The Impact of Sex Hormones on Collagen Fiber Diameter and Density
UPDATE

Previous Findings

1. Estradiol was the only hormone or SHBG to be independently related to in-vivo ACL stiffness in young women
    a. Estradiol was negatively related to ACL stiffness in healthy women (Romani, et al, 2003)
    b. Testosterone and Progesterone were significantly related to ACL stiffness however, these relationships were dependent upon Estradiol concentrations. (Romani, et al, 2003; Lovering and Romani, 2005)
2. Sex specific differences in type I and type III collagen mRNA expression may influence the mechanical properties of the rat anterior cruciate ligament. (Romani, et al, 2010)
3. Male rats with normally circulating testosterone had stronger anterior cruciate ligaments than rats with testosterone removed. (Romani, et al, 2016)

Our initial trials on healthy, active women were consistent with the early in-vitro worked that found estradiol to be antagonistic to collagen fibroblast proliferation and that the effects of progesterone were negated in the presence of estradiol. Anterior cruciate ligament stiffness in our subjects was negatively related to E2 concentrations near ovulation and the positive relationship between progesterone and estrone and ACL stiffness was not significant in the presence of E2. These findings suggest that estradiol was the only sex hormone of SHBG measured that was independently related to ACL stiffness.

Testosterone was positively related to ACL stiffness in human and rat trials. What was unclear was the mechanism by which sex hormones influenced the remodeling and strength of the collagen that accounts for the strength of the ligaments. We identified gender specific differences in the expression of type I and type III mRNA, a precursor to the proteins that comprise the tensile (type I) and elastic (type III) properties of the ACL. What was still unanswered, however, was the downstream influence of sex hormones on the expression of collagen proteins that comprise the structure and strength of the ligament.

The current study used a model of male and female rats with intact sex organs and normally circulating sex hormones, as well as castrated rats that were deprived of hormones for 3 or 6 weeks. Our hypothesis was that sex hormones would have a significant influence on the cross sectional area and fiber density of collagen fibers. Consistent with our hypothesis and earlier trials, removing E2 resulted in larger collagen fibers, while removing testosterone resulted in smaller collagen fibers, indicating that E2 appears to be antagonistic to fiber size and testosterone is likely anabolic. These findings suggest that sex hormones may influence the strength of the ACL by influencing the metabolism of collagen and ultimately, the size of strength of the ligaments.

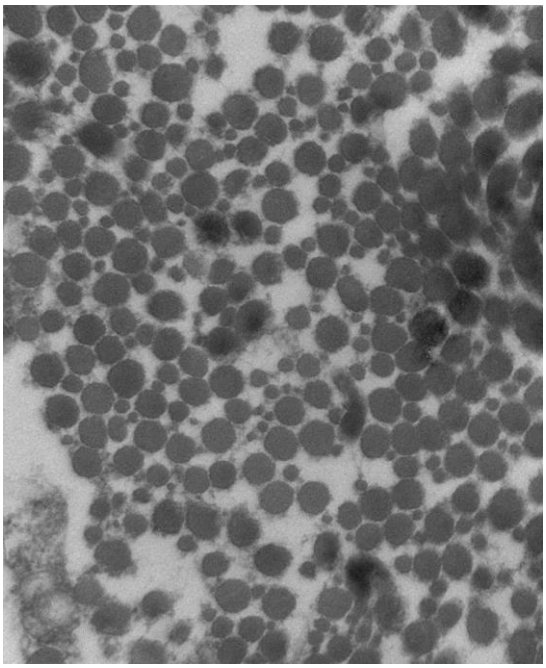
Animals: Male and female Sprague Dawley rats sacked between 118 (17 weeks) and 142 days (20 weeks) old. All groups underwent an initial 2 week clearance period to insure removal of any circulating hormones

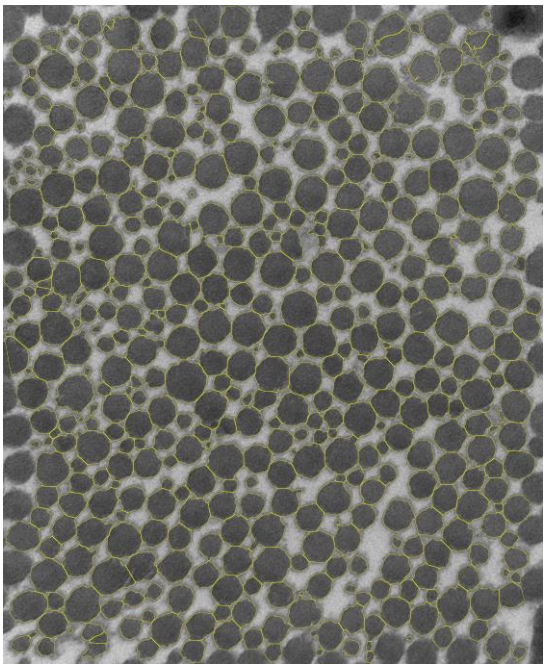
Female Control (FC):	Intact ovaries
Female Short Castrated (FSC):	Castrated 3 weeks after delivery
Female Long Castrated (FLC):	Castrated 6 weeks after delivery
Male Control (MC):	Intact ovaries
Male Short Castrated (MSC):	Castrated 3 weeks after delivery
Male Long Castrated (MLC):	Castrated 6 weeks after delivery

Methods

Imaging: 5 ACL samples per group were fixed and sectioned for TEM analysis. Magnification and resolution was optimized for analysis with ImageJ software (NIH). In order to analyze a representative sample of collagen fibers in each specimen 479 total images were evaluated (range = 58 to 109/sample)(Left)

Since past attempts to automate the measurement of collagen fiber diameter were confounded by the irregular shape and close approximation of individual fibers ImageJ software was modified by the JHU Physiology Lab. Images (Right) were evaluated with ImageJ software to determine the mean area (nm2) of each fiber in the image field. See yellow outline of fibers. The density of the fibers was determined by calculating the average numbers of fibers per image. Area of the fields measured 2333nm x 2848nm. Images with poor resolution or that did not result in consistent measures of fiber diameter were not used for analysis. (~20%)





Statistical Analysis: A one-way analysis of variance was conducted to determine the relationship between sex hormone exposure in one control group (C) with in-tact sex organs and two experimental groups that had been castrated for three (SC) and six (LC) weeks and the mean fiber area and number of collagen fibers within a series of TEM images of a fixed area (2333 x 2848 microns). Table 1 is a summary of the numbers of images and fibers used for each group.

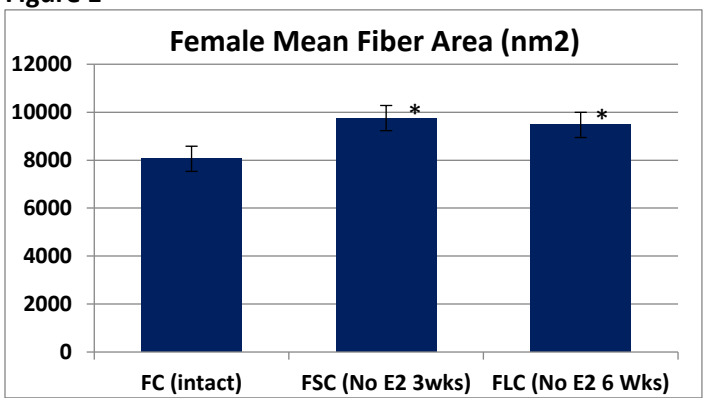
Table 1

	TOTAL FIBERS	Total Image Count	Mean Fiber Area (nm^2)	AveFibers/Image
FControl	26680.00	58.00	8057.13	460.00
FShortCastrated	30245.00	69.00	9755.19	438.33
FLongCastrated	33257.00	89.00	9469.85	373.67
MControl	31861.00	71.00	8452.55	448.75
MShortCastrated	42325.00	109.00	8996.77	388.30
MLongCastrated	42337.00	83.00	8349.83	510.08

Mean Fiber Area: *The diameter of the fibers in each ligament*
The mean fiber areas for each image in each group was used for a gender specific analyses of the impact of group on mean fiber area.

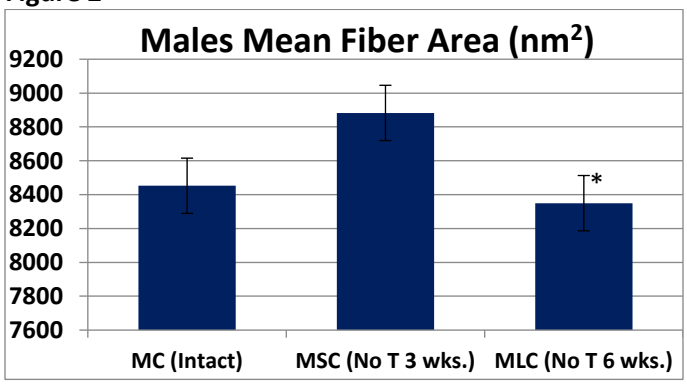
Females: Removing E2 results in increased fiber area
Figure 1 shows a statistically significant between group difference in mean fiber area (P=0.00046). * = significant differences between the FC and FSC samples that were without E2 for 3 weeks (P= 0.002) and FLC samples that were without E2 for 6 weeks (P=0.0004). These findings suggest that removing E2 through castration resulted in an increase in mean collagen fiber area.

Figure 1



Males: Removing T results in decreased fiber area after 6 weeks
Figure 2 shows no statistically significant between group difference in mean fiber area (P=0.26). * = significant differences between the MSC and MLC samples that were without E2 for 3 weeks and 6 weeks respectively (P=0.037). These findings suggest that removing testosterone through castration resulted in a decrease in mean collagen fiber area after 6 weeks. This delayed response that comes after a statistically insignificant initial increase in fiber area may suggest a collagen synthesis remodeling cascade that is initiated by testosterone and continues protein/fiber synthesis as long as three weeks after initiation. It may be possible that our data are beginning to reflect the longer term impact of removing testosterone on collagen remodeling that may take as long as six weeks to impact the ultrastructure of collagen fibers.

Figure 2

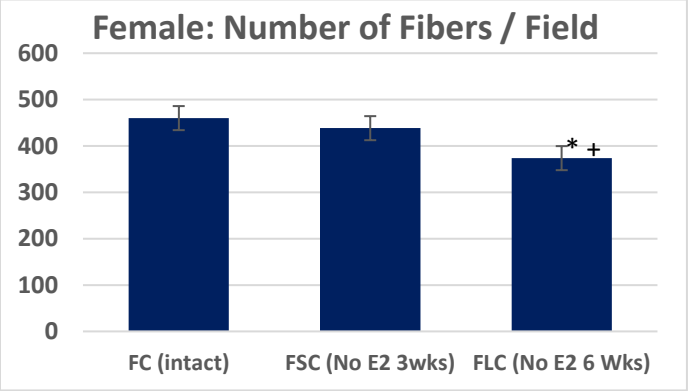


Mean Fiber Number: *Number fibers within field of view as a measure of fiber density.*

The mean fiber number calculated for each image in each group was used for a gender specific analyses of the impact of group on mean fiber number. Since the number of fibers were calculated from a fixed field of view, the calculation of number of fibers / field of view was used as a measure of fiber density.

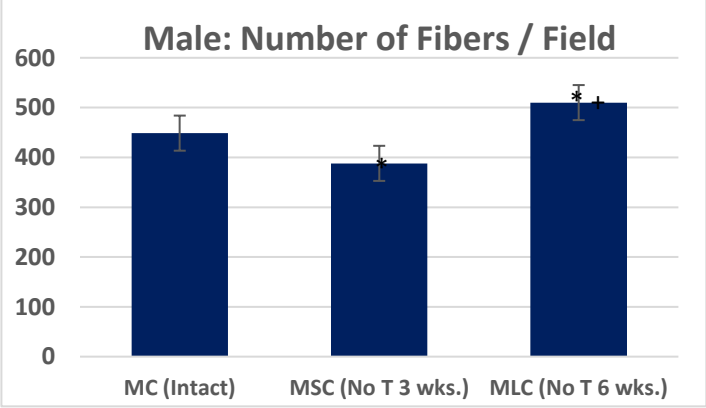
Females: Decreased number of fibers after removing E2 for 6 weeks
Figure 3 shows a statistically significant between group difference in mean fiber number (P=0.0051). * = significant differences between the FLC and FC samples (P= 0.0003) that were exposed to normal levels of E2. + = significant difference between FLC and FSC samples that were without E2 for 3 weeks (P=0.0014). These findings suggest that after removing E2 through castration for six weeks, there was a decrease in the density of collagen fibers. These findings are consistent with data in Figure 1 that demonstrates a statistically significant increase in fiber area and with our hypothesis that as E2 was removed, larger fibers with increased diameter would develop thus, reducing the density of fibers within a fixed area.

Figure 3



Males: Number of fibers initially decreases after 3 weeks than increases after 6 weeks without T
Figure 4 shows a statistically significant between group difference in mean fiber number (P<0.001). * = significant differences between the MC and MSC samples (P= 0.0002) where T and was removed for 3 weeks and MLC samples that were without T for 6 weeks (P=0.00086). + indicates significant differences between the MSC and MLC groups (P<0.001) These findings suggest that after removing T through castration for three and six weeks, there was an increase in the number of collagen fibers. These findings are consistent with data in Figure 2 that demonstrates a statistically significant decrease in fiber area and with our hypothesis that as T was removed, there would be more "space" for smaller, perhaps weaker fibers with decreased diameter.

Figure 4



What is not clear from these findings is whether these fibers are collagen fibers that have "shrunk" in an environment absent of the anabolic testosterone or are the early development of new fibers that may be smaller, immature, or with a different ultrastructure or morphology. In our earlier work (Romani, et al, 2010) we demonstrated gender specific differences in the amount and ratio of Type 1 and Type 3 collagen mRNA, an early precursor to collagen synthesis. That study was followed by our more recent findings in 2016 that showed a decrease in the strength of the ACL following the removal of testosterone for 5 weeks. Where there are several possible mechanism that may impact collagen formation including tensile loading (Kurosowa, 2002) matrix metalloproteinases, their inhibitors, growth factors, and the aromatases that have been shown to influence collagen remodeling in ligament and other collagen-rich tissues.

Ultimately however, any of these factors would have to impact the structure of the ligament. As a result, this study set out to determine what the gender specific downstream impact of sex hormones would have on the collagen diameter and density. We found that removing the previously demonstrated deleterious E2 resulted in larger fibers within 6 weeks with a concurrent decrease in fiber density. We also showed that removing the anabolic effects of testosterone showed an initial (albeit non-significant) increase in fiber diameter that may be the residual cascade of collagen synthesis. That initial increase was followed by a decrease in collagen fiber diameter.

As collagen provides the primary tensile restrain to ligament loads, it makes sense that larger fibers would be better able to withstand that load. Our findings demonstrate that sex hormones may, in fact, impact the ability of the ACL to withstand tensile loads by impacting the size and density of collagen fibers.

Conclusions:

1. Removing E2 from female rats results in an increase in cross sectional area of collagen fibers. This finding is consistent with our hypothesis and previous work in our and other labs that E2 is detrimental to collagen cell proliferation, collagen remodeling and ligament strength
2. Removing T from male rats for 6 weeks results in a decrease in collagen fiber cross sectional area. These findings are consistent with earlier work in our lab that showed ACL tissue to have androgen receptors and in-vivo rat models that demonstrated decreased ACL strength after the removal of T for 5 weeks
3. Removing E2 from female rats showed a corresponding decrease in the number of fibers in each field of view. Given the larger fiber cross sectional area of the fibers in each field it would make sense that a field of view (area) with larger fibers that take up more space would result in fewer fibers within that space.
4. Removing T resulted in an initial decrease then increase in number of fibers in male rats. These results are consistent with our data in Figure 2 that show an initial increase then decrease in fiber cross sectional area that we would expect to result in fewer larger then more, smaller fibers, respectively.