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1 **GnRH-agonist implantation of pre-pubertal male cats affects their reproductive**
2 **performance and testicular LHR and FSHR expression**

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16

17 **Abstract**

18 This study was conducted to investigate the effect of GnRH-agonist implantation in
19 pre-pubertal tomcats on sexual behavior, reproductive performance and expression of
20 testicular LHR and FSHR, and also to compare the testicular characteristics, LHR and FSHR
21 expression between pre-pubertal and adult tomcats. In Exp1, 3 months-old tomcats
22 (n=6/group) were either treated with or left without 4.7 mg Deslorelin implants. Semen
23 collection and evaluation were performed just before castration at 48 wks after treatment;
24 removed testes were analyzed for mRNA and protein expression of LHR and FSHR. We
25 were able to collect semen from six non-treated cats, whereas in treated cats, semen was
26 uncollectable. The results revealed that sexual behavior was absent in the implanted cats
27 throughout the study period. Testicular volume was decreased found from 30 wks after
28 treatment onwards in the implanted cats compared to the controls ($P < 0.05$). Semen
29 production was found only in non-implanted cats. Testicular tissue score, seminiferous tubule
30 diameter and LHR protein expression was found lower in the implanted cats ($P < 0.05$) but no
31 differences were observed in mRNA expression of LHR and protein expression of FSHR
32 between groups. The mRNA expression of FSHR was higher in the implanted ($P < 0.05$)
33 compared to control cats. In Exp2, testes from pre-pubertal (n=6) and adult (n=6) male cats
34 were collected after castration and analyzed for mRNA and protein expression of LHR and
35 FSHR. No differences were observed in the protein expression of LHR and FSHR between
36 the two groups, while mRNA expression of FSHR was higher in pre-pubertal cats ($P < 0.05$).
37 Testicular and epididymal weight, diameter of seminiferous tubules and the testicular grade
38 were higher in the adult compared to pre-pubertal cats ($P < 0.05$). In conclusion, deslorelin
39 implants suppressed protein expression of LHR and enhanced mRNA expression of FSHR
40 along with suppression of reproductive function without any adverse effects for at least 48
41 wks in male cats.

42

43 **Key Words:** GnRH-agonist, Pre-pubertal cat, LHR, FSHR, Reproductive behaviour

44 **Introduction**

45 Overpopulation of cats is a serious global problem and in big towns/cities roaming of
46 tomcats is reported to be out of owners' control. The result is unwanted pregnancies with
47 undesirable consequences in this species. Free roaming cats without any care are also
48 subjected to higher risks like suffering from diseases (including zoonotic diseases),
49 malnutrition and accidents. The number of cats euthanized in shelters is also on the rise every
50 year mainly due to overpopulation [1] which needs to be controlled in order to address the
51 welfare problems associated with it.

52 Contraception is one of the most successful methods for population control in many
53 animal species. Traditional way of contraception by castration is presently in practice in cats
54 as well. However, castration is an invasive surgical procedure and can only be performed on
55 anesthetized animals, whereas anesthesia poses serious problems in juvenile and senile cats
56 and in cats with health problems. Cats reach puberty by the age of 4 month [2] with a
57 possibility of mating soon after. However, surgical neutering in early age may pose risks like
58 higher sensitivity to many drugs including the anesthetics [3]. Therefore, nonsurgical
59 neutering could be a welfare-friendly and viable alternative to surgical methods of neutering
60 [4].

61 Reproduction in mammals is controlled by the hypothalamic-pituitary-gonadal (HPG)
62 axis and it has been shown that long-term continuous administration of GnRH
63 desensitizes/downregulates the pituitary gland, profoundly suppresses the gonadotrophins
64 release and impairs the reproductive function [5]. Accordingly, a contraceptive method has
65 been developed; it is employed by GnRH-agonist implantation (Suprelorin[®]; Peptech Animal
66 Health), and has been proven effective in pubertal tomcats [5, 6] and female domestic cats [6-

67 8]. This method results into long-term reversible contraception without any negative effects
68 to the animals. The contraceptive effects of GnRH-agonist have also been reported in other
69 species such as dogs, wild felids, gilts, flying fox and giraffes [9-15]. Moreover, Trigg, Doyle
70 [16] have reported that when 4 months old female pups were implanted with 9.4 mg of
71 deslorelin, contraceptive effect was prolonged and lasted for at least 36 wks while the
72 contraceptive effect in pubertal dogs was varied from 24 to 48 wks. It is a possibility that this
73 longevity effect might have been achieved by a delay in the age of puberty in these animals.
74 Moreover, there are reports to suggest that early-age neutering could reduce undesirable
75 behaviour of cats especially in adopted cats and could help reduce the unwanted litters in
76 many pet shelters. Although GnRH implantation has been used in cats to suppress the
77 reproductive function but the studies in pre-pubertal cats are rare and with variable results
78 [17, 18].

79 The effects of GnRH implantation on the gonadotrophins' release along with the
80 suppression of reproductive function are well documented [5, 19]. However, it is not known
81 whether such effects are achieved through an alteration in the gonadal expression of receptors
82 for LH and FSH and/or testosterone production. The present study was, therefore, designed
83 to investigate the effects of long-term GnRH implantation [4.7 mg GnRH-agonist
84 (Deslorelin)] on the reproductive performance, testicular morphology and expression of LHR
85 and FSHR in pre-pubertal male cats. Testicular morphology and expression of LHR and
86 FSHR were also compared between pre-pubertal and adult male cats.

87

88 **2. Materials and methods**

89 *2.1 Experiment design and animals*

90 Experiment 1: Three months old tomcats that were proven to be clinically healthy and
91 had attended a complete vaccination program were either implanted with 4.7 mg deslorelin

92 GnRH-agonist (Suprelorin[®] 4.7mg, Virbac Animal Health, France) in the interscapular area
93 (Deslorelin implanted; n=6) or left without any implantation and served as controls (Non-
94 implanted; n=6). The cats were housed together in an open-air room with natural daylight in
95 the Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science,
96 Chulalongkorn University, Thailand. During the study period animals were fed with a
97 commercial diet twice daily with water always available *ad libitum*. The study had ethical
98 approval and was performed under the license of Chulalongkorn University Laboratory
99 Animal Center number13310056.

100 Implanted animals were monitored for any potential adverse effects like tissue reaction at
101 the implantation site and/or infection, rashes, oedema, erythema of implantation area etc for a
102 period of one week. Body temperature was measured daily for one week after the hormonal
103 implantation to monitor any infection and if found, blood was collected for profile
104 monitoring.

105 Body weight of all the cats in both the groups was recorded fortnightly until the end of
106 the experiment (48 wks) when both the testes were collected after surgical castration.
107 Throughout the experimental period, functional evaluations of the reproductive organs such
108 as penile spines, testicular volume and consistency, and male sexual behavioural
109 characteristics were monitored at 2-weekly intervals in all the cats. Presence of penile spine
110 was taken as a criterion of puberty [5]. Length, width and depth of the scrotum/testis were
111 measured using vernier calipers and testicular volume was calculated with a modified
112 spherical equation; volume (cm³) = 4/3 x π x (1/2 length x 1/2 width x 1/2 depth) [5].
113 Testicular consistency was recorded by palpation by one observer and was noted as soft, firm
114 or hard. Male sexual behaviour such as marking, mounting (with or without intromission),
115 and fighting [20] were observed for at least 30 min at 2-weekly intervals in all the cats.
116 Faeces were collected at 2-weekly intervals to measure testosterone concentrations. An

117 attempt was made to collect semen from all the cats before surgical castration by using the
118 electro-ejaculator; which was performed 48 wks after implantation. Soon after collection,
119 semen was evaluated for its volume, colour, motility, concentration, viability and sperm
120 morphology. If semen ejaculation could not be accomplished, epididymal sperms were
121 collected immediately after castration and evaluated.

122 Experiment 2: Testes were collected from 4 to 6 months old (pre-pubertal, n = 6) or 1 to
123 3 years old (adult, n=6) normal healthy male cats after surgical castration at the Small Animal
124 Hospital, Faculty of Veterinary Science, Chulalongkorn University, Thailand.

125 In both the experiments, after castration, weight of each testis and epididymis was
126 recorded. Each testis was divided into two parts; one part was fixed in 4% (w/v)
127 paraformaldehyde for 48 to 72 hours and then stored in 70% ethanol until processing for
128 cytology/morphology and immunohistochemistry, whereas the other part was snap frozen in
129 liquid nitrogen and stored in -80 °C until RNA extraction.

130 *2.2 Luteinizing hormone receptor (LHR) and Follicle stimulating hormone receptor (FSHR)*

131 *Expression*

132 Fixed testicular tissues were embedded in paraffin wax and cut into 5µm sections by a
133 rotor microtome, applied to gelatin-coated slides and left to dry in an incubator at 37°C.
134 Sections were deparaffinized with Xylene (J.T. Baker, PA, USA) and rehydrated through
135 ascending concentrations of alcohol (50%, 70%, 90%, 99.7% and 100%). The
136 immunohistochemical staining was performed as described previously by Ponglowhapan *et al.*
137 [21]. Briefly, the tissue sections/slides were placed in boiling 0.01M sodium citrate solution,
138 then cooled down to room temperature for 35 mins to de-mask epitopes. Slides were then rinsed
139 three times in phosphate buffered saline (PBS). Endogenous peroxidase activity was
140 inactivated by immersing slides in 1% (v/v) hydrogen peroxide in methanol for 10 min, then
141 rinsed again three times in PBS. Sections were subsequently blocked for 60 min in a humidified

142 chamber using a blocking solution, comprising 1% normal horse serum (Vector Laboratories,
143 CA, USA) diluted in PBS and 20% (v/v) avidin solution (Avidin/Biotin blocking kit; Vector
144 Laboratories, CA, USA). After washing slides three times in PBS, the slides were incubated
145 overnight at 4°C in a humidified chamber with LHR (H-50) polyclonal antibody (Santa Cruz
146 biotechnology, Inc., USA) at a dilution of 1:50 or with FSHR (N-20) polyclonal antibody
147 (Santa Cruz biotechnology, Inc., USA) at a dilution of 1:50. The primary antibodies were
148 diluted in PBS to which 20% (v/v) biotin solution (Avidin/Biotin blocking kit; Vector
149 Laboratories, CA, USA) was added. The negative control sections were treated in the same
150 manner with PBS and biotin mixture in the absence of primary antibodies. After incubation,
151 sections were washed with PBS three times (3 x 10 minutes). Then, secondary antibody
152 (Biotinylated anti-mouse anti-rabbit IgG, Vector Laboratories, Inc., USA for LHR localization
153 and Biotinylated anti-goat IgG, Vector Laboratories, Inc., USA for FSHR localization) were
154 applied to the sections and incubated for 30 min. Sections were washed again three times in
155 PBS and incubated at room temperature with 20% (v/v) avidin-biotin complex solution
156 (VECTASTAIN® Vector Laboratories, Inc., USA) for 30 min. Tissue sections were then
157 incubated with DAB peroxidase substrate (Vector Laboratories, Inc., USA) until colour
158 development. All slides were counterstained with Mayer's hematoxylin. Brown staining was
159 observed on tissue sections with positive staining for both LHR and FSHR and no staining was
160 observed for negative controls for either receptor.

161 At least two sections for both positive antibody staining and negative controls were
162 examined from each animal.

163

164 *2.3 Quantification of immunohistochemical staining*

165 The pattern and intensity of protein staining for LHR and FSHR were determined
166 semi-quantitatively using a histochemical score (HSCORE) method. Ten fields per

167 section of each tissue sample were assessed blind by one assessor using a light
168 microscope at X 200 magnification. The intensity of staining was classified on a scale of
169 1-3, where 1 = weak staining, 2 = moderate staining and 3 = strong staining [21, 22].
170 Histochemical score (H-SCORE) was assessed as percent of each level (weak, moderate
171 or strong) of staining in each tissue area with the Image-pro plus 7.0 program (Media
172 Cybernetics, Inc. MD, USA). An expression index (EI) was calculated for each tissue
173 sample based on the percentage of positively stained cells and the intensity of staining
174 using the following formula:

$$175 \quad EI = \% \text{ total stained cells} \times [(1 \times \% \text{ weak}) + (2 \times \% \text{ medium}) + (3 \times \% \\ 176 \text{ strong})] / 100$$

177 A mean expression index was calculated to represent the protein expression of LHR
178 or FSHR in each testicular section of every testis from an individual animal [22-24].

179

180 *2.4 Morphology of Testes*

181 Testes collected in both the experiments were fixed, embedded in paraffin wax, cut into
182 5µm sections and stained with hematoxylin and eosin. The stained sections (5 sections/testis)
183 were evaluated for seminiferous tubules; those with normal basement membrane (basement
184 membrane with a continuous line and germ cells well attached) [19] were considered as normal
185 and functional. Diameter of seminiferous tubules in each tissue section was measured using
186 ocular micrometer at X 200 magnification. Using the criteria of Novotny *et al.* [19] a total of
187 200 seminiferous tubules per section were classified as st0, st1, st2, st3 and st4 if they had only
188 spermatogonia (st0), only spermatogonia and spermatocytes (st1), or with spermatids (st2), or
189 with elongating spermatids (st3) or with elongated spermatids (st4) present in the lumen. Each
190 tissue section was graded into 5 grades (0 to 4), based on the majority of seminiferous tubules

191 found in the tissue sections; Grade 0: Testicular tissue with the majority of st0 seminiferous
192 tubules, Grade 1: Testicular tissue with the majority of st1 seminiferous tubules, Grade 2:
193 Testicular tissue with the majority of st2 seminiferous tubules, Grade 3: Testicular tissue with
194 the majority of st3 seminiferous tubules, and Grade 4: Testicular tissue with the majority of st4
195 seminiferous tubules. Any pathological changes in the testes, if present, were investigated and
196 recorded.

197

198 *2.5 Quantitative real-time polymerase chain reaction (qPCR) for the LHR and FSHR mRNA* 199 *in the testicular tissue*

200 *2.5.1 Extraction and reverse transcription of mRNA*

201 Frozen testicular tissue was ground with a homogenizer at 10,000 to 20,000 RPM for 10
202 to 20s and used to extract the total RNA by the RNeasy mini kit (QIAGEN®, Alameda, CA,
203 USA) following the manufacturer's instructions. Concentration and purity of the extracted
204 RNA were assessed by spectrophotometer (ND-2000, NanoDrop, Wilmington, DE, USA).
205 The RNA samples were stored at -70°C before qPCR analysis.

206 *2.5.2 Quantitative real-time PCR*

207 Conventional PCR was performed and the PCR product was used for the preparation of
208 standards and analyzing the optimal melting and annealing temperature for each gene [LHR,
209 FSHR and GAPDH (reference gene)]. The thermal cycler (G-Storm Thermal Cycler,
210 Somerset, United Kingdom) was set at the conditions of 15 min at 95°C to activate Taq DNA
211 polymerase, 30 cycles of 30s at 94°C for denaturing, 90s at 57°C for annealing, 30s at 72°C
212 for extension and 10 min at 72° C for the final extension. Previously published sequences of
213 forward and reverse primers for feline LHR and FSHR, and GAPDH were used [25, 26] and
214 are shown in Table 1. Each reaction was contained with Qiagen Multiplex PCR Kit
215 (QIAGEN®, Alameda, CA, USA). Amplified products were run on 1.2% agarose gel

216 (SIGMA-ALDRICH[®], St, Louis, MO, USA) and visualized under UV gel document and
217 analysis (SYNGENE[®] Cambridge, United Kingdom) to confirm the presence of single
218 products without dimers. Purification of the amplified products was performed with the
219 QIAquik PCR purification kit (QIAGEN[®], Alameda, CA, USA). Purified products were
220 quantified by spectrophotometer (ND-2000, NanoDrop, Wilmington, DE, USA) and used to
221 prepare standards for use in qPCR assessment.

222 Real-time qPCR amplification was performed using CFX96 Thermal cycler (Bio-Rad
223 Laboratories, Inc., Hercules, CA, USA) with the Bio-Rad CFX manager 3.1 software (Bio-Rad
224 Laboratories, Inc., Hercules, CA, USA). Each reaction (20 μ l) was contained with 10 μ l of 2x
225 qPCR BIO SyGreen Mix Lo-ROX (PCR Biosystems Ltd, London, United Kingdom), 0.8 μ l of
226 each forward and reverse primer, 5 μ l of a DNA template (5ng/ μ l), and the volume made up to
227 20 μ l with RNase free water. RNase free water was added instead of cDNA template in the
228 Non-template control (NTC). Thermocycler was set for 38 cycles of denaturing at 95°C for 5s
229 following with the optimum annealing temperature of 61.4°C, 60°C and 61.4°C for 25s and
230 melting temperature of 82°C, 80°C and 76°C for 10s for GAPDH, FSHR and LHR, respectively
231 with a gradient from 50 to 95°C to investigate the gene expression. Standards of each gene
232 were used as controls to determine the absolute quantity of mRNA (fg/ μ g of total RNA).

233 *2.6 Statistical analysis*

234 Body weight and testicular volume were compared between the deslorelin implanted and
235 non-implanted (Expt 1) animals using Independent T-test.

236 General linear model (GLM) was performed to compare the protein and mRNA expression
237 of LHR and FSHR and the epididymal weight between the deslorelin implanted and non-
238 implanted (Expt 1) and between the pre-pubertal and adult (Expt 2) animals. Wilcoxon rank
239 sum test was performed to compare the testicular weight, the mean diameter of seminiferous

240 tubules and the grade of seminiferous tubules between the deslorelin implanted and non-
241 implanted cats (Expt 1) and between the pre-pubertal and adult cats (Expt 2)

242 **3. Results**

243 No tissue reaction and/or infection were observed after deslorelin implantation.

244 No difference in body weight was recorded between deslorelin implanted and non-implanted
245 cats (Figure 1). The implanted cats had significantly lower ($P<0.05$) testicular volume from wk
246 30 of study onwards (Figure 2). Male sexual behaviour was absent in implanted cats but was
247 present in non-implanted cats from 28 wks onwards of the study period. However, testicular
248 consistency was soft in both groups and remained soft in deslorelin implanted cats throughout
249 the study while from the 28th wk until the end of study period non-implanted cats had
250 comparatively firmer testicular consistency. Penile spines in non-implanted cats were present
251 from the 28th wk onwards of study period and were absent in implanted cats throughout the
252 study period of 48 wks. Higher faecal testosterone levels ($P<0.05$) were observed in non-
253 implanted compared to deslorelin implanted cats from 20 wks onwards of study period (Figure
254 3). It was possible to collect semen from all of non-implanted cats., semen volume were 10 –
255 73 μ l with 40 – 60% of sperm motility, 49 – 72% of sperm viability and sperm concentration
256 at 0.15×10^6 – 18×10^6 sperms/ml. Not only implanted cats failed to ejaculate but also we were
257 not able to collect epididymal sperm from them.

258 Testicular and epididymal weight, testicular grade and mean seminiferous tubule diameter were
259 higher in the adult as well as non-implanted cats compared to pre-pubertal or implanted cats (P
260 < 0.05) (Table 2).

261 LHR and FSHR were localized in the cytoplasm of Leydig and germ cells, respectively. LHR
262 expression was significantly higher ($p<0.05$) in the non-implanted compared to deslorelin
263 implanted tomcats whereas no difference was found between adult and pre-pubertal animals.

264 FSHR expression was not different between adult and pre-pubertal cats or between implanted
265 and non-implanted cats (Figures 4, 5).

266 LHR and FSHR mRNA were expressed in all the testicular samples collected in both the
267 experiments. No differences were observed in the expression of LHR mRNA expression
268 between the groups in either experiment. The expression of FSHR mRNA, however, was
269 significantly higher ($P < 0.05$) in the pre-pubertal and deslorelin implanted cats compared to
270 adult and non-implanted cats, respectively (Figures 6 and 7).

271

272 **4. Discussion**

273 The objectives of this study were to compare 1) the testicular characteristics, and both
274 protein and mRNA expression of LHR and FSHR between pre-pubertal and adult tomcats and
275 2) to investigate the effect of GnRH-agonist implantation on the sexual behavior, reproductive
276 performance and the testicular LHR and FSHR expression in pre-pubertal tomcats.

277 Deslorelin implantation which was done without any anesthesia, local or general, was
278 very well tolerated by male pre-pubertal cats as has been reported in previous studies [5, 17].
279 The sexual behaviour of implanted cats was suppressed and many unwanted behaviours such
280 as spraying, fighting and roaming were totally absent in these cats. This suppression of
281 behaviors resulting from Deslorelin implantation was comparable with behaviours eliminated
282 by surgical castration [27]. Moreover, suppression of physiology of the reproductive organs
283 such as, the grade of testicular tissue, the seminiferous tubules diameter, and the weight of
284 testes and epididymides of implanted cats, which were significantly lower compared to non-
285 implanted cats, confirms the action of Deslorelin implantation on suppressing the function of
286 male reproductive tract.

287 The physiology and sexual behavior of male mammals is mainly controlled by
288 testosterone which is produced by the activation of Leydig cells by the LH released from the

289 pituitary gland [28, 29]. Aromatase and 5- α reductase transform testosterone into estrogen and
290 dihydrotestosterone, respectively and these two hormones are considered to be responsible for
291 the change in the male behaviour. However, testosterone itself acts on the Sertoli cells of the
292 testis to support spermatogenesis. FSH is believed to have an important role in the first wave
293 of spermatogenesis in pre-pubertal mammals but its role in spermatogenesis in adults remains
294 to be confirmed. However, FSH is considered to induce meiosis during spermatogenesis
295 process and therefore, is responsible for increasing the number of spermatogonia in the
296 seminiferous tubules [30].

297 The HPG axis is activated by the release of GnRH from the hypothalamus which
298 stimulates the release of gonadotropins from the pituitary gland to regulate the reproductive
299 function. The chronic administration of GnRH down regulates the pituitary GnRH receptors
300 and suppresses the release of gonadotropins and the reproductive function [31]. It is for this
301 reason that GnRH-agonist implantation is used as an alternative to surgical castration in a
302 number of species including felines. In the present study, we have tried to confirm this in pre-
303 pubertal male cats and to explore whether such a GnRH therapy suppresses the reproductive
304 function via an involvement of testicular expression of LHR and FSHR. FSHR is expressed in
305 the Sertoli cells of the testes and is responsible to control spermatogenesis after activation by
306 the FSH [32], whereas LHR being expressed in the Leydig cells, is responsible to stimulate
307 androgen secretion [33] by activating the biosynthetic pathway that changes cholesterol into
308 testosterone [34].

309 In the present study, the significantly higher expression of FSHR mRNA in the testicular
310 tissue of deslorelin implanted male cats compared to untreated controls could be a result of the
311 compensatory mechanism resulting from the suppression of the endogenous release of GnRH
312 (and/or FSH) due to implantation of deslorelin (GnRH-agonist). It seems that translational
313 pathway has also been affected by deslorelin implantation as no difference was observed in the

314 protein expression of FSHR, even though the mRNA expression of the FSHR was significantly
315 higher in the implanted group. As in pre-pubertal mammals FSH is known to play a major
316 role in the first wave of spermatogenesis [30, 35] but in pubertal mammals, spermatogenesis is
317 mainly androgen-dependent and the effect of FSH is limited mainly to support the production
318 of spermatogonia [30], the absence of sperm production observed in deslorelin-implanted cats
319 therefore seems to result from the suppression of testosterone production due to the
320 downregulation of LHR in the Leydig cells of deslorelin-treated cats.

321 In this study, we observed that faecal testosterone concentrations and testosterone-
322 dependent sexual behaviour were both suppressed in deslorelin implanted compared with the
323 non-implanted cats. We also found that the LHR protein expression in deslorelin implanted
324 cats was suppressed compared with the non-implanted cats. As testosterone production
325 depends on the activation of LHR in the Leydig cells, it therefore, seems highly likely that the
326 observed suppression of faecal testosterone and the testosterone-dependent behaviours may be
327 the result of observed testicular suppression of LHR protein in the implanted cats.

328 Both mRNA and protein expression of the LHR were studied in deslorelin-implanted and
329 control cats. Deslorelin suppressed the LHR protein expression but was without any effect on
330 the LHR mRNA. These results suggest that deslorelin downregulates the LHR by interfering
331 at the translational level but do not interfere transcription of the gene. Surprisingly, no
332 difference was observed in the protein or mRNA expression of LHR between the adult and pre-
333 pubertal cats. This may indicate that at the age of 3 months cats already had active Leydig cells
334 that are capable to produce testosterone. It is difficult to estimate the exact time period after
335 which deslorelin might have been effective to suppress the LHR in the implanted cats because
336 the testes were collected only 48 wk after the implantation. However, there was no difference
337 in the faecal testosterone concentrations between the two groups at the time of implantation
338 but from wk20 onwards testosterone concentrations in the implanted group started to be

339 significantly lower than the controls. This may suggest that the LHR suppression might have
340 resulted sometimes within the 20 wks period of deslorelin implantation. However, it remains
341 to be determined whether testosterone suppression in deslorelin-implanted cats was the result
342 of LHR suppression only or was a result of the combined effect of suppression in the LH
343 concentrations and LHR. Moreover, this study was not designed to investigate how the
344 deslorelin implantation directly or indirectly might have affected the biosynthetic pathway of
345 testosterone production that may involve changes in the cAMP to stimulate the transport of
346 cholesterol into the mitochondria and changes in the activities of different enzymes responsible
347 for the pregnenolone, progesterone, androstenedione and finally testosterone production [29]. We
348 speculate that the effect of deslorelin may not be only at the level of LH production and/or
349 testicular LHR expression but may also be at other sites in the biosynthetic pathway of
350 testosterone production. A suppression of pulsatile LH secretion from long-term GnRH-agonist
351 (Goserelin) treatment has already been reported in gilts [15] and several male characteristics
352 such as the presence of penile spines and male behaviours such as roaming, fighting and
353 spraying are gonadal steroid hormones (especially testosterone) dependent and could be
354 eliminated via the suppression of testosterone [27].

355 Normally GnRH-agonist implantation presents an upregulation effect in the first period
356 after hormonal implantation followed by a downregulation effect after long-term
357 administration in pubertal tomcats [5]. However, in this study we did not observe any
358 upregulation effect when male cats were implanted with GnRH-agonist at the age of 3 months
359 possibly because of an immature HPG axis at this age [17].

360 This study was not designed to investigate whether the observed suppression of the
361 reproductive function could be reversed or not. However, non-reversibility of reproductive
362 function has been reported in dogs that were implanted with GnRH agonist before the age of 4
363 months [16, 36]. Reversibility of reproductive function has been reported in studies using 1.6

364 mg deslorelin in post-natal cats and 4.7 mg deslorelin in 114 days old female cats, puberty was
365 postponed until the age of 16 months and 134 to 286 days, respectively [17, 18] . However, in
366 this study it remains to be seen whether reproductive function could be reinstated or not,
367 nevertheless, the results obtained show that reproductive function remained suppressed for a
368 period of at least 48 wks after 4.7 mg deslorelin administration at the age of 3 months.

369 In conclusion, the results of the present study have shown that implantation of 4.7 mg
370 GnRH-agonist (Deslorelin®) in male cats at the age of 12 wks suppresses the reproductive
371 function for at least for 48 wks without any adverse effects on the general health. Moreover,
372 this suppression of reproductive function may be achieved partly by down-regulation of LHR
373 in the Leydig cells while maintaining the FSHR expression at the pre-pubertal levels in the
374 Sertoli cells of the testis.

375

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483 2.
- 484

486 **Table 1:** Description of forward and reverse primers for GAPDH as housekeeping gene and
 487 feline LH receptor (LHR) and FSH receptor (FSHR) as target genes.

Gene	Primer sequence (5'–3')	Length (bp)	Reference
GAPDH	F: GGAGAAAGCTGCCAAATATG	20	[25] and [26]
	R: AGGAAATGAGCTTGACAAAGTGG	23	
LHR	F: CTAATGCCTTTGACAACCTAATA	23	[25]
	R: CCCATTGAATGCATGACTTTGTA	23	
FSHR	F: CATGCTGCTAGGCTGGATCTT	21	[25]
	R: CTTGGCGATCTTGGTGTCACT	21	

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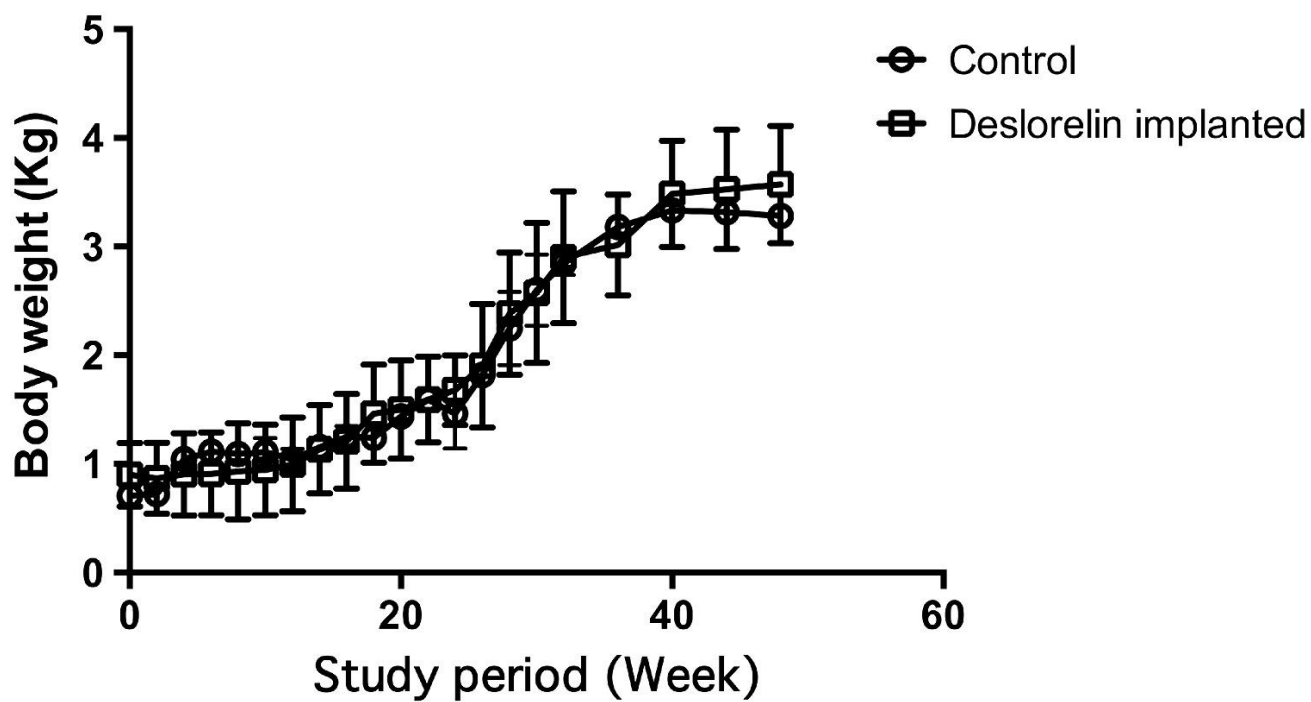
491 **Table 2:** Mean \pm SEM values for weight (g) of the testes and epididymides, seminiferous
 492 tubule diameter (μm), and grade of testicular tissues obtained from tomcats in both the
 493 experiments.

Expt	Groups	Weight (g)		Seminiferous tubule diameter (μm)	Testicular tissue grade
		Testes	Epididymides		
1	Implanted	0.09 \pm 0.02 ^a	0.03 \pm 0.01 ^a	62.02 \pm 2.88 ^a	0.00 \pm 0.00 ^a
	Non-implanted	1.54 \pm 0.20 ^b	0.26 \pm 0.03 ^b	98.48 \pm 3.59 ^b	3.86 \pm 0.17 ^b
2	Adult	1.24 \pm 0.20 ^x	0.24 \pm 0.03 ^x	95.31 \pm 3.94 ^x	3.83 \pm 0.41 ^x
	Prepubertal	0.19 \pm 0.04 ^y	0.08 \pm 0.02 ^y	57.85 \pm 8.50 ^y	1.33 \pm 0.21 ^y

494

495 Values within a column with different superscript letters (a, b and x, y) in an experiment differ
 496 significantly ($P < 0.05$).

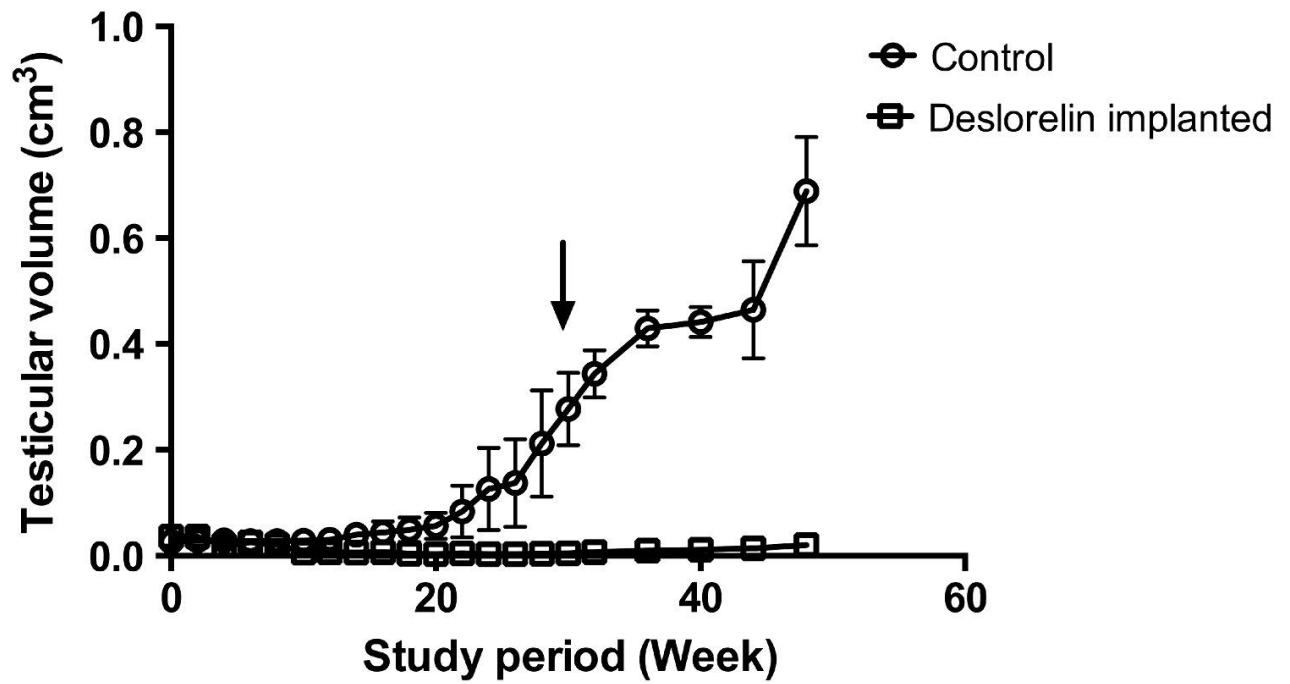
497 **Figure legends**



498

499 **Figure 1:** The mean (\pm SEM) body weight (kg) in cats (n=6/group) with or without deslorelin
500 implantation. Cats were implanted with deslorelin at the age of 3 months for a period of 48
501 weeks.

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503

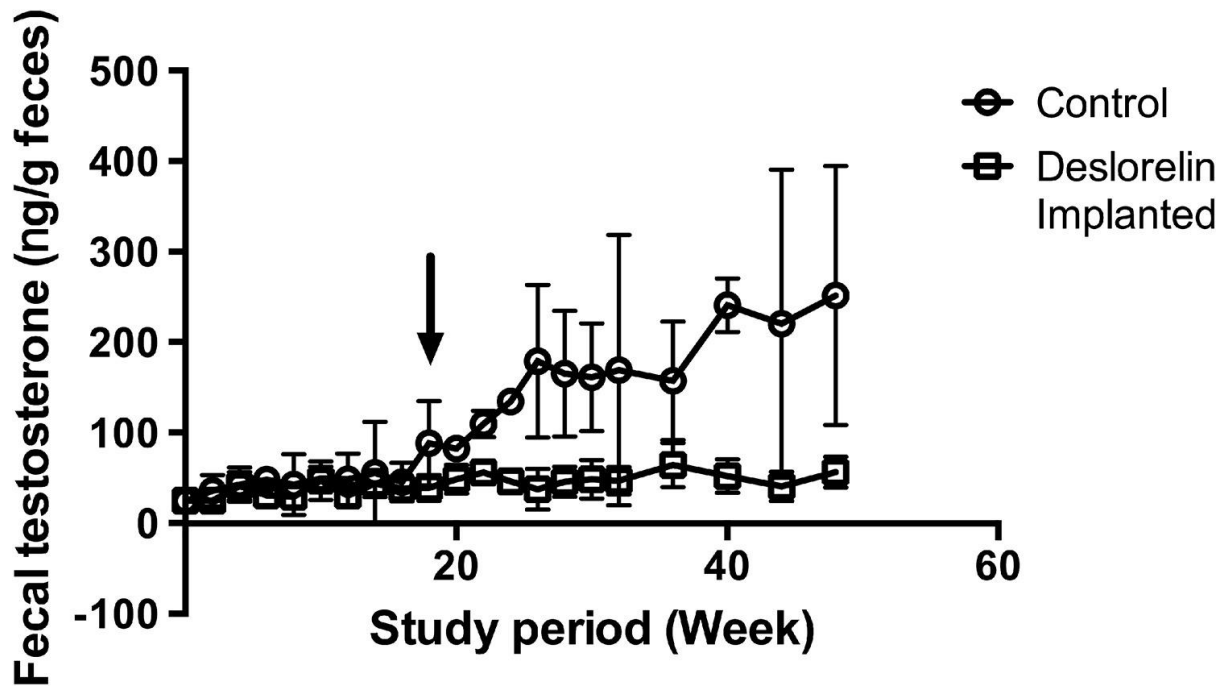
504 **Figure 2:** The mean (\pm SEM) testicular volume (cm³) in cats (n=6/group) with or without

505 deslorelin implantation. Cats were implanted with deslorelin at the age of 3 months for a

506 period of 48 weeks. Black arrow indicates the week that the control group has significantly

507 higher ($p < 0.05$) testicular volume than the implanted group.

508

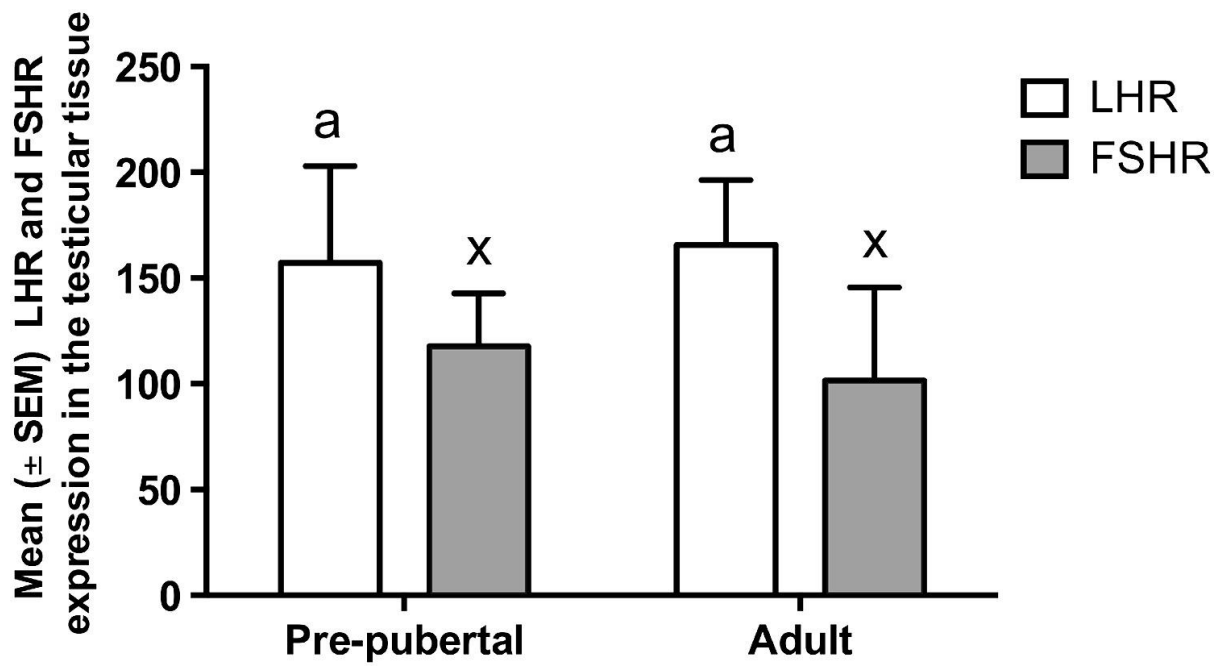


509

510 **Figure 3.** The mean (\pm SEM) faecal testosterone levels (ng/g of faeces) in cats (n=6/group)
 511 with or without deslorelin implantation. Cats were implanted with deslorelin at the age of 3
 512 months for a period of 48 weeks. Black arrow indicates the week when the control group
 513 started to have significantly higher faecal testosterone levels than the implanted group ($P <$
 514 0.05).

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517

518 **Figure 4.** The mean expression index (± SEM) for LHR and FSHR in testicular tissue of

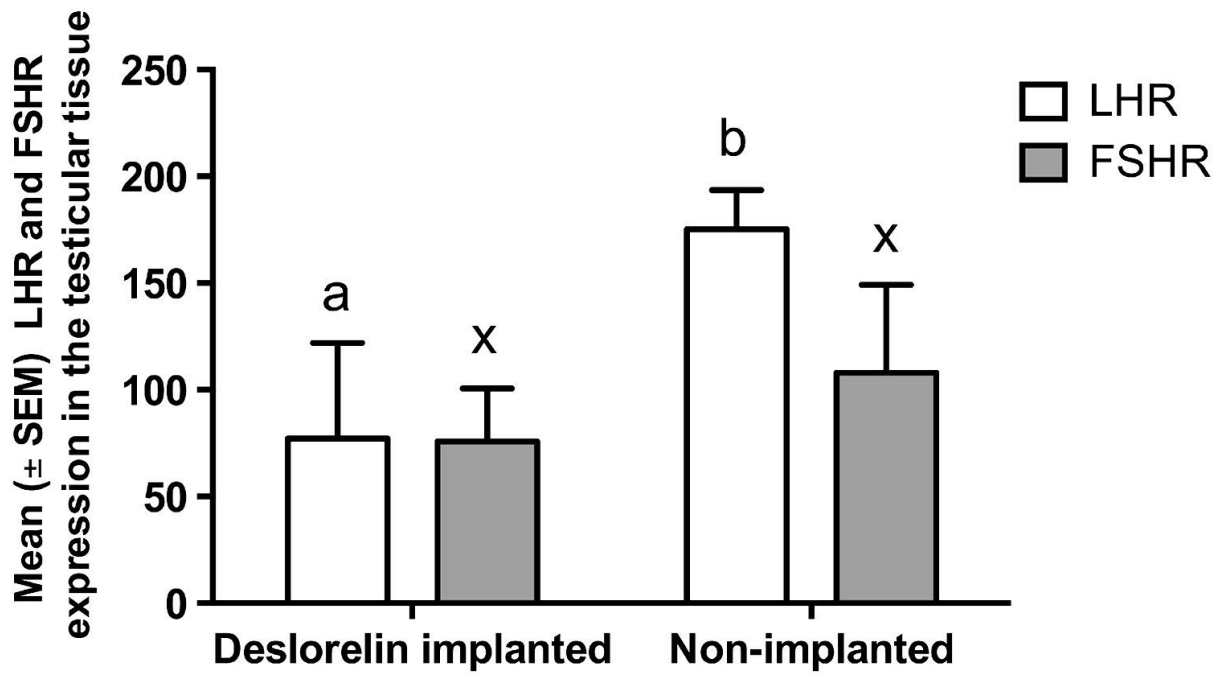
519 pubertal and pre-pubertal cats (n=6/group). Different letters on bars for a certain receptor

520 indicate significant differences ($P \leq 0.05$).

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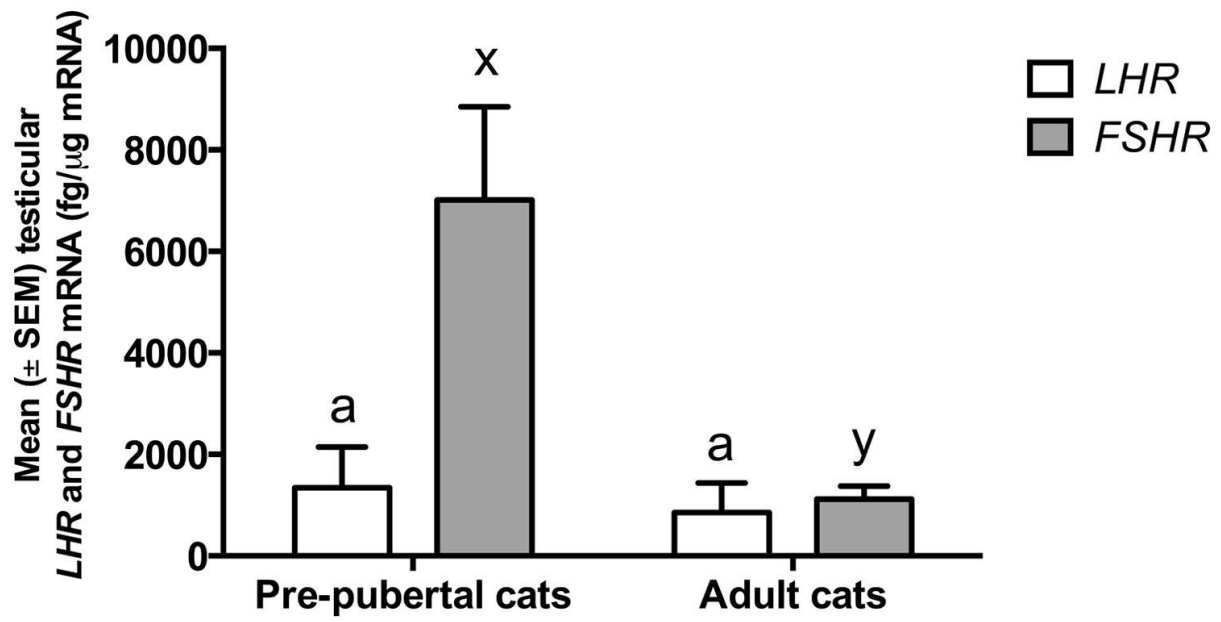


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526 **Figure 5.** The mean expression index (± SEM) for LHR and FSHR in testicular tissue of cats
527 (n=6/group) with or without deslorelin implantation. Cats were implanted with deslorelin at
528 the age of 3 months for 48 weeks. Different letters on bars for a certain receptor indicate
529 significant differences ($P \leq 0.05$).

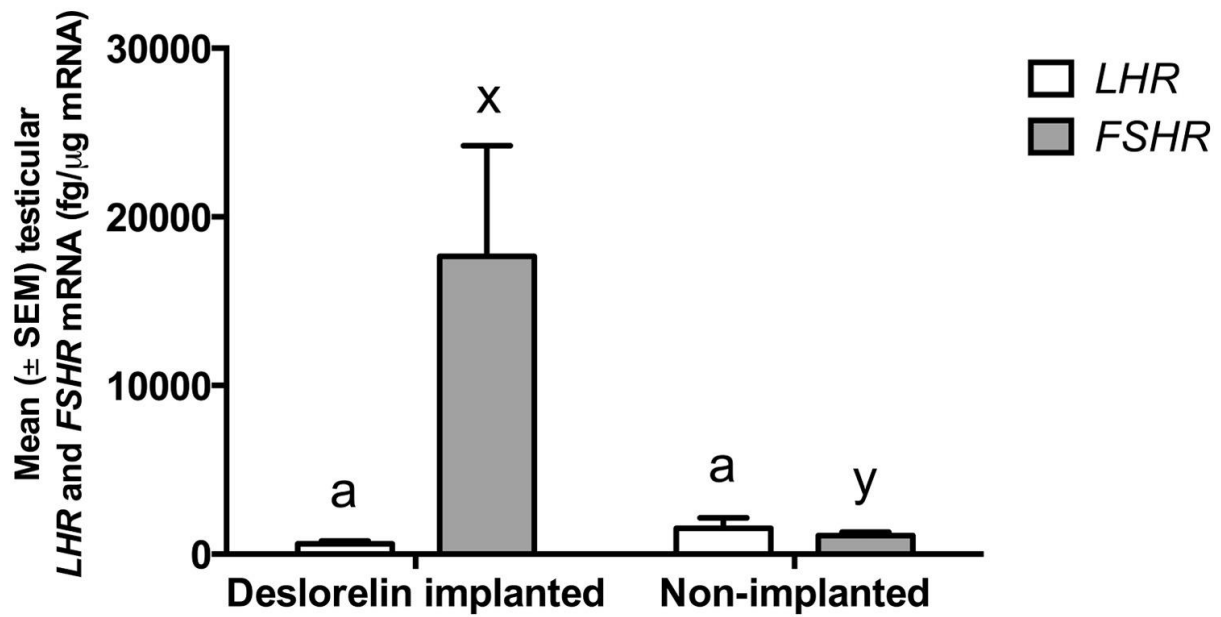
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531

532 **Figure 6.** The mean mRNA concentration (fg/μg mRNA) (± SEM) for LHR and FSHR in
 533 testicular tissue of pubertal and pre-pubertal cats (n=6/group). Different letters on bars for a
 534 certain receptor indicate significant differences ($P \leq 0.05$).

535



536

537 **Figure 7.** The mean mRNA concentration (fg/μg mRNA) (± SEM) for LHR and FSHR in
 538 testicular tissue of cats (n=6/group) with or without deslorelin implantation. Cats were
 539 implanted with deslorelin at the age of 3 months for 48 weeks. Different letters on bars for a
 540 certain receptor indicate significant differences ($P \leq 0.05$).

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