

# Sex hormone regulation of collagen concentrations in cranial cruciate ligaments of sexually immature male rabbits

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**Objective**—To investigate the effects of gonadectomy on collagen homeostasis in cranial cruciate ligaments of male rabbits.

**Animals**—30 sexually immature (16-week-old) male New Zealand White rabbits.

**Procedures**—Rabbits were randomly assigned to 5 groups of 6 rabbits each: sexually intact, placebo (control group); castrated, placebo; castrated, testosterone; castrated, dihydrotestosterone; and castrated, 17 $\beta$ -estradiol (E<sub>2</sub>). Control rabbits underwent a sham operation, and all other rabbits underwent gonadectomy. At the time of gonadectomy, the placebo and sex hormones were administered via slow-release pellets implanted subcutaneously as assigned. After 21 days of hormone supplementation, measurements were obtained of serum testosterone and E<sub>2</sub> concentrations, ligament collagen characteristics, and androgen receptor, estrogen receptor  $\alpha$ , and matrix metalloproteinase expression.

**Results**—Following gonadectomy and hormone supplementation, the treatment groups differed in serum testosterone and E<sub>2</sub> concentrations to various degrees. Collagen concentrations were lower and fiber diameters higher in the absence of sex hormones, in association with the degrees of estrogen receptor  $\alpha$  and androgen receptor expression. Although differences were detected among the groups in matrix metalloproteinase expression, these differences were not significant.

**Conclusions and Clinical Relevance**—Sex hormones appeared to play a role in cranial cruciate ligament homeostasis in male rabbits. Physiologic changes triggered by the lack of sex hormones following gonadectomy in sexually immature rabbits may potentially predispose those rabbits to orthopedic injuries. (*Am J Vet Res* 2012;73:1186–1193)

Rupture of the CCL is the leading cause of orthopedic disorders of the canine stifle joint,<sup>1</sup> and 20% of dogs admitted to university hospitals for lameness have this condition.<sup>2–4</sup> The economic impact associated with the treatment of CCL rupture in the United States in 2003 was estimated at > \$1.3 billion.<sup>4</sup>

The pathogenesis of CCL rupture has been linked to degenerative changes within the ligament and may result from conformational abnormalities and traumatic events.<sup>2,5–7</sup> Studies<sup>5,8,9</sup> have demonstrated a positive correlation between CCL rupture, body weight, gonadectomy status, and age-related factors, such as development of synovitis and degenerative or adaptive changes in the ligament.

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## ABBREVIATIONS

AR	Androgen receptor
CCL	Cranial cruciate ligament
DHT	Dihydrotestosterone
E <sub>2</sub>	17 $\beta$ -estradiol
ESR1	Estrogen receptor $\alpha$
MMP	Matrix metalloproteinase
TEM	Transmission electron microscopy

The possibility exists that sex hormones impact CCL integrity. A review of data from > 1,000 dogs with CCL injuries at 23 veterinary clinics across North America revealed that gonadectomized dogs of both sexes had a higher prevalence of such injuries than did sexually intact dogs.<sup>5</sup> Another study<sup>10</sup> found that the prevalence of CCL injuries in sexually intact female dogs (5.15%) was higher than in sexually intact male dogs (2.09%). These findings were supported by those of observational studies<sup>10,11</sup> that found gonadectomy predicted an increased prevalence of CCL injuries in both sexes and in all sizes and breeds of dogs. Altogether, these observations suggest that the incidence of CCL rupture may be related, at least in part, to sex hormone action.

The mechanisms by which sex hormones affect CCL integrity are not clear, but studies<sup>12-14</sup> have shown that CCL remodeling is subject to sex hormone action. For example, high serum E<sub>2</sub> concentrations in women can decrease collagen production in anterior cruciate ligament cultures.<sup>15</sup> Other studies<sup>16-19</sup> found that expression of the MMP group of enzymes, which play a role in collagen metabolism, is regulated by hormones. Thus, it is possible that sex differences affecting ligament remodeling have an influence on the size, shape, and internal structure of the CCL and hence its ability to sustain loads.<sup>20</sup> It follows that altered ligament remodeling triggered by changes in blood sex hormone concentrations potentially predisposes CCLs to injury.<sup>20</sup>

Although castration has historically been performed as early as 4 weeks of age,<sup>21</sup> veterinarians in the United States now recommend that gonadectomy in dogs and cats be performed between 6 and 9 months of age.<sup>22</sup> Incidentally, gonadectomy prior to the attainment of sexual maturity has been suggested to result in sexual and developmental anomalies or to predispose dogs to certain diseases.<sup>23,24</sup> The interaction between E<sub>2</sub> and relaxin is believed to selectively contribute to degeneration of specific synovial joints in women, such as the knee joint, pubic symphysis, and temporomandibular joint.<sup>25</sup> Indeed, sex hormones appear to play a role in matrix turnover and collagen loss.<sup>26</sup> However, there is little or no information on the role of sex hormones in collagen homeostasis in males. The purpose of the study reported here was to investigate the effects of gonadectomy on collagen homeostasis in the CCLs of male rabbits.

## Materials and Methods

**Animals**—Thirty 16-week-old sexually intact male New Zealand White rabbits<sup>a</sup> were obtained and allowed 3 days to acclimate to new housing conditions. This study protocol was approved by the Institutional Animal Care and Use Committee of Auburn University.

**Procedures**—Rabbits were randomly assigned to 5 groups of 6 rabbits each as follows: sexually intact–placebo (control group), castrated–placebo, castrated–testosterone, castrated–DHT, and castrated–E<sub>2</sub>. On the fourth day after arrival, rabbits were anesthetized by IM administration of ketamine<sup>b</sup> (15 mg/kg) and dexmedetomidine<sup>c</sup> (0.5 mg/kg). To establish baseline values for serum sex hormone concentrations, a blood sample was obtained from a marginal ear vein and separated to obtain serum, which was stored at –20°C until analyzed.

Rabbits in the control group underwent a sham operation, whereas all remaining rabbits underwent gonadectomy. At the time of surgery, placebo and slow-release treatment pellets containing steroid hormones<sup>d</sup> were implanted subcutaneously in the lateral aspect of the neck and a suture was placed over the incision. Treatment pellets contained 50 mg of testosterone or DHT or 25 mg of E<sub>2</sub>. Implants were left in place for a period of 3 weeks (21 days), at the end of which the rabbits were anesthetized for blood sample collection followed by euthanasia with a solution of pentobarbital sodium and phenytoin sodium<sup>e</sup> (150 mg/kg, IV).

The right CCL of each rabbit was aseptically harvested, snap frozen in liquid nitrogen, and stored at –80°C until immunoblot analysis of gene expression (n = 6 ligaments). The left CCL was collected and processed for electron microscopy to determine collagen fiber diameters (n = 3) or for collagen assays to measure collagen concentrations (n = 3).

**Radioimmunoassays**—Serum concentrations of testosterone and E<sub>2</sub> were determined with a tritium-labeled testosterone and E<sub>2</sub> radioimmunoassay<sup>f</sup> that involved use of antibodies against testosterone and E<sub>2</sub><sup>g</sup> (anti–testosterone-6 bovine serum albumin and anti–estradiol-6 bovine serum albumin, respectively). Interassay variation for the testosterone assay was 7.8%, with a lower limit of detection of 0.01 ng/mL, whereas the interassay variation for E<sub>2</sub> was 8.5%, with a lower limit of detection of 0.005 ng/mL.

**Collagen assay**—Concentrations of soluble collagen in CCLs were determined with a dye collagen assay kit<sup>h</sup> in accordance with manufacturer's instructions. This assay is based on the binding of Sirius red, which is an anionic dye, to the side chains of amino acids present in collagens fibers (I to IV). Briefly, ligament samples (50 mg) were placed in digestion solution containing a protease inhibitor mixture (10 µL/mL) and pepsin (1:10) in 0.5M acetic acid at a pH of 3.0 and incubated overnight on a rocker at room temperature (approx 22°C). The following morning, fresh solution was added and incubation was continued for 4 hours. At the end of incubation, 100 µL of the pepsin digest was mixed with 1 mL of dye and incubated for 30 minutes at room temperature. The collagen-dye complex was then precipitated by centrifugation at 12,879 × g for 10 minutes. The supernatants were drained and discarded, and the collagen-dye pellet was dissolved in 1 mL of alkali buffer provided by the manufacturer.

Aliquots of the dissolved collagen-dye complex (200 µL) for each sample were transferred to a 96-well plate and measured at a wavelength of 540 nm on a plate reader.<sup>i</sup> Ligament collagen concentrations were calculated by use of collagen standards provided by the manufacturer. The assays were performed in triplicate, and means were calculated for results in each treatment group.

**Western blot procedures**—Degrees of steroid hormone receptor and MMP expression were determined in CCL samples from all 6 rabbits in all 6 groups. Briefly, ligament samples (25 mg) were homogenized in lysis buffer<sup>j</sup> supplemented with 10 µL of protease inhibitor<sup>k</sup> in preparation for evaluation with the Bradford protein assay.<sup>l</sup> Ten- to 20-µg samples of the homogenized product were applied to 10% glycine-SDS-PAGE gels in a vertical electrophoresis system<sup>l</sup> and transferred by electrophoresis to nitrocellulose membranes<sup>l</sup> (0.45 µm). Subsequently, nitrocellulose membranes were preincubated for 60 minutes at room temperature in blocking buffer (5% whole milk in Tris-buffered saline [0.9% NaCl] solution containing 0.1% Tween 20). The nitrocellulose blots were incubated overnight at 4°C with primary antibodies against ESR1,<sup>m</sup> AR,<sup>n</sup> MMP-1,<sup>n</sup> MMP-2,<sup>m</sup> MMP-9,<sup>m</sup> and β-actin<sup>n</sup> at a dilution of 1:1,000 or 1:2,000 (β-actin). Subsequently, blots were

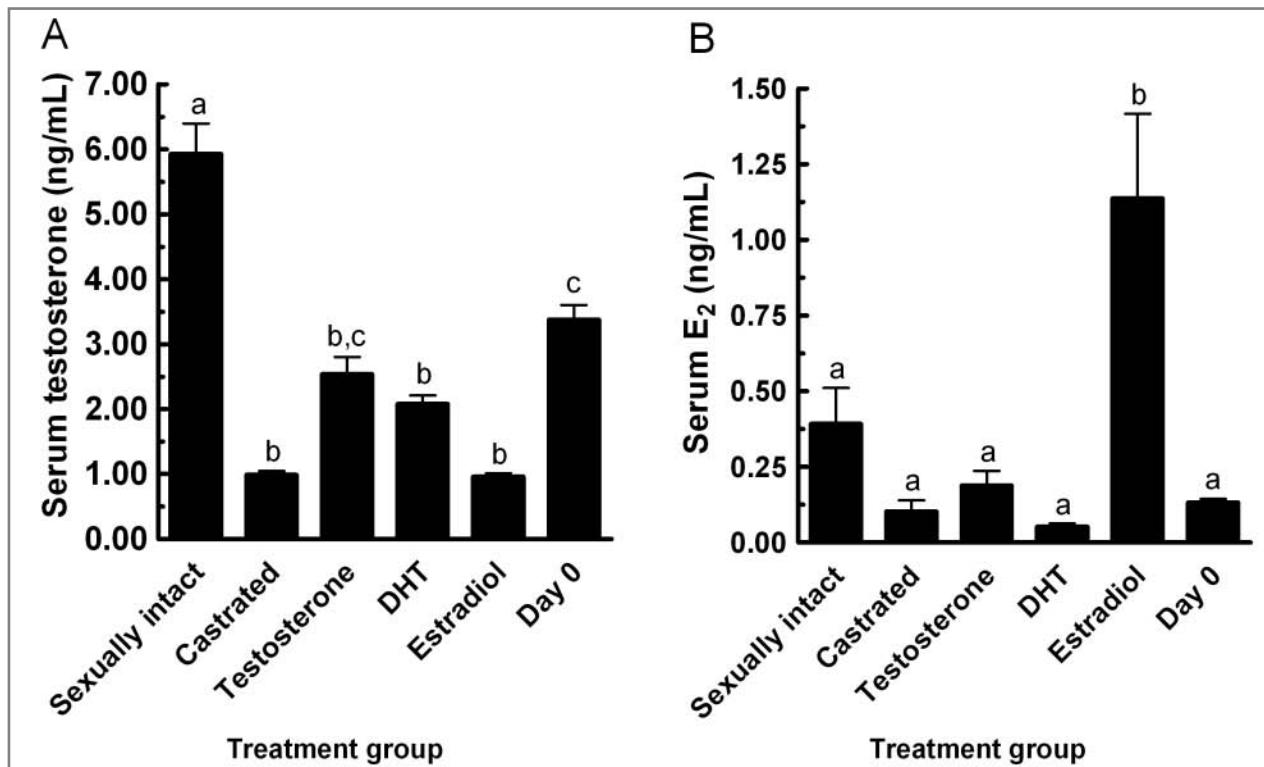


Figure 1—Mean  $\pm$  SD serum concentrations of testosterone and E<sub>2</sub> in sexually intact rabbits that received a placebo implant and in castrated rabbits that were implanted with placebo, DHT, testosterone, or E<sub>2</sub> pellets (n = 6 rabbits/group), as measured with a radioimmunoassay. Data for day 0 represent precastration values. <sup>a-c</sup>Values with different letters are significantly ( $P < 0.05$ ) different.

washed 3 times for 5 minutes each time and incubated in the appropriate horseradish peroxidase-conjugated secondary antibody<sup>n</sup> at a dilution of 1:2,000 in the blocking buffer for 90 minutes.

The resulting bands were made visible via chemiluminescence.<sup>n</sup> Relative protein amounts in identified immunoblots were estimated by measuring optical density of the bands displayed on exposed autoradiography films<sup>o</sup> with the aid of computer software<sup>p</sup> and were normalized to the degree of  $\beta$ -actin expression as an internal control. Six western blots were performed for each desired protein, with 1 sample from each of the 5 groups along with 2 positive control samples.

**TEM**—The diameter of collagen fibers was determined from ultraphotomicrographs after CCL samples were processed for TEM by use of standard procedures. Briefly, CCL samples were collected and immediately placed in a mixture of 4% paraformaldehyde-PBS solution and 0.5% glutaraldehyde solution. The samples were subsequently immersion-fixed in 2% O<sub>3</sub>O<sub>4</sub>-PBS solution overnight at 4°C. Following dehydration in a graded series of ethanol, samples were infiltrated and embedded with an epoxy resin.<sup>q</sup> Polymerization of capsules was performed at 60°C for 48 hours. Ultrathin sections (approx 85 to 90 nm) were collected on copper mesh grids, and photomicrographs were obtained via TEM<sup>r</sup> at a magnification of 45,000 $\times$ . Three photomicrographs were obtained per sample, and 20 random fiber diameters were measured on each photomicrograph. Thus, 60 measurements/sample and 180 measurements/treatment group were obtained.

**Statistical analysis**—Data are reported as mean  $\pm$  SD. Data were analyzed with 1-way ANOVA followed by the Tukey-Kramer test for multiple group comparisons.<sup>s</sup> Values of  $P < 0.05$  were considered significant. Pearson interclass correlation coefficients ( $r$ ) were determined for the relationship between collagen concentrations and fiber diameters.

## Results

Following gonadectomy and implantation of hormone pellets (placebo, DHT, testosterone, and E<sub>2</sub>) in the rabbits, several differences were evident in serum sex hormone concentrations among the various treatment groups (Figure 1;  $P < 0.05$ ). For example, serum testosterone concentrations were low in the castrated-placebo, castrated-DHT, and castrated-E<sub>2</sub> groups and were high in the castrated-testosterone group, although concentrations were lower than in the sexually intact control group ( $P < 0.05$ ). On the other hand, serum E<sub>2</sub> concentrations were higher in the castrated-E<sub>2</sub> group than in other groups ( $P < 0.05$ ). Mean  $\pm$  SD baseline serum testosterone and E<sub>2</sub> concentrations were  $3.38 \pm 1.73$  ng/mL and  $0.12 \pm 0.09$  ng/mL, respectively. Data on precastration serum testosterone and E<sub>2</sub> concentrations for all groups were pooled because there were no differences among groups ( $P > 0.05$ ).

The collagen assay revealed that the lack of sex hormones had an effect on collagen concentrations in CCL samples (Figure 2;  $P < 0.05$ ). For example, collagen concentrations in CCL samples from castrated rabbits were low, compared with concentrations in sexually in-

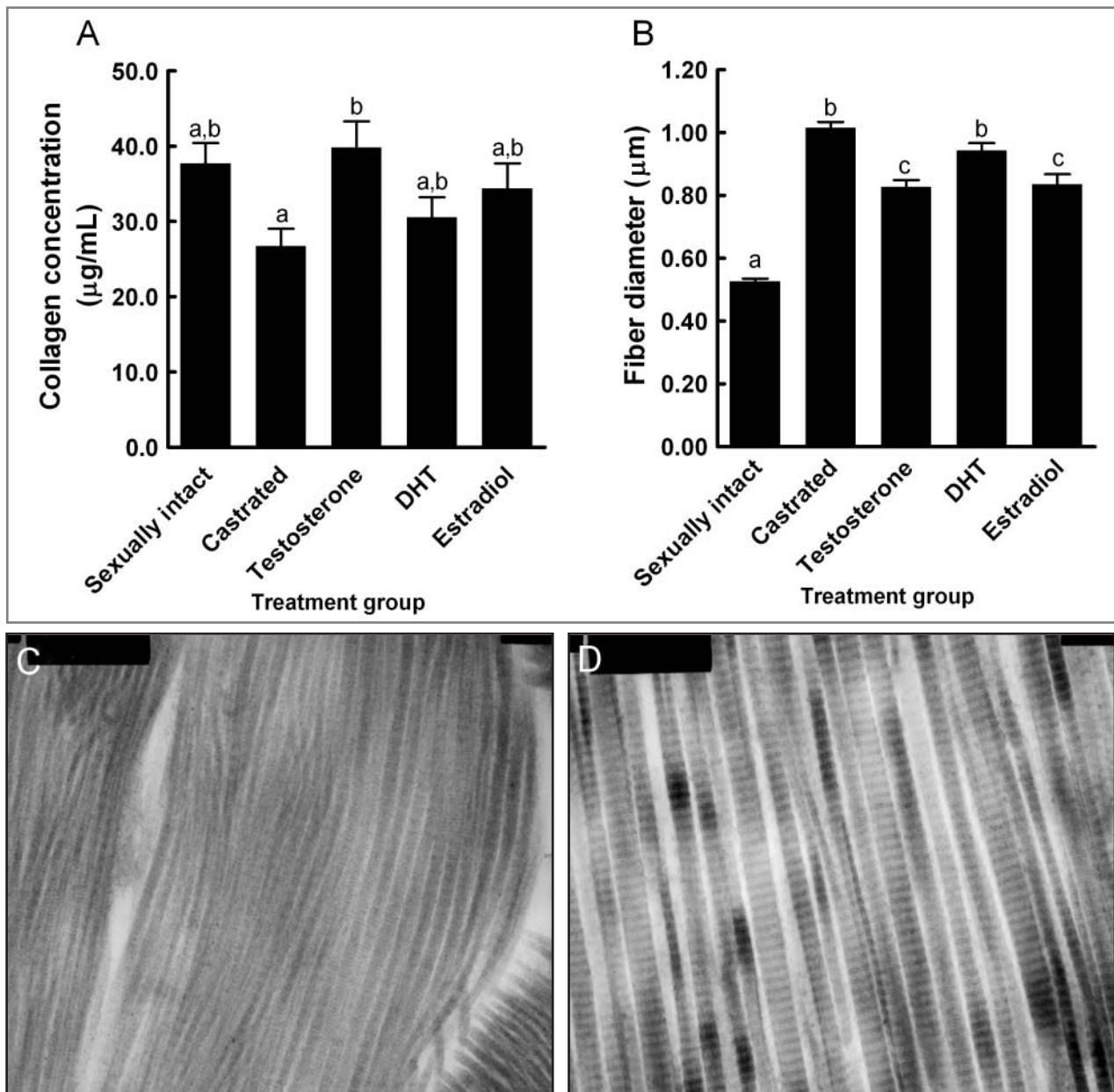


Figure 2—Mean  $\pm$  SD collagen concentrations (A) and collagen fiber diameters (B) as well as representative photomicrographs (C, sexually intact control; D, castrated) of CCL samples from the rabbits in Figure 1. See Figure 1 for remainder of key.

tact rabbits in the control group. These concentrations were partially restored to baseline values in rabbits in the castrated-DHT and castrated-E<sub>2</sub> groups and were fully restored in rabbits in the castrated-testosterone group ( $P < 0.05$ ). On the other hand, collagen fiber diameters were smallest in the sexually intact control group, were greatest in the castrated-placebo and castrated-DHT group, and were intermediate in castrated-testosterone and castrated-E<sub>2</sub> groups ( $P < 0.05$ ).

The changes in collagen concentrations and fiber diameters were associated with differences in the degree of steroid hormone receptor (AR and ESR1) expression in CCL samples. For example, the degree of AR expression was greater in the castrated-placebo group than in castrated rabbits that received sex hormone implants (Figure 3;  $P < 0.05$ ). Although ESR1 expression in the

castrated-placebo group appeared greater than in the castrated-testosterone group, the difference was not significant. Similar to AR expression, the degree of ESR1 expression was greater in the castrated-placebo group than in the castrated-DHT and castrated-E<sub>2</sub> groups ( $P < 0.01$ ). Furthermore, ESR1 expression in castrated-placebo rabbits was greater than in sexually intact rabbits ( $P < 0.05$ ).

Differences in serum sex hormone concentrations appeared to affect MMP expression, although these findings were not significant ( $P > 0.05$ ). However, the degree of MMP-1, -2, and -9 expression was similar in CCL samples from sexually intact control animals and in castrated rabbits that received testosterone and DHT implants but was higher in the castrated-placebo and castrated-E<sub>2</sub> groups (Figure 4). The degree of MMP ex-



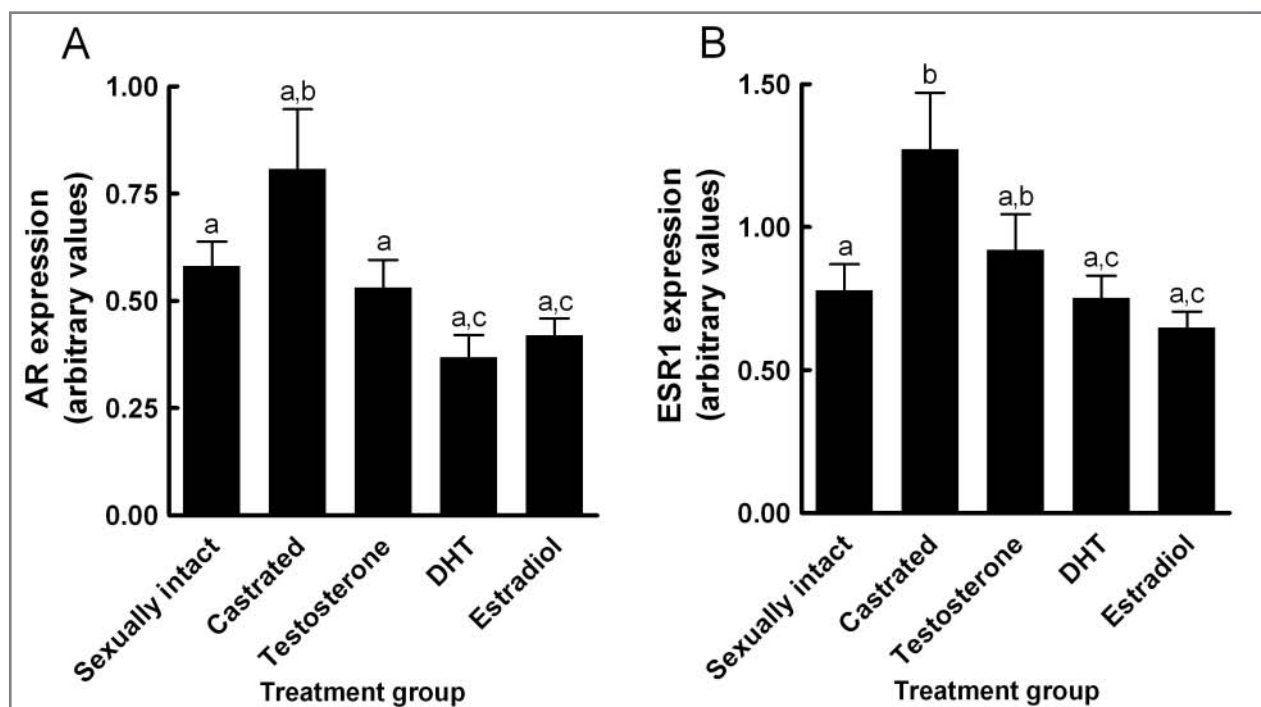


Figure 3—Mean ± SD degree of protein expression for AR (A) and ESR1 (B) in CCL samples (n = 6/group) from the rabbits in Figure 1. Values were calculated in relation to  $\beta$ -actin expression and are reported in arbitrary units. See Figure 1 for remainder of key.

pression was inversely related ( $r = -0.73$ ;  $P < 0.05$ ) to collagen concentrations in the castrated-placebo group (Figure 3).

## Discussion

Understanding any effect that sex hormones have on CCL growth during sexual maturation could provide an informed basis for determining the appropriate age for gonadectomy in young animals. Through use of sexually immature male rabbits, the present study was designed to investigate the effects of sex hormones on collagen content in CCLs. Although castrated rabbits that received  $E_2$  implants had higher serum concentrations of these hormones than did sexually intact control rabbits, the concentrations were within the physiologic range of  $< 20$  pg/mL for male rabbits.<sup>27</sup>

The results of the present study suggest that gonadectomy decreases serum testosterone concentration and collagen concentrations but increases ESR1 expression and fiber diameters in the CCLs of rabbits. Sex hormone implantation resulted in partial or full alleviation of these effects. Nevertheless, the finding that collagen concentration was decreased after gonadectomy is important because collagen is known to be the main load-bearing component of ligaments. We also observed that following gonadectomy, rabbits that received testosterone or  $E_2$  implants had collagen concentrations more similar to those of sexually intact control rabbits; this effect was not observed in DHT-supplemented rabbits. Consequently, aromatization of testosterone to  $E_2$  could be an important regulating factor in CCL homeostasis. Although changes in MMP protein expression following gonadectomy were subtle, further elucidation of the interaction between sex hormones and MMP activity

is warranted. Altogether, these results suggest that sex hormones are necessary for CCL homeostasis in male rabbits during sexual maturation.

In a study<sup>28</sup> of collagen fiber diameter in CCLs of 2 breeds of dogs with or without a predisposition to CCL rupture, investigators found that collagen fiber diameters of the predisposed dogs were significantly smaller than those of the nonpredisposed dogs. This finding led to the supposition that collagen fiber diameter might be an important factor in CCL insufficiency in dogs. In contrast, the present study showed that sexually intact rabbits, which are presumably less likely to be predisposed to CCL insufficiency, had smaller fiber dimensions than any castrated rabbit, including those that received hormone implants. An inverse relationship between collagen concentrations and fiber diameters was also evident. Although the mechanisms that bring about the increase in fiber diameter are not yet understood, the results of the present study do not suggest that fiber diameters predict CCL insufficiency.

The principal function of sex hormones in males is to support development of sexual characteristics and behavior and to maintain male fertility.<sup>29</sup> Although testosterone is the primary endocrine product of the testes,  $E_2$ , which is the predominant sex hormone in females, is present in small amounts in males (approx 20% of the concentration in nonpregnant females).<sup>29</sup> Conversion of testosterone to  $E_2$  is due to action of the aromatase enzyme, which is expressed in several body tissues.<sup>29</sup> However, there is general consensus that, in addition to regulating reproductive activity, sex hormones affect development of the musculoskeletal system.<sup>29,30</sup>

Because blood testosterone concentrations gradually increase in parallel with other changes

that occur during sexual development in males, it is reasonable to expect that the surge in sex hormone

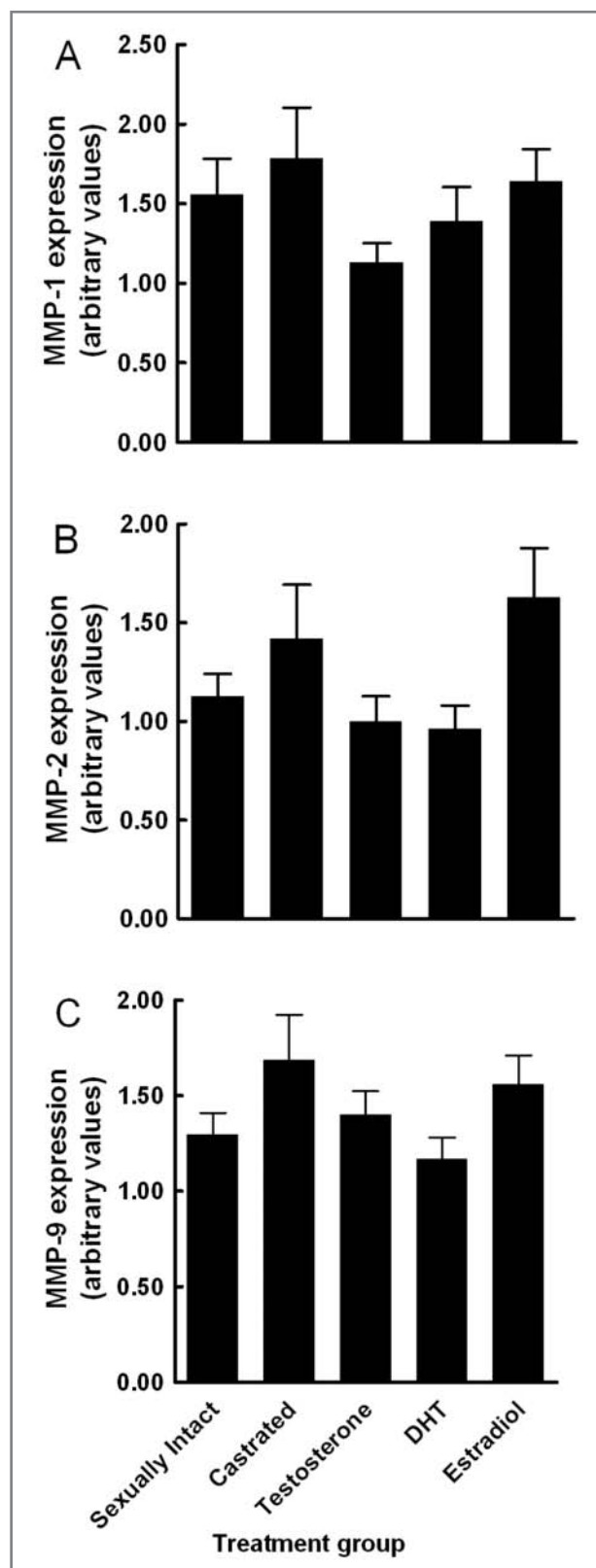


Figure 4— Mean  $\pm$  SD degree of protein expression for MMP-1 (A), MMP-2 (B), and MMP-9 (C) in CCL samples from the rabbits in Figure 1. Differences between groups were not significant ( $P > 0.05$ ). See Figures 1 and 3 for remainder of key.

concentrations that occurs during sexual maturation impacts physical development. Indeed, long-standing evidence suggests that testosterone influences collagen remodeling and structure.<sup>31–33</sup> There is also clear evidence that estrogens regulate the collagen content of CCLs<sup>15</sup> and affect the load needed for the ligament to rupture in women.<sup>34</sup> In this regard, several studies<sup>35</sup> have demonstrated that  $E_2$  can modulate changes in collagen turnover and MMP expression. Therefore, an interesting observation in the present study was the increase in the degree of AR and ESRI expression in CCLs from castrated rabbits. Although the basis for the increase in receptor expression after castration remains unclear, it is possible that increased degree of AR and ESRI expression represents an adaptive response mechanism that serves to augment sex hormone signaling after gonadectomy.<sup>36</sup>

The MMP family of proteolytic enzymes mediates the degradation and turnover of extracellular matrix macromolecules and collagen fibers.<sup>19</sup> Analysis of MMP expression in the presence and absence of sex hormones was important to the objectives of the present study because estrogens and estrogen receptor agonists are known to affect collagen content<sup>37</sup> as well as MMP-2 and MMP-9 activity.<sup>38</sup> However, a major limitation of the study was our inability to measure MMP enzyme activity or to analyze a wide spectrum of collagenases. Although increases in MMP-1, -2, and -9 expression were detected after gonadectomy, these changes were not significant. The lack of robust changes in MMP expression may have been due in part to the small sample size, which might have limited our ability to detect associations, or may have been the result of variations in the responses of individual rabbits to sex hormone implantation and action. Other uninvestigated MMPs might have had a greater role in mediating sex hormone action in CCLs.<sup>31</sup> This possibility remains to be investigated. Also considered was the idea that a longer period of sex hormone deprivation than used in the present study (3 weeks) might have yielded a different outcome.

In the United States, veterinarians commonly castrate nonbreeding dogs at  $< 6$  months of age. However, results of several studies<sup>21,39–41</sup> in which the effects of early-age gonadectomy on skeletal development were investigated suggest that this standard practice may adversely affect dogs involved in sports (eg, racing or agility trials). For example, bone growth plate closure occurs later in dogs castrated at 7 weeks than in those castrated at 7 months of age.<sup>21</sup> In concert with growth factors, sex hormones promote closure of growth plates at sexual maturity,<sup>40</sup> suggesting that bones in dogs castrated before sexual maturity may continue to grow. Thus, dogs castrated at an early age can have longer limbs than dogs castrated later in life.<sup>40</sup> Abnormal growth in bone length commonly results in modifications in body proportions and distortions of bone-to-bone relationships. Excessive growth increases the lever arm (distance from the fulcrum or point of angular movement) in the lower portion of the hind limb, which exerts stress on the CCL. Together, these observations suggest that structural and physiologic changes triggered by the lack

of sex hormones following gonadectomy predispose these animals to orthopedic injuries.

Other evidence exists that castrated dogs have a higher incidence of CCL rupture,<sup>10</sup> which is potentially detrimental to performance in dogs used in sports. Additional studies are warranted to evaluate the effects of gonadectomy prior to sexual maturity on musculoskeletal function, particularly in canine athletes. Studies are also required to determine the relative roles and interactions of sex hormones and other physiologic factors in the regulation of CCL development prior to sexual maturity or cessation of physical development.

- a. Myrtle's Rabbitry Inc, Thompsons Station, Tenn.
- b. Fort Dodge Animal Health, Charles City, Iowa.
- c. Orion Corp, Espoo, Finland.
- d. Innovative Research of America, Sarasota, Fla.
- e. Euthasol, Virbac AH Inc, Fort Worth, Tex.
- f. T-Net 370, E<sub>2</sub>-Net 317, Perkin Elmer Life and Analytical Sciences, Boston, Mass.
- g. Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colo.
- h. Sircol Collagen Assay Kit, Accurate Chemical and Scientific Corp, Westbury, NY.
- i. SPECTRAMax Plus 384, Molecular Devices, Sunnyvale, Calif.
- j. Lysis buffer, product No. 78510, Thermo Scientific, Rockford, Ill.
- k. Protease inhibitor, product No. 78410, Thermo Scientific, Rockford, Ill.
- l. Mini-PROTEAN, Bio-Rad Laboratories, Hercules, Calif.
- m. Abcam, Cambridge, Mass.
- n. Santa Cruz Biotechnology, Santa Cruz, Calif.
- o. Denville Scientific, Metuchen, NJ.
- p. Doc-It LS software, version 5.5.4, UVP Inc, Upland, Calif.
- q. Ducopan Epoxy Resin, EMS, Hatfield, Pa.
- r. Phillips 301, 60 kV, Eindhoven, The Netherlands.
- s. Prism, version 4.0, GraphPad Inc, San Diego, Calif.

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