertilization by evaluating the number
311 of 2-cell embryos that were observed 24 hrs after insemination. Overall, cleavage to the 2-cell
312 stage was comparable between T-treated and control mice, and was similar to percentages of
313 fertilized eggs obtained from the washout group (Fig. 7).
314

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315 Discussion

316

317 Transgender men who have been undergoing testosterone therapy and who wish to obtain eggs
318 for fertilization or freezing are generally stimulated with gonadotropins. This is usually done
319 following a period of T cessation that is sufficient for the menstrual cycle to resume (Adeleye et
320 al., 2019; Broughton and Omurtag, 2017; Leung et al., 2018). There is strong evidence that
321 transgender men who have taken T can produce viable, developmentally competent eggs after
322 discontinuing its use (Adeleye et al., 2019; Broughton and Omurtag, 2017; Leung et al., 2018;
323 Light et al., 2014), but to date, there are no publications regarding the quality and
324 developmental capacity of eggs retrieved from transgender men who remain on HT. Here, we
325 show that treating female mice with T cypionate weekly for 6 weeks does not impair the
326 fertilizability of their eggs, and our results suggest that T treatment does not need to stop before
327 gonadotropic stimulation.

328

329 T cypionate elevated blood T levels to those found in male mice, stopped the estrous cycle, and
330 caused significant clitoral growth, changes that are commonly observed in transgender males
331 on HT (Unger, 2016). It was noteworthy that the clitoromegaly did not persist in the washout
332 group, which was surprising because it is generally recognized that this is a permanent change
333 in human females exposed to high levels of T (Cabrera and Rogol, 2013; Sielert et al., 2013). It
334 is possible that the regression of clitoromegaly upon T withdrawal in our mice represents a
335 difference between mice and humans; however, careful measurements using a larger sample
336 size would need to be done to determine this definitively.

337

338 T-treated mice had smaller ovaries than controls, which is likely due to the greatly reduced
339 number of CLs in these mice. A recent study reported a complete absence of CLs in T-treated
340 female mice (Kinnear et al., 2019). The finding that our T-treated mice had CLs at all was

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341 unexpected, as we used a concentration of T cypionate that was similar to the mid-range
342 effective dose used by Kinnear et al. In general, the eosinophilic staining of CLs we observed in
343 T-treated mice was consistent with residual rather than freshly ovulated CLs (Gaytan et al.,
344 2017) and there were considerably fewer CLs in our T-treated mice than in controls, suggesting
345 that the estrous cycle was indeed inhibited in our mice, as was also shown by vaginal cytology.
346 Smaller ovaries were also apparent in T-treated mice in the washout group, which likewise
347 contained significantly fewer CLs than controls, suggesting that these mice had only recently
348 begun cycling prior to ovary harvest and therefore had many fewer ovulations than controls.
349
350 Although they weighed less than controls, the ovaries from T-treated mice contained a
351 complement of histologically normal antral follicles that were similar to controls. Unlike the
352 study by Kinnear et al. (2019), who reported a higher incidence of late-stage atretic, cyst-like
353 follicles, we only observed a single preovulatory atretic follicle in 3/6 ovaries examined, which
354 was not significantly different from controls. Rather, the majority of atretic antral follicles we
355 observed came from follicles that were in the ~250-320 μm diameter size range, not yet
356 preovulatory, and the percentages were not different between control and T-treated ovaries. T-
357 treated ovaries that were stimulated by eCG had more preovulatory follicles than unstimulated
358 ovaries, and these numbers were comparable to the number of preovulatory follicles observed
359 in control mice. One of the T-treated mice unexpectedly ovulated in response to eCG
360 stimulation. There is evidence that T induces the expression of FSH receptors in granulosa
361 cells (Garcia-Velasco et al., 2012; Liu et al., 2015; Sen et al., 2014), and if this occurred in our
362 mice, then it is possible that the T-treated follicles were sensitized to eCG such that
363 spontaneous ovulation occurred prior to the administration of hCG.
364
365 T-treated mice responded to eCG treatment by producing E2. Interestingly, T-treated mice in
366 the active exposure group did not elevate E2 to the same extent as controls. The difference in

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367 E2 levels after eCG was surprising because a similar number of oocytes were collected after
368 stimulation. In human IVF cycles, the peak E2 level achieved directly correlates with the
369 number of oocytes collected at the time of retrieval (Chenette et al., 1990), and during reported
370 IVF cycles in transgender males, the peak E2 levels, as well as the number of oocytes
371 collected, have been reported to be similar to female controls, although higher doses of
372 gonadotropins were used for transgender males (Leung et al., 2018). Lower E2 levels here may
373 reflect the lower number of CLs in the T group. Though it is thought that CLs are rapidly
374 inactivated after each cycle in mice (Accialini et al., 2017), it is possible that the residual CLs
375 retain some ability to produce E2 in response to eCG.
376
377 Ovaries from T-treated mice produced meiotically competent oocytes. Oocytes retrieved from
378 unstimulated and eCG-stimulated, T-treated ovaries were able to mature to the metaphase II
379 stage in culture, forming morphologically normal meiotic spindles. This is consistent with a
380 descriptive study of human oocytes collected from the ovarian cortex of HT-exposed
381 transgender males, in which oocyte meiotic spindle structure after in vitro maturation was found
382 to be normal (Lierman et al., 2017). In addition, T-treated mice in both the active exposure and
383 washout groups ovulated similar numbers of eggs in response to eCG and hCG injection as
384 controls. These eggs fertilized to the same extent as controls and cleaved to the 2-cell stage.
385 One exception in the active exposure group was a single T-treated mouse that only produced 3
386 poor-quality eggs, none of which fertilized. One hypothesis is that this mouse ovulated
387 prematurely, as there was some histological evidence in a different T-treated mouse of
388 premature ovulation after eCG. There was insufficient data to fully explore this isolated
389 scenario, and the other T-treated mice produced fertilizable eggs.
390

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391 In conclusion, we provide evidence showing that female mice produce normal, fertilizable eggs
392 after testosterone exposure, whether T levels are low after a washout period or high during
393 active exposure. One limitation in our study could be that we only exposed mice to T for 6
394 weeks. While this length of exposure was sufficient to produce phenotypes characteristic of
395 human transgender males exposed to T, it may not completely mimic the human situation, in
396 which many transgender males seeking fertility treatment have been on HT for several
397 years. Further studies that expose mice to T for longer periods of time would help confirm that
398 its effects are not detrimental to the reproductive process. Despite this concern, our results
399 provide promising data that could help influence the treatment options for transgender men
400 seeking fertility treatment. If testosterone has no detrimental impact on ovarian function,
401 transgender males will have greater flexibility in making reproductive decisions. It is important
402 to note, however, that if a transgender male plans to become pregnant by spontaneous
403 pregnancy or use of assisted reproductive technology, testosterone must be discontinued due to
404 its teratogenic effects (De Roo et al., 2016). Our study suggests that the current practice of T
405 cessation prior to ovarian stimulation and surgical oocyte retrieval may not be necessary when
406 the transgender male does not plan to carry the pregnancy at that time, and could potentially
407 help serve as the basis for human trials to examine this current clinical practice.

408 Authors' Roles

409 C.B.B. designed the study, acquired and interpreted the data, and helped draft the article.
410 T.F.U. contributed to the study design, data acquisition, and article review. L.L. contributed to
411 data acquisition and article review. L.M.M. designed the study, contributed to data acquisition
412 and interpretation, and helped draft the article.

413

414

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423 Conflict of interest

424 The authors have no competing interests.

425

426

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Figure legends.

523 Figure 1. T treatment induces virilization in female mice. A) Clitoromegaly was apparent in the
524 active exposure group (a=control; b=T-treated), but was no longer apparent in the washout
525 group (c=control; d=T-treated). B) Vaginal smears from a cycling, control mouse (a-e) and a T-
526 treated mouse (f-j). Smears were obtained during the fifth week of T injections. Shown here is
527 5 sequential days of a representative control and T-injected mouse. a=diestrus; b=diestrus into
528 proestrus; c=proestrus; d=estrus; e=metestrus from a control mouse. f-j = T-injected mouse in
529 diestrus.

530 Figure 2. Testosterone cypionate transiently elevates T levels to those of untreated adult
531 males. T levels were significantly higher in the active exposure mice, which were tested
532 following the 6th T injection. T levels declined to baseline levels by 5 weeks after the last
533 injection. Different letters above the bars indicate statistical significance; $P<0.0001$, as
534 determined by one-way ANOVA.

535

536 Figure 3. Ovaries from T-treated mice weigh less than control ovaries. A) Ovary weights from
537 mice in the active exposure group. B) Ovary weights from mice in the washout group. Bars with
538 different letters are significantly different ($P<0.05$), as determined using 2-way ANOVA followed
539 by Bonferroni's multiple comparison test.

540

541 Figure 4. Ovaries from T-treated mice have normal complements of antral follicles but fewer
542 corpora lutea. A) Histology sections showing representative images through ovaries from
543 unstimulated and eCG-stimulated control and T-treated mice from the active exposure group
544 using 2X, 4X, or 10X objectives. C=control; T=testosterone; * = corpus luteum. The bottommost
545 image is from a T-treated mouse that ovulated in response to eCG stimulation, showing fresh
546 CLs (f*) and residual CLs (r*). B) Quantification of antral follicles in unstimulated and eCG-

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547 stimulated control and T-treated mice. "Small antral" follicles measured ~250-320 μm in
548 diameter; "preovulatory" follicles measured >320 μm in diameter. ** P<0.01 (unpaired t-test); ***
549 P<0.0001 (unpaired t-test). The increase in preovulatory follicles from eCG-stimulated ovaries
550 compared to unstimulated ovaries is significant (P<0.05; 2-way ANOVA followed by Bonferroni's
551 multiple comparison test). Data are mean ± SEM.

552

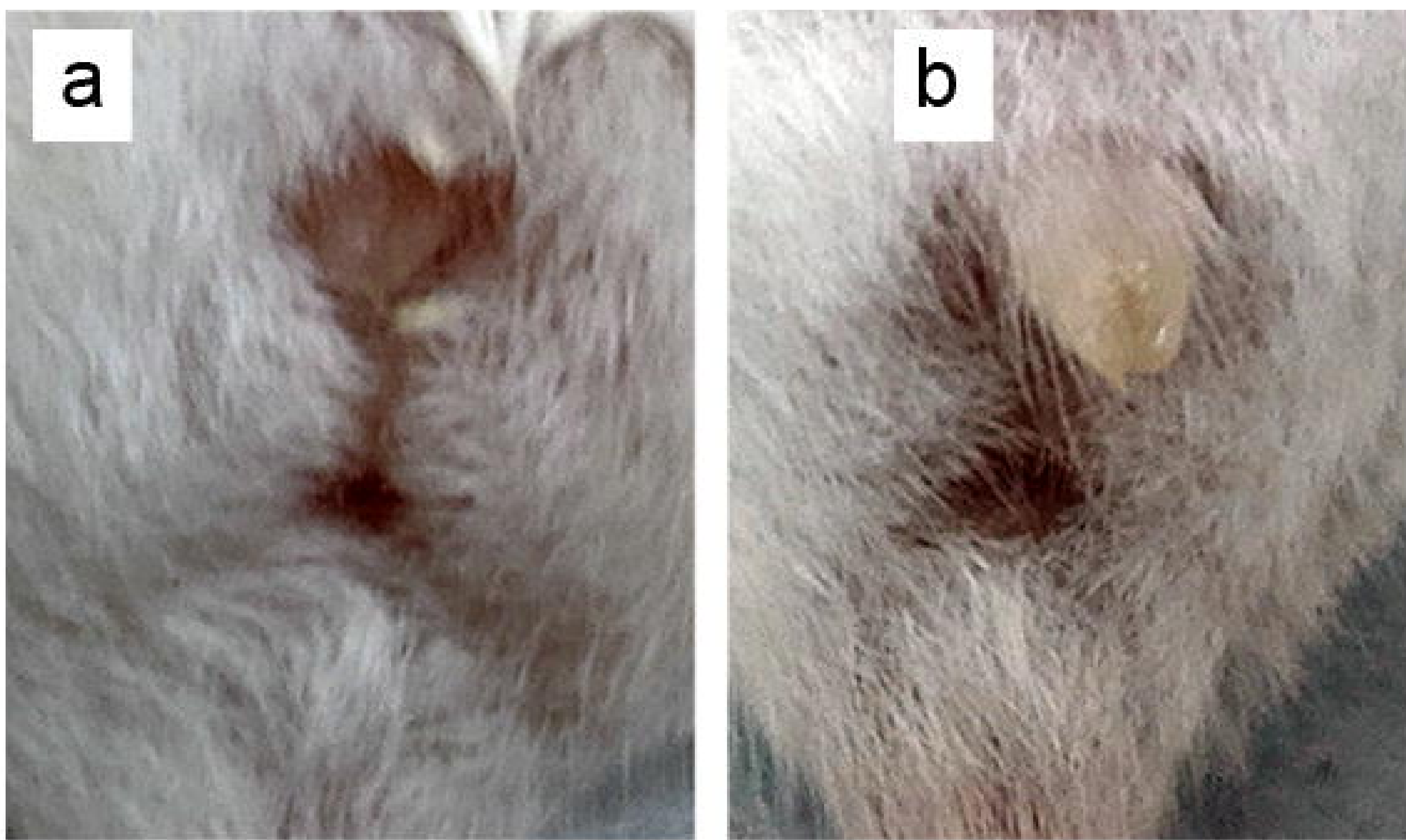
553 Figure 5. eCG stimulates E2 production in control and T-treated mice in both the active
554 exposure and washout groups. Bars with different letters are significantly different (P<0.05; 2-
555 way ANOVA, Bonferroni's multiple comparison post-test). Data are mean ± SEM.

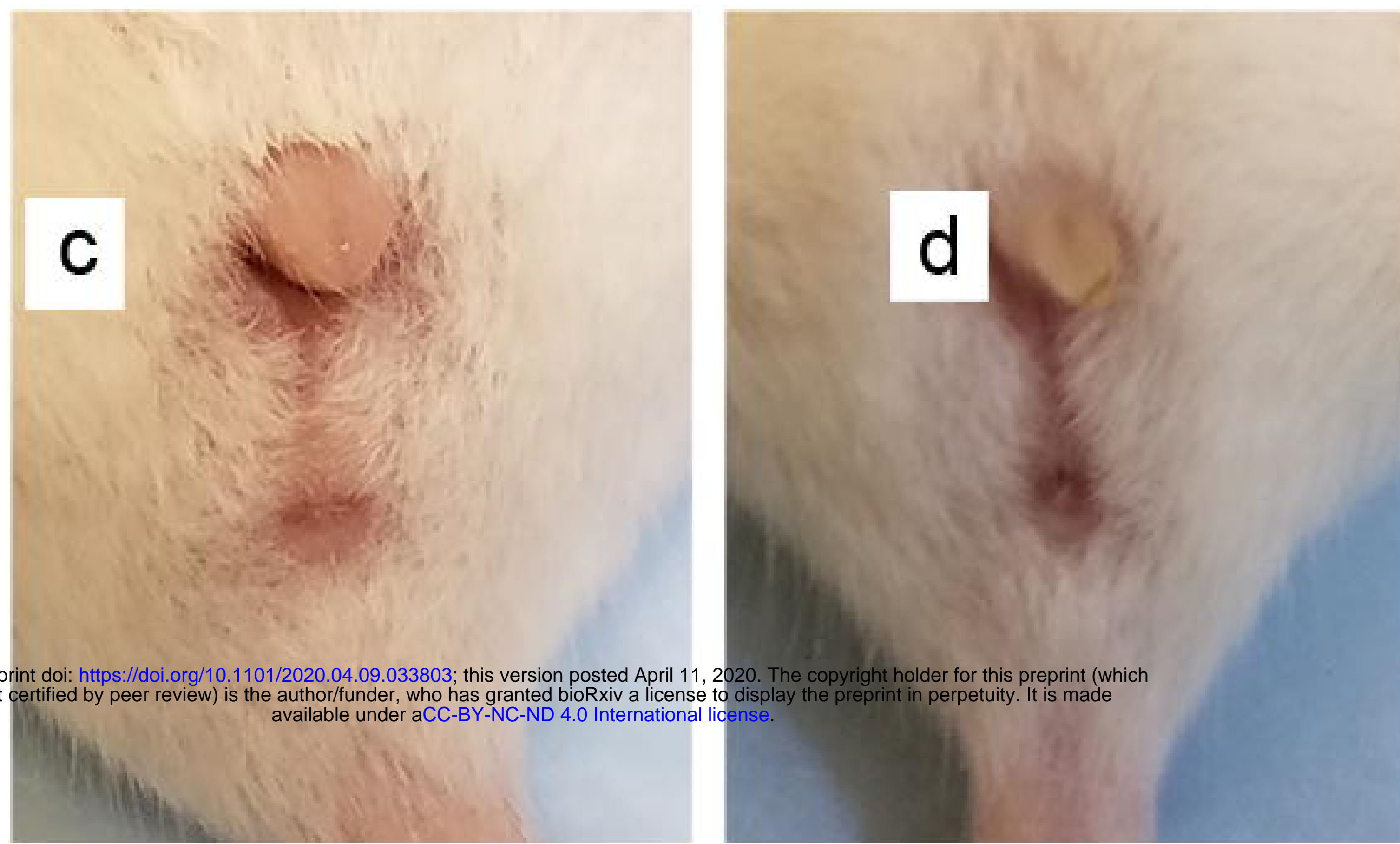
556

557 Figure 6. T-treated mice produce meiotically competent oocytes and ovulate comparable
558 numbers of eggs as controls. A,B) Numbers of oocytes and ovulated eggs recovered per ovary
559 in the active exposure (A) and washout group (B). Bars are mean ± SEM; P<0.05 was
560 considered significant, as determined by two-way ANOVA followed by Bonferroni's multiple
561 comparison test. C) Representative meiotic spindles from in vitro matured, eCG-stimulated
562 ovaries from the active exposure group. Green = tubulin; Red = DNA. D) Percentage of eggs
563 that formed normal meiotic spindles following in vitro maturation. Bars are mean ± SEM.
564 Numbers over each bar are the total number of in vitro matured eggs. AE = active exposure;
565 WO = washout. E) Diameters of in vitro matured eggs in control vs. T-treated mice.

566

567 Figure 7. Eggs from T-treated mice are fertilizable. Completed fertilization rates, defined as the
568 number of 2-cell embryos per the number of inseminated eggs, in control vs. T-treated mice in
569 both active exposure (A) and washout (B) groups. Each dot represents a single mouse.
570 Horizontal bars are the mean and the vertical bars are ± SEM.





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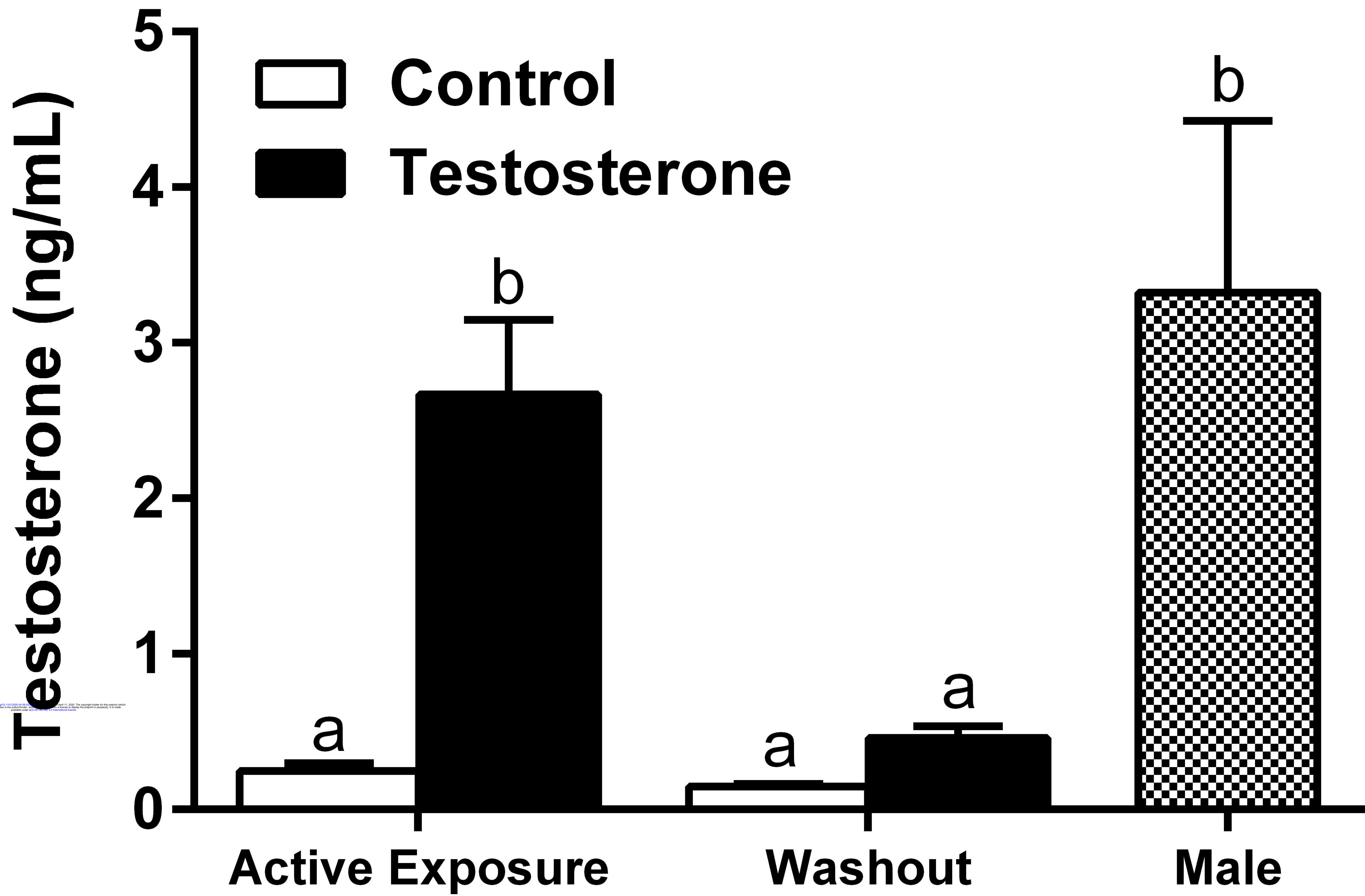

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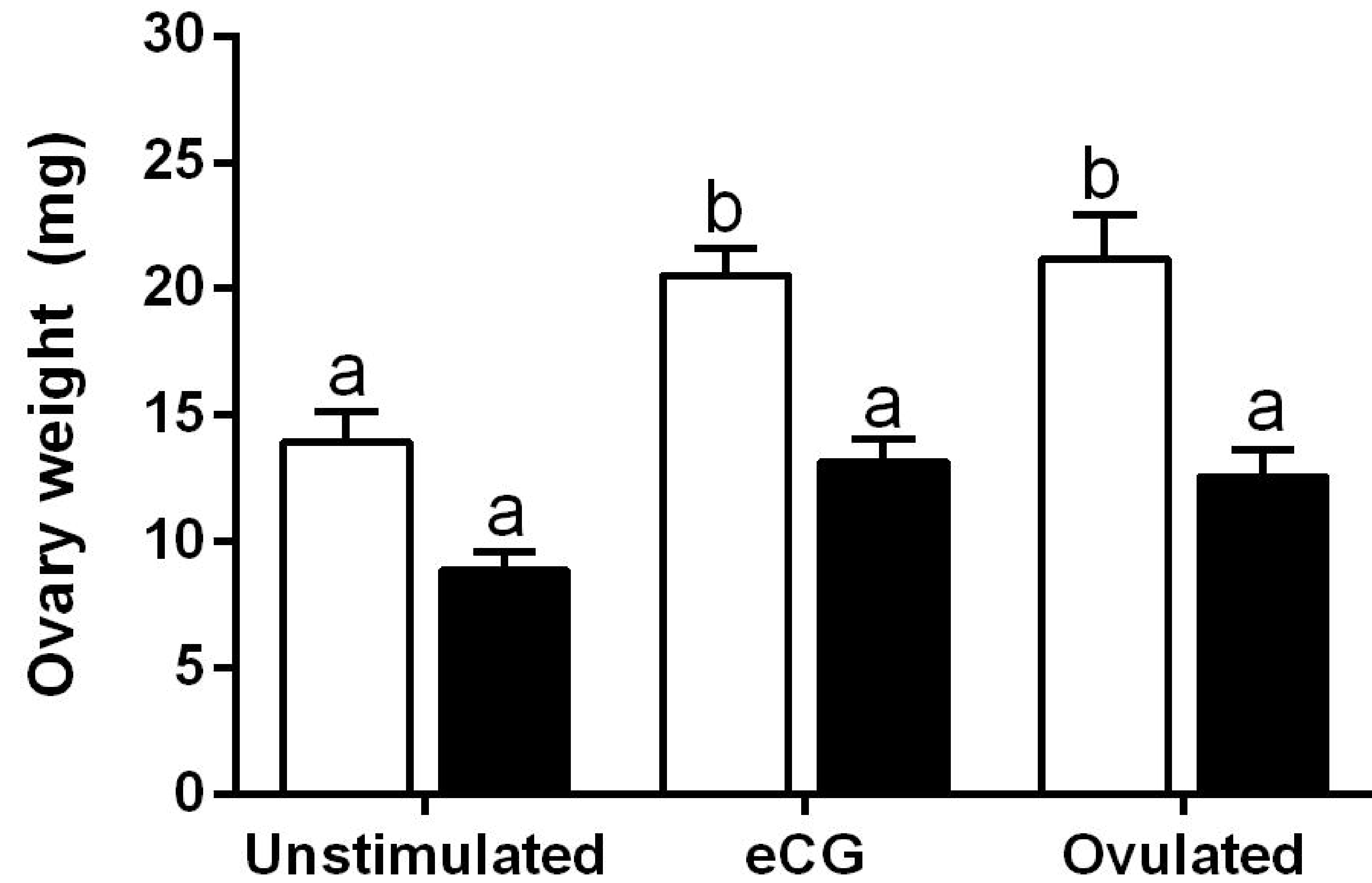

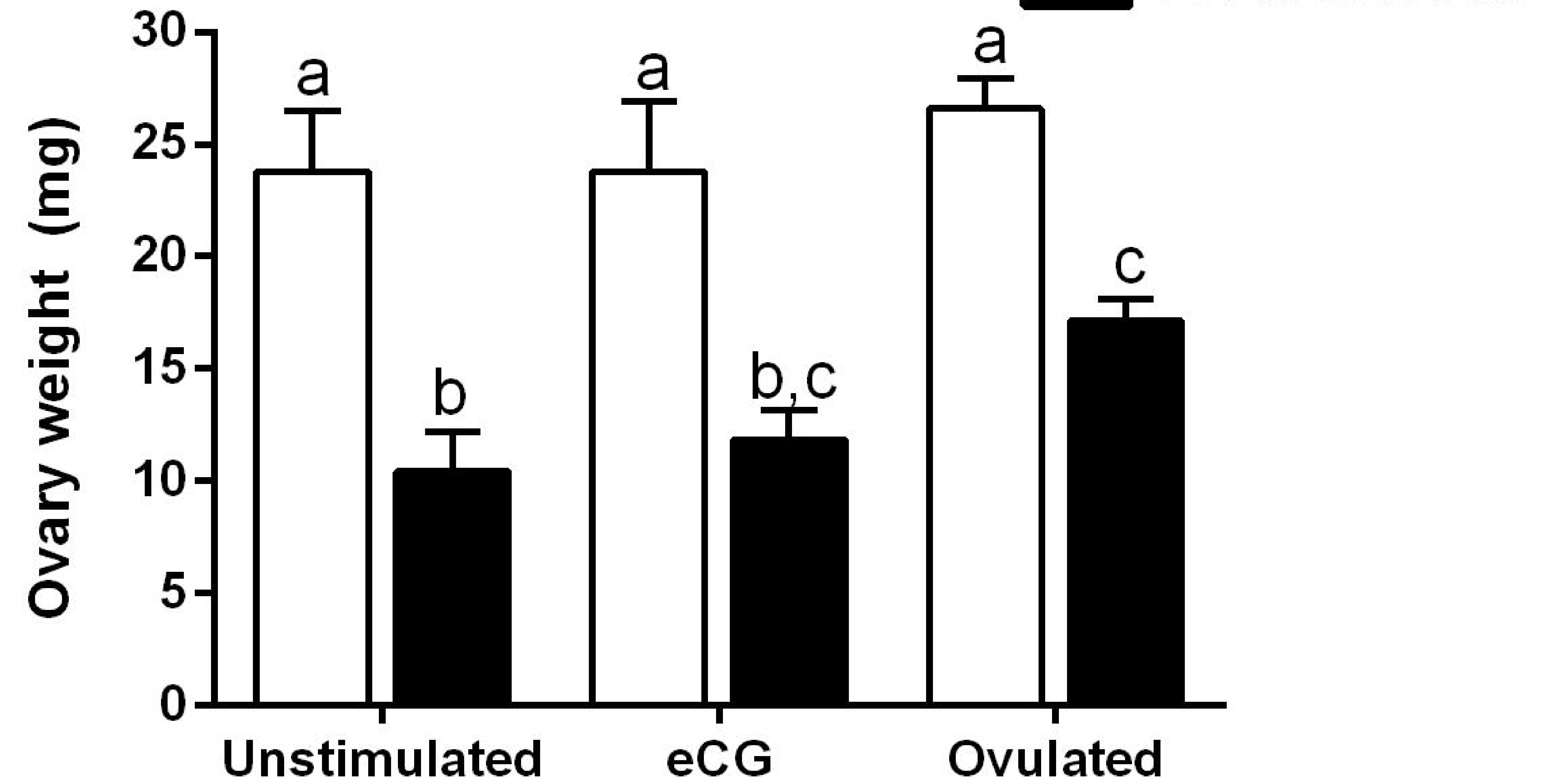

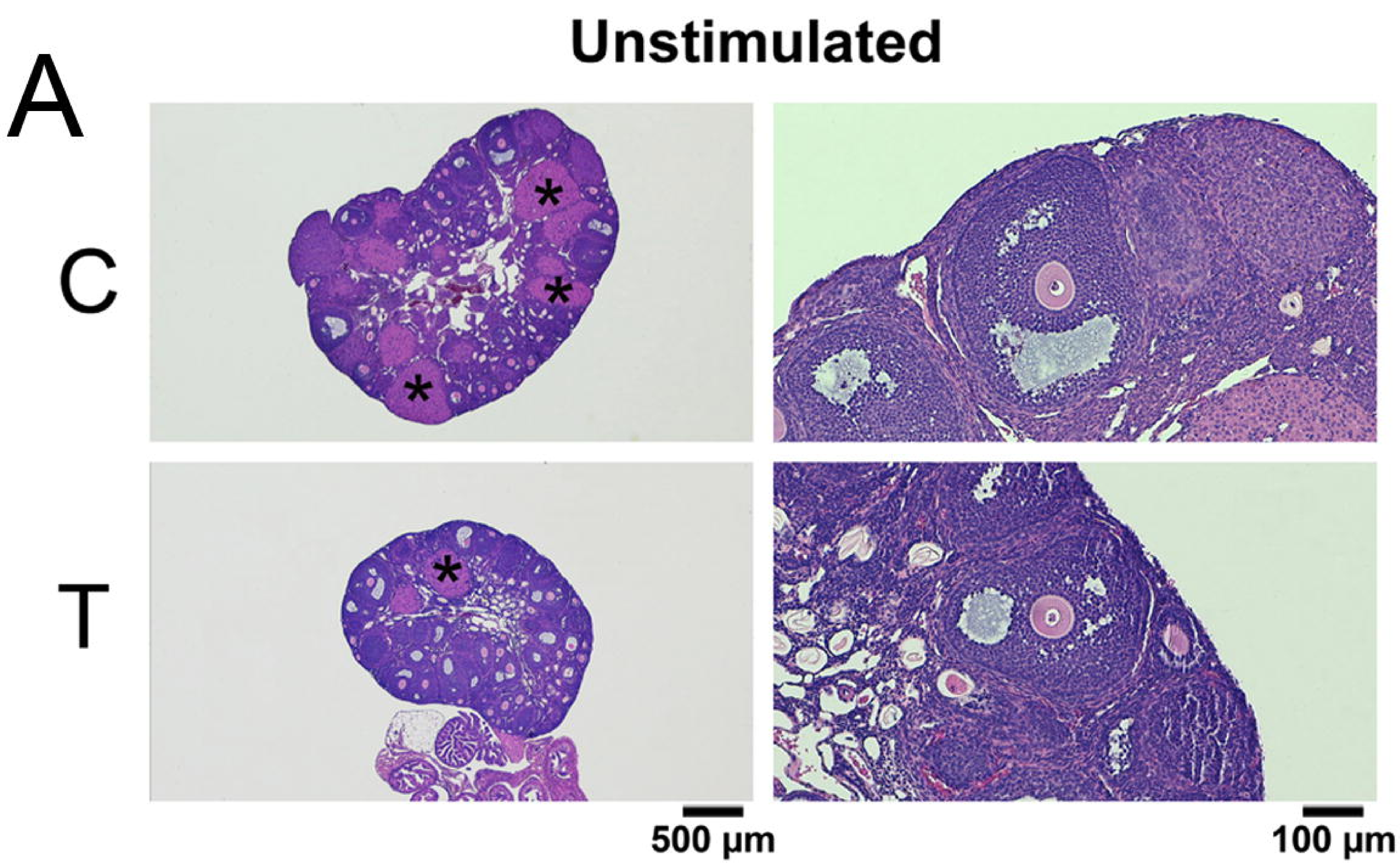

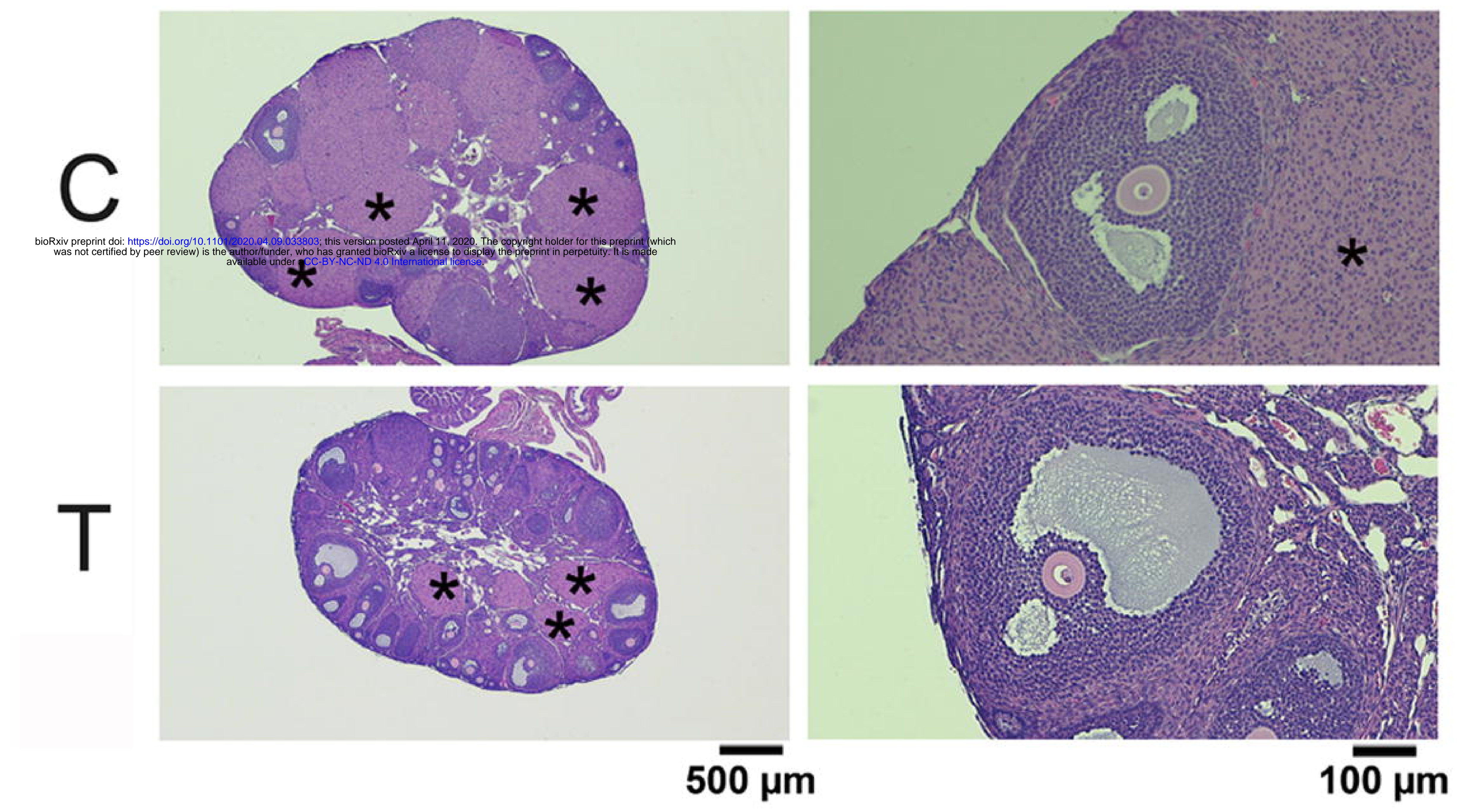

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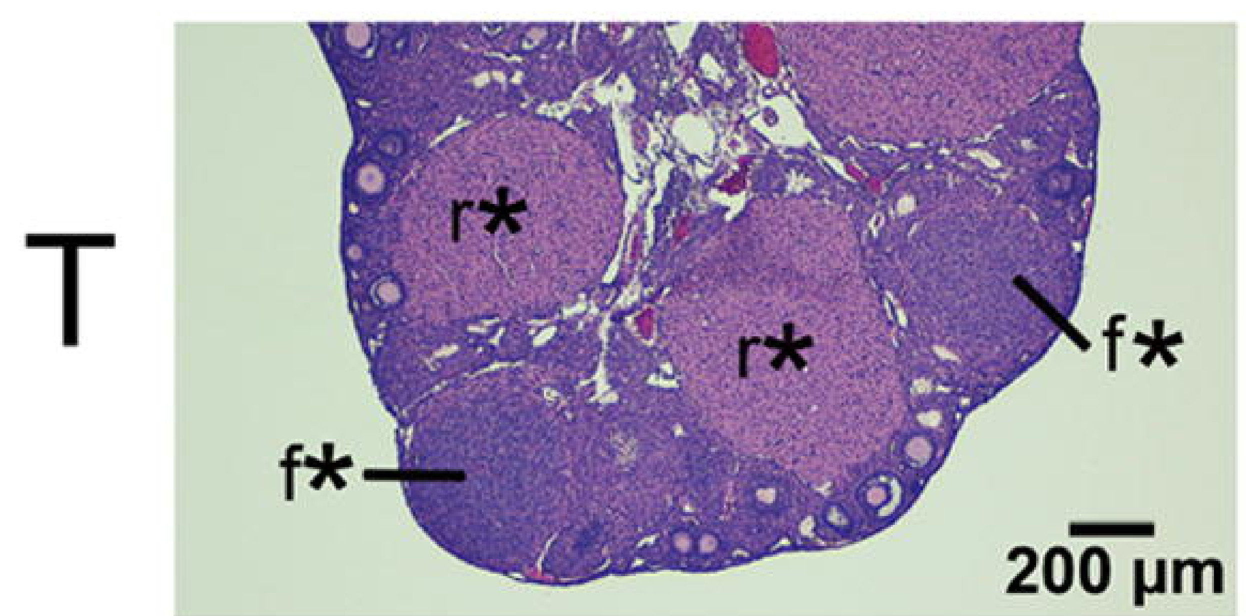

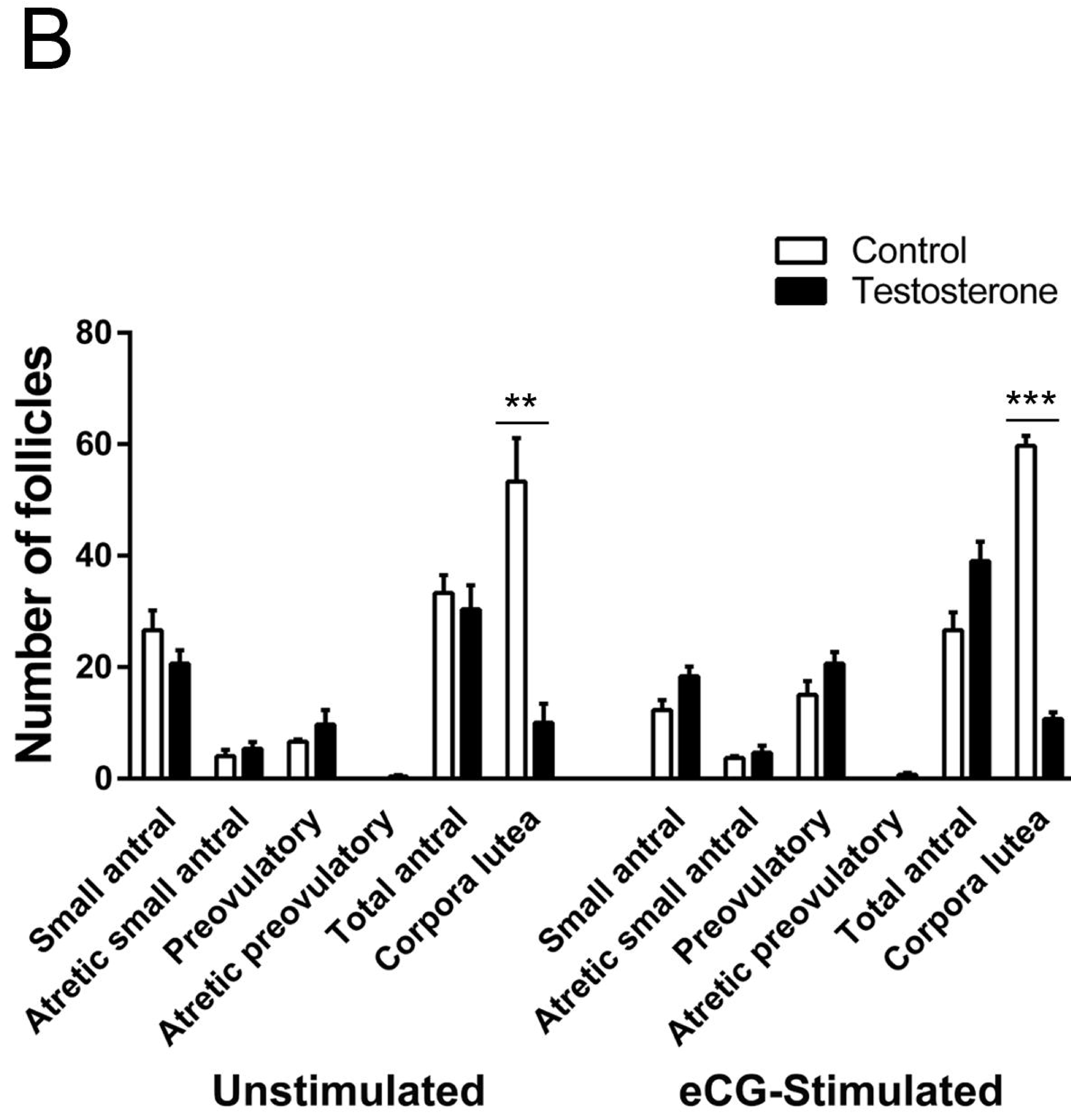

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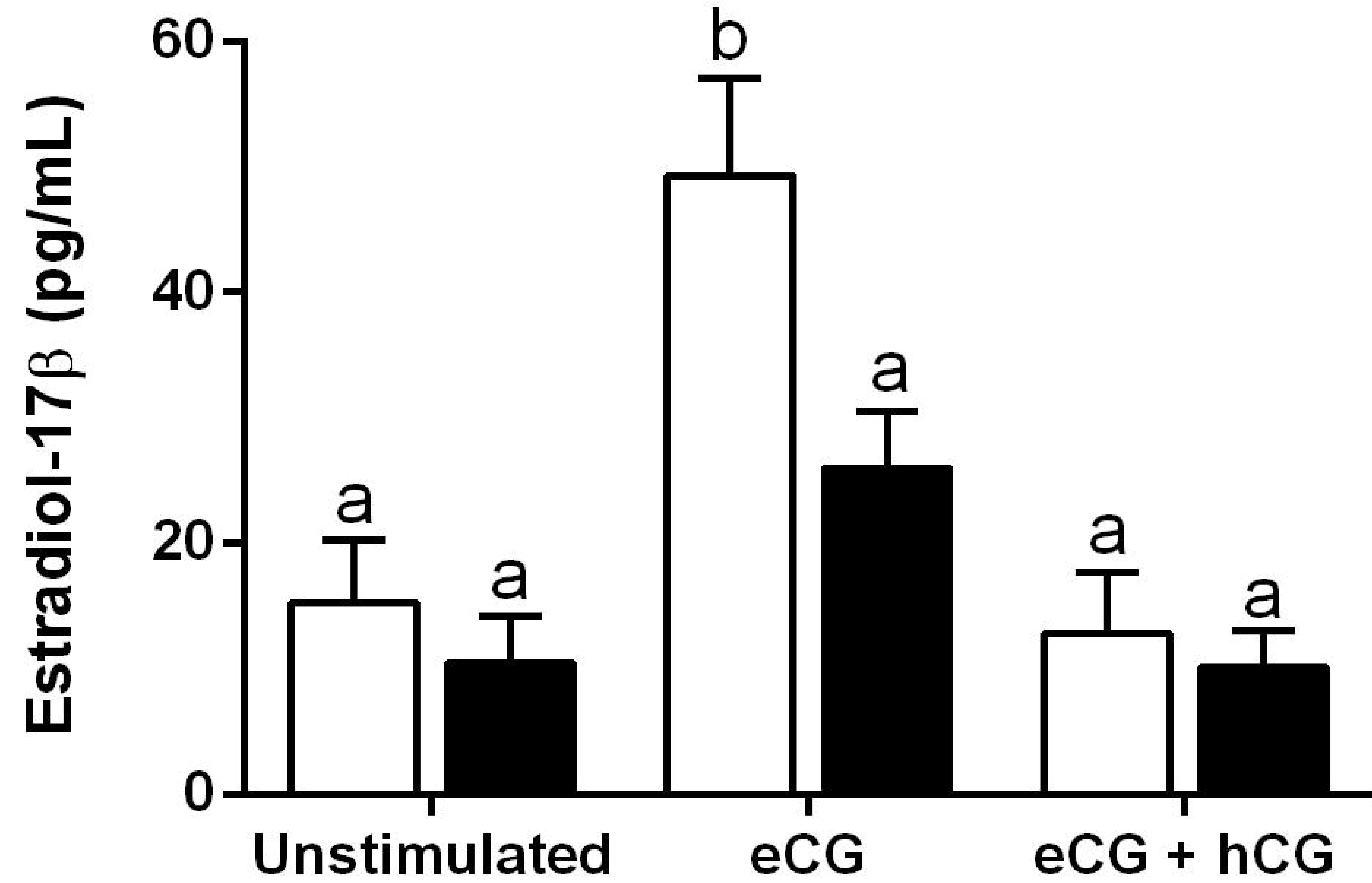


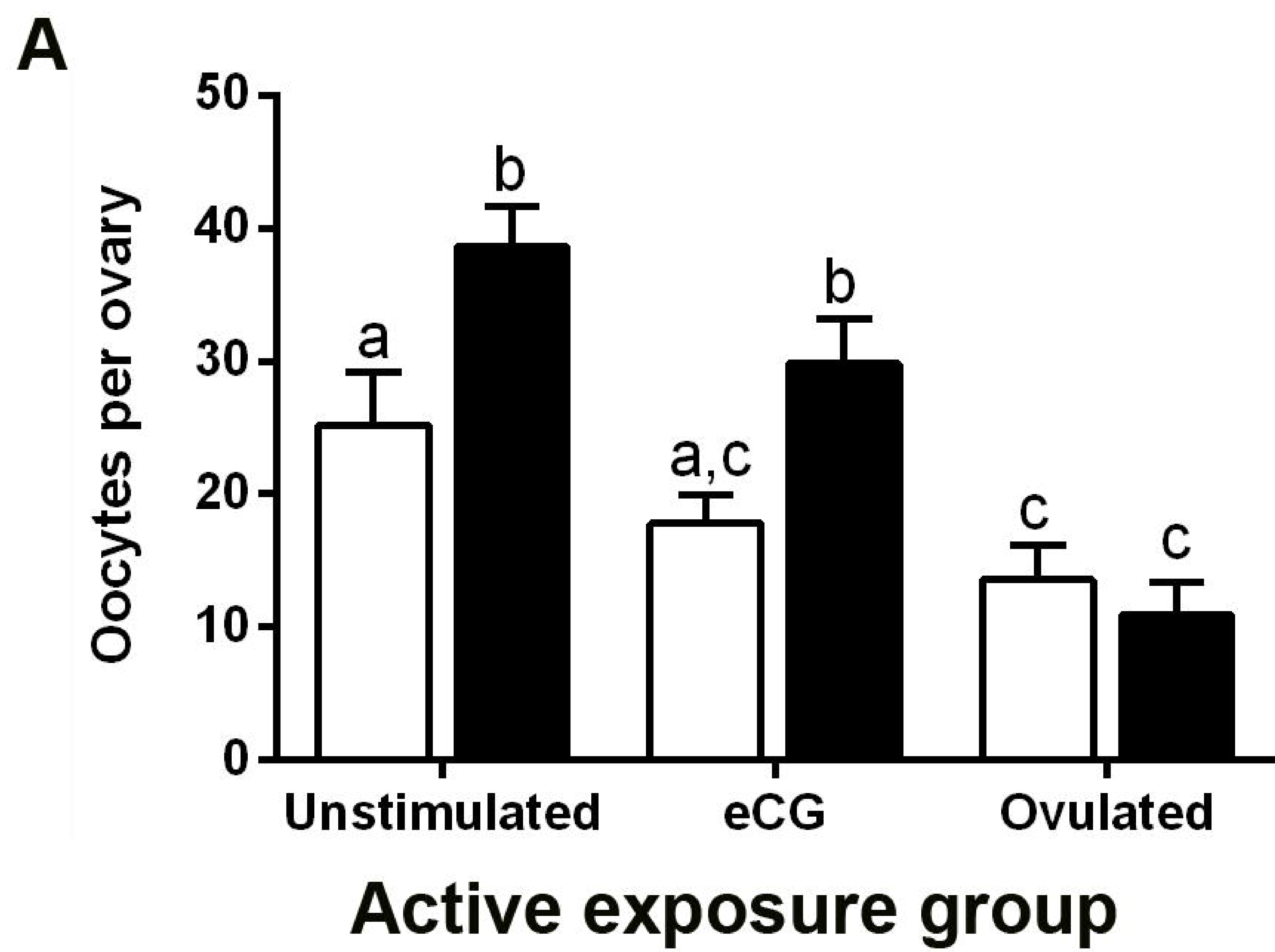
B

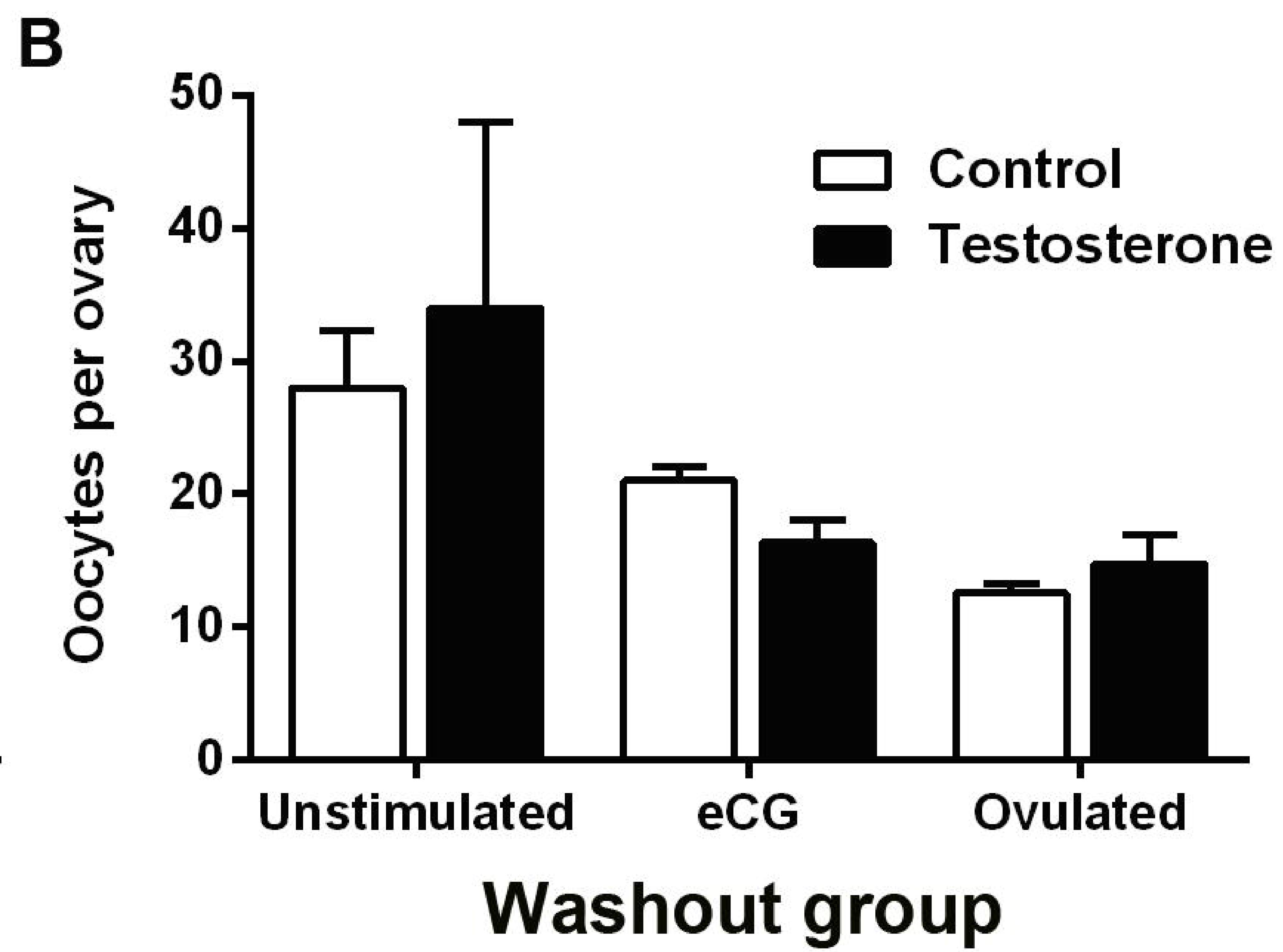
Control
Testosterone

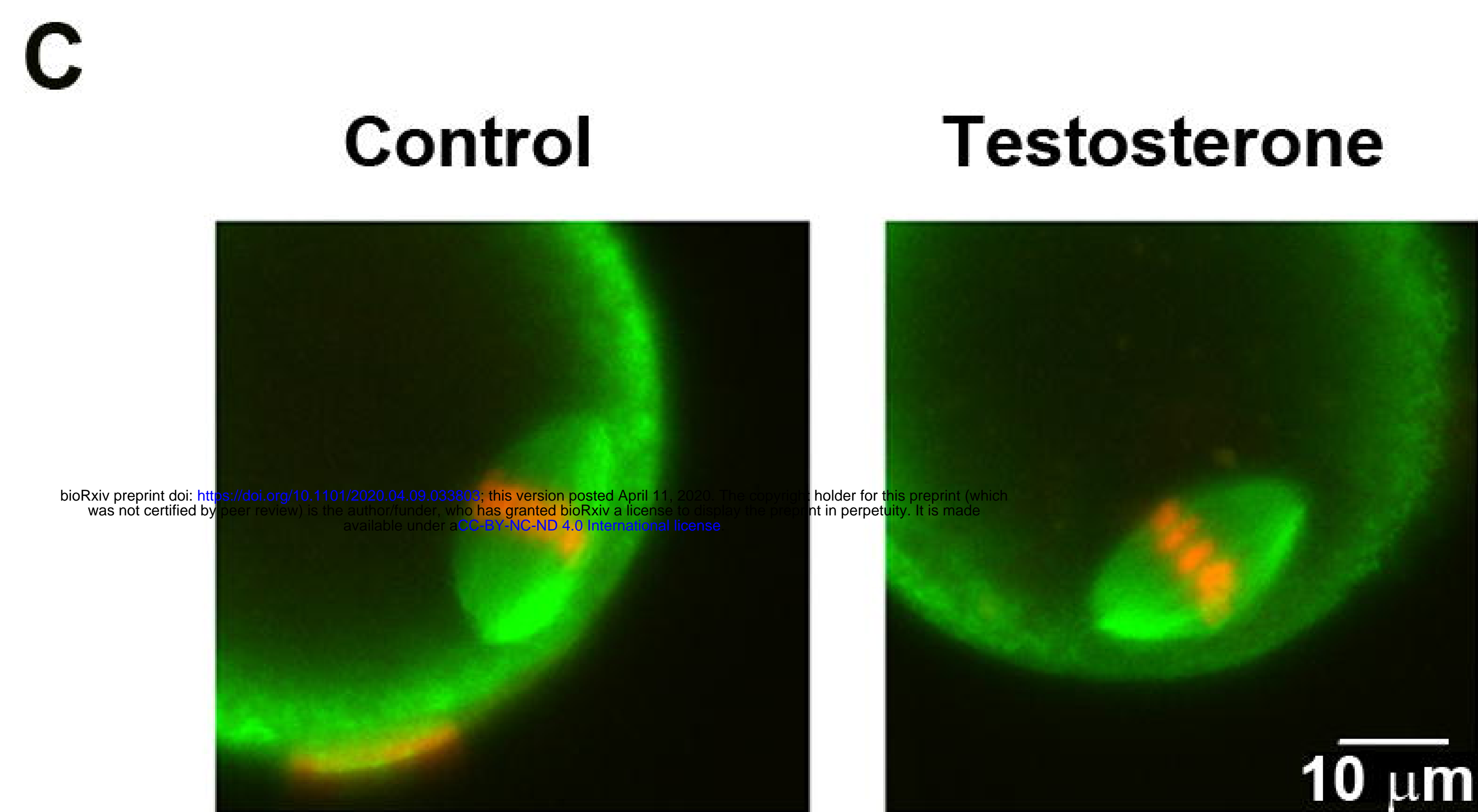
Estradiol-17β (pg/mL)

200
150
100
50
0

a,b
a
Unstimulated
eCG
eCG + hCG







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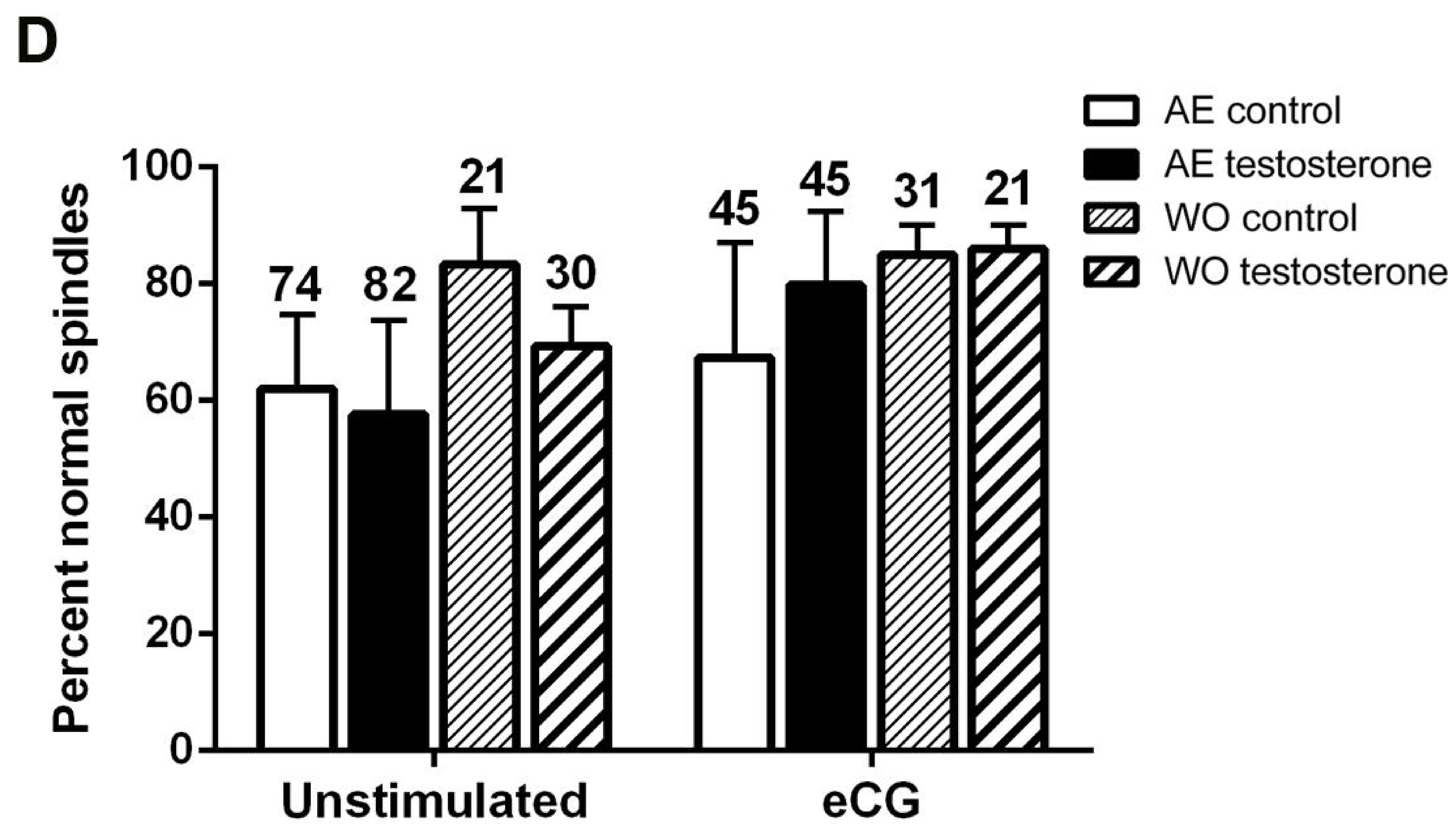
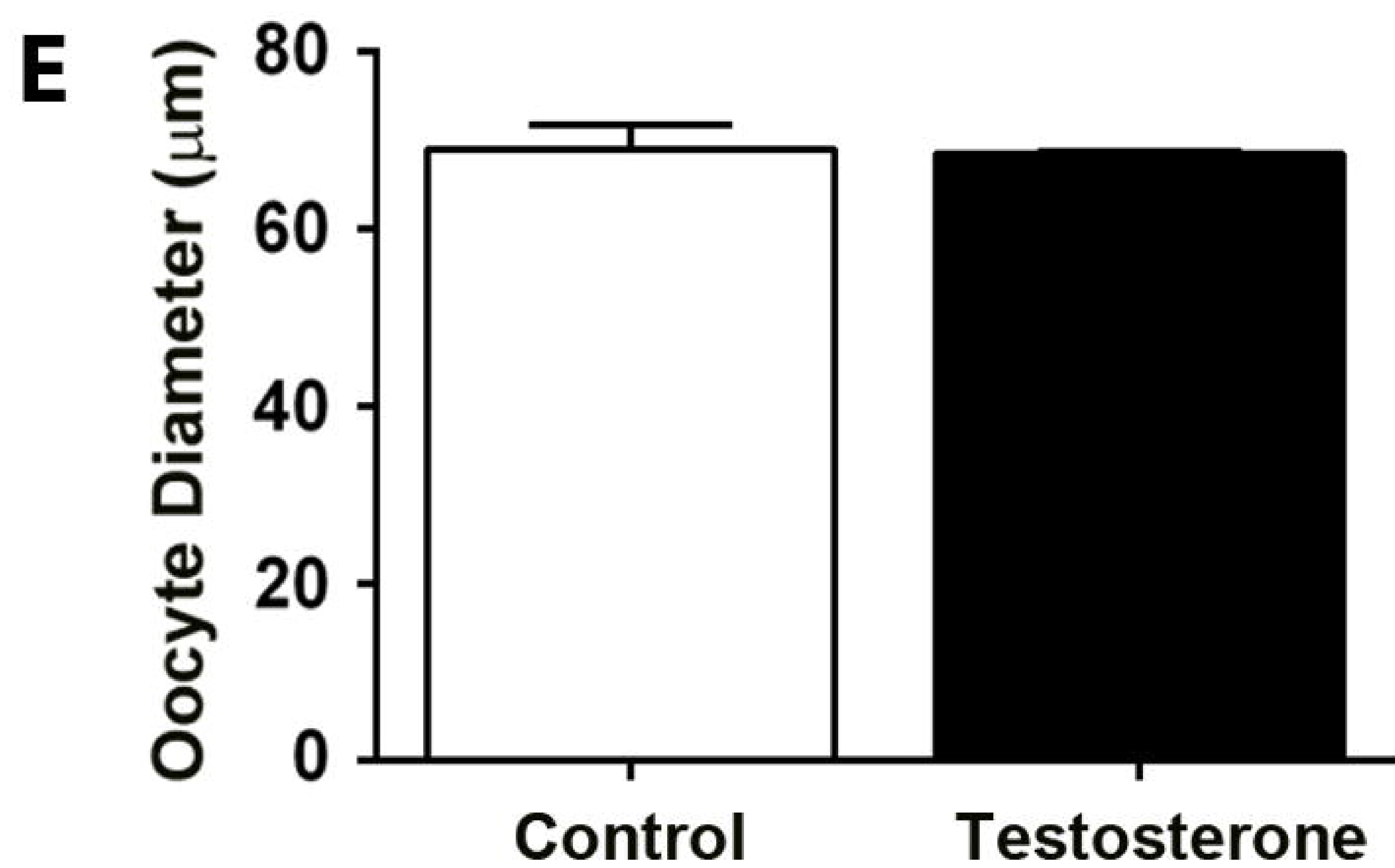
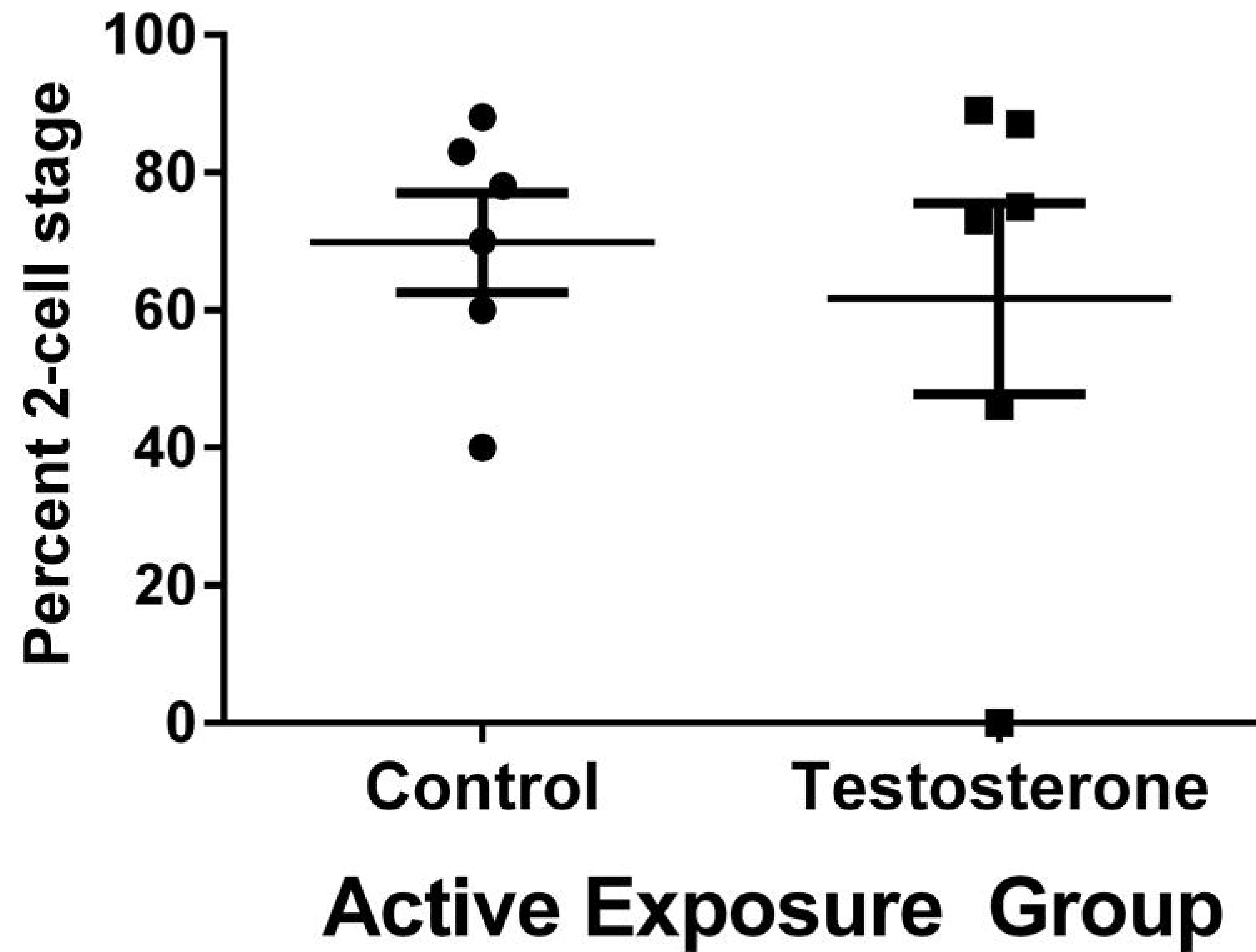
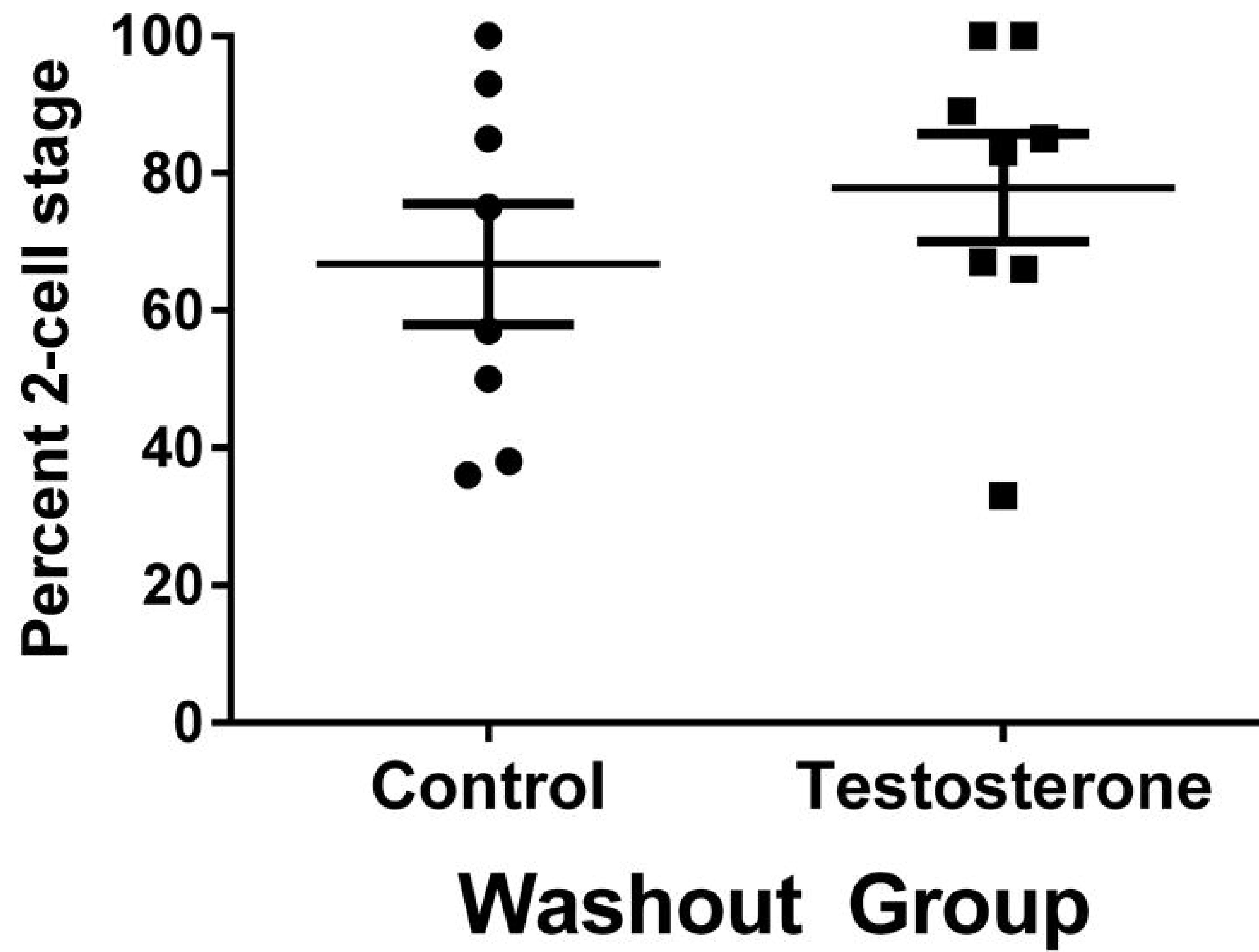





A
Percent 2-cell stage
100
80
60
40
20
0
Control
Testosterone
Active Exposure Group


B
Percent 2-cell stage
100
80
60
40
20
0
Control
Testosterone
Washout Group