

The Effects of Sex Hormones on Physical Characteristics of the Medial Collateral
Ligament in Prepubescent Female Rats

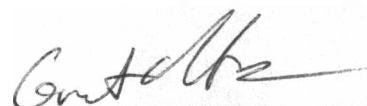
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Abstract

About 100,000 injuries to anterior cruciate ligaments (ACL) occur annually in the United States, with females reporting an ACL injury rate two to eight times higher than their male counterparts. Many intrinsic and extrinsic factors have been cited to explain the disparity between rates of injury between males and females, such as anatomical differences, joint laxity, training technique, and sex hormones, the focus of the present research. Estrogen has been shown to decrease collagen content within ligament tissue whereas progesterone has been shown to inhibit estrogen and in some cases promote the production of collagen. Testosterone has been linked with increased collagen content, but its effects upon female knee ligaments have not been studied in detail.

The present research focused on the role that estrogen, progesterone, and testosterone had, both singularly and in combination, on the physical properties of ligament tissue. Peak levels of estrogen, progesterone, and testosterone were simulated in prepubescent female rats, with treatment groups as follows: estrogen, progesterone, testosterone, estrogen and progesterone, estrogen and testosterone, and control. Mechanical tests on ligaments were subsequently performed after termination to measure ligament laxity and breaking point strength.

The results show that estrogen in combination with testosterone or progesterone significantly decreases the breaking point strength in ligaments. Estrogen in combination with testosterone or progesterone and testosterone alone significantly decreased ligament laxity.

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Introduction

About 100,000 injuries to anterior cruciate ligaments (ACL) occur per year in the United States (Huston *et al.*, 2000). In 2001, the National Collegiate Athletic Association reported that females have a two to eight times higher incidence of ACL injuries than their male counterparts, translating to greater than one injury sustained for every ten female athletes participating in collegiate sports. Also in 2001, knee surgeries accounted for 70% of all surgeries performed on female athletes, and at an estimated \$17,000 per athlete for surgical treatment and rehabilitation, potentially an annual sum of \$646 million could be spent on female athletes. While female ACL injuries cause a significant financial burden due to the sheer number of reported injuries, (over 38,000 ACL tears in females per year), ACL injuries also impose significant trauma on female athletes, as they can potentially lose participation in sports seasons as well as future scholarship funding and professional earnings (Toth and Cordasco, 2001).

Although many factors are involved in female ACL injuries, the role of cyclic fluctuations of sex hormones associated with the female menstrual cycle has recently come under observation. Yu *et al.* (1999) suggests that cyclic variations of endogenous hormones, in particular estrogen, during menstruation may predispose females to ligament injury. Liu *et al.* (1996) and Slauterbeck *et al.* (1999) also demonstrate the effects of estrogen upon the structural and compositional integrity of molecular elements comprising ligaments, further suggesting hormonal influence may compromise female ligaments and contribute to injury. While the present research examined hormonal effects on rat medial collateral ligaments (MCL), Yiannakopoulos *et al.* (2005) confirmed the biomechanical symmetry of the ACL and MCL, allowing for results obtained from

MCL studies to be applied to ACLs and vice versa. The present research focused on the role that sex hormones play in increasing the propensity for a female athlete to tear her ACL.

Knee Anatomy

The knee is a compound, complex, and insecure joint. Located between the femur and the tibia, it is subject to much leverage, but its composition of powerful ligaments and strong muscles make it one of the strongest joints in the body. The knee maintains stability during its vast range of motion primarily through the posterior and anterior cruciate ligaments (PCL and ACL, respectively). The PCL, composed of short fibers, is stronger than the ACL, and is attached to the posterior intercondylar area of the lateral meniscus, ascending anteromedially to attach to the lateral surface of the medial femoral condyle (Williams *et al.*, 1989). The ACL is composed primarily of two bands: the anteromedial, which is tightest when the knee is in flexion, and the posterolateral, which is tightest when the knee is in extension. The ACL runs from the posteromedial portion of the lateral femoral condyle to an area just lateral to the medial tibial anterior intercondylar area (Moeller and Lamb, 1997 and Williams *et al.*, 1989). The ACL plays a dynamic role in the knee, serving primarily as a restraint to anterior tibial subluxation and secondarily as a restraint against internal rotation and varus and valgus angulation when the knee is in full extension (Toth and Cordasco, 2001 and Moeller and Lamb, 1997).

Along with the PCL, the ACL provides the axis for knee rotation, linking rotation with flexion and extension (Moeller and Lamb, 1997). In any knee position, varying between full flexion and extension, knee stability results from a balance between forces

extending the joint and passive mechanisms resisting such motion. In all knee positions, one cruciate ligament is taut, as it acts as a direct bond between the tibia and femur, allowing for restraints to be placed on the back and forward translation between the tibia and femur bones (Williams *et al.*, 1989). Therefore, compromising the integrity of a cruciate ligament is detrimental to the overall stability of the knee joint.

Composition of Ligaments

Collagen is critical to the functional integrity of tissues such as bone, cartilage, skin, and tendon. Connective tissue mechanical behavior is primarily determined by the composition and organization of collagen, as collagen transmits force between bones or bone and muscle (Provenzano and Vanderby, 2006). Collagen's characteristic assemblage of fibers depends on cross-links between adjacent molecules, which are responsible for bearing the physical stresses to which they are exposed. The precursor molecules, procollagen, are enzymatically trimmed to give rise to collagen molecules. These collagen molecules spontaneously form macromolecular aggregates in the extracellular space resulting in the five-stranded collagen microfibril (Burgeson and Nimni, 1992). These microfibrils are then recognized by lysyl oxidase, an enzyme that initiates the formation of cross-links in collagen (Burgeson and Nimni, 1992) which allows the long, filamentous microfibrils to aggregate and form collagen fibers (Provenzano and Vanderby, 2006). During collagen fibrillogenesis, proteoglycans and several members of the small leucine-rich proteoglycan (SLRP) family guide and stabilize collagen fibril formation and maturation (Provenzano and Vanderby, 2006).

Procollagen is the precursor to three interstitial collagens: Types I, II, and III. Type I collagen comprises about 80 to 99% of the total collagen found in tendons and

ligaments, whereas Type II collagen can be found in connective tissues but is most abundant in cartilage. Type III collagen is found in many connective tissues, and has been cited as having the ability to rapidly cross-link through intermolecular disulfide bridges, proffering a great advantage to wound healing as collagen is deposited in these areas at a rapid rate. Type III collagens have also been correlated with tissue extensibility, contributing unique biomechanical properties to the collagen fibers incorporating this type of collagen. This may be due to Type III collagen's location within the fibril: it resides at the fibril surface, which may provide an interactive edge for other fibril or matrix molecule associations and therefore underlie the mechanical properties of the tissue (Burgeson and Nimni, 1992). Therefore, the most important collagens to the present study are Types I and III. Fibroblasts, which are embedded within connective tissue, are responsible for the synthesis of Type I and III collagen (Weiss 1983).

Provenzano and Vanderby (2006) determined that force within tissues is directly transferred through collagen fibrils and not through interfibrillar couplings, such as proteoglycan bridges or SLRP connections, so the strength of a ligament is directly derived from collagen composition and structure. Therefore, if collagen structure or composition is compromised by hormones that inhibit fibroblast proliferation and as a result, collagen synthesis, the result would be substantially weakened ligaments (Yu *et al.*, 1999, Liu *et al.*, 1997, and Provenzano and Vanderby, 2006).

Factors Contributing to ACL Injury

The majority of ACL injuries are non-contact and can be attributed to three main mechanisms: planting and cutting, straight-knee landing, and one-step stop landing with

the knee hyper-extended (deceleration activities) (Toth and Cordasco, 2001 and Moeller and Lamb, 1997). While the mechanism contributes to injury occurrence, a number of extrinsic and intrinsic factors acting concomitantly may also significantly affect the incidence of ACL injury. Extrinsic factors relate to environmental and biochemical aspects and include muscle imbalances, frictional playing surfaces, the use of braces, and joint positions (Moeller and Lamb, 1997). A noted muscle imbalance between females and males is that of the quadriceps/hamstring ratio. Females utilize their quadriceps, an ACL antagonist, and ligaments as the dominant muscle groups to control knee stability whereas males employ their hamstrings to perform the majority of knee stability work (Toth and Cordasco, 2001 and Moeller and Lamb, 1997). In females, then, if excessive pull by the quadriceps occurs without significant co-contraction of the hamstring, their knee joint may become imbalanced, resulting in subluxation of the tibia anteriorly and a torn ACL (Toth and Cordasco, 2001). While females have been shown to have decreased hamstring to quadriceps strength ratios relative to men, no significant correlation between such strength ratios and knee injuries has been determined. Playing surfaces have also been cited as factors in knee injuries due to the high coefficient of friction found on basketball courts and turf fields. Prophylactic braces are also potential contributors to injury, especially ankle-braces, as the increased stability of the ankle due to bracing transmits the forces once absorbed by the ankle to the knee (Moeller and Lamb, 1997). Females also often perform cutting and landing maneuvers with unsafe joint positions, reflected by a more erect hip-trunk posture combined with greater knee valgus, due to weakened or decreased activation of gluteus musculature while standing, and less knee flexion in a female as opposed to her male counterparts. These unsafe techniques, then,

result in amplified ground-contact forces which translate into increased knee joint loads (Toth and Cordasco, 2001).

Intrinsic factors originate in and are related to knee joint anatomy and include intercondylar notch configuration, joint laxity, anatomic alignment differences, pelvic structure, and hormones (Moeller and Lamb, 1997). If an intercondylar notch width is small, the ACL is more likely to contact the medial femoral condyle when the knee is in flexion and the anterior notch when the knee is in full extension, resulting in increased friction and stress on the ACL. Literature suggests that the intercondylar notch width is smaller in females than in males (Toth and Cordasco, 2001), but no definitive studies have demonstrated a significant linkage between notch width and female ACL injury (Moeller and Lamb, 1997). While joint laxity, defined as a combination of joint hypermobility and musculotendinous flexibility, has been shown to be more prevalent in females than males (Toth and Cordasco, 2001), experimental data attempting to correlate joint laxity to ACL injury have been contradictory (Moeller and Lamb, 1997).

Anatomical differences between women and men are numerous, but one of the most studied differences regards the quadriceps (Q) angle, which is the angle formed by the intersection of a line from the anterior superior iliac spine to the center of the patella with the tibial tubercle, simply referred to as the tibia-femoral angle (Moeller and Lamb, 1997). While studies establish that an increased Q-angle contribute to patello-femoral tracking problems and anterior knee pain, large Q-angles have only been associated, not significantly correlated, with ACL injury (Moeller and Lamb, 1997 and Toth and Cordasco, 2001). Women also have wider pelves, which leads to an increased forward slope or tilt of their pelves and may contribute to a female's tendency to hyperextend her

knees in landing and balance attempts, one of the main mechanisms of non-contact-ACL injury. Hormones have also been cited to affect ACL injury, as combinations of relaxin and estrogen increase ligament laxity and therefore may be responsible for decreases in static and dynamic knee stability in female athletes. Estrogen acting alone decreases human ACL fibroblast proliferation and the rate of collagen synthesis, important structural components of the ACL (Toth and Cordasco, 2001). Oral administration of estrogen reduced levels of insulin-like growth factor-1 (IGF-1), a growth factor that promotes the proliferation of multiple body tissues, including connective tissue and collagen synthesis (Ho and Weissberger, 1992). The present research focused on the role that sex hormones play in increasing the propensity for a female athlete to tear her ACL.

Hormonal Impact on Ligaments

Normal ovulatory menstrual cycles consist of a well-defined cascade of hormonal events, triggered when gonadotropins interact with female sex steroids that function to direct changes in the uterine endometrium. The first phase of the menstrual cycle is the follicular, or proliferative, phase in which concentrations of both estrogen and progesterone are low. Ovulation is preceded by a mid-cycle surge of estradiol, and estrogen levels remain high for the rest of the cycle. The second phase is the luteal, or secretory, phase where progesterone levels rise. Without implantation of a fertilized egg into the uterine wall, hormone levels decline which results in menstruation (Lebrun, 1994).

The presence of receptors for estrogen and progesterone have been identified in female ACLs (Liu *et al.*, 1996), suggesting that female sex hormones may have an effect on the structure and composition of the ACL (Yu *et al.*, 1999). Sciore *et al.* (1998) have

determined that when estrogen and progesterone bind to their respective receptors, they modify gene expression within hormone-responsive tissues, thereby altering the structure and composition of a ligament. The effects of estradiol on the structural and compositional changes on a molecular level could result in decreased strength of the ACL overall and therefore play a significant role in predisposing female athletes to ligament injury (Yu *et al.*, 1999 and Liu *et al.*, 1997).

Connective tissues, e.g. ligaments, are primarily comprised of Type I and Type III collagen fibers, which are synthesized by fibroblasts (Weiss 1983). Lui *et al.* (1996) determined that increasing levels of estradiol significantly decrease fibroblast proliferation and therefore the rate of collagen synthesis. The cross-sectional area in ligaments also decreases with estradiol treatments, an effect that may stem from decreased proteoglycan content (Räsänen and Messner, 2000) due to the inhibitory effects of estradiol on proteoglycan synthesis (Rosner *et al.*, 1979). Through experiments on the fibroblasts derived from rabbit ACLs, Liu *et al.* (1997) found that collagen synthesis and fibroblast proliferation were significantly reduced with increasing estradiol concentrations (Liu *et al.*, 1997). Yu *et al.* (1999) assessed Types I and III procollagen from a human ACL and also determined that Type I procollagen synthesis and ACL fibroblast proliferation decreased in a dose-dependent manner with increasing estradiol concentrations. Yu *et al.* (1999) observed these changes on days one and three of the study; therefore, early physiologic change induced by estradiol suggests that the acute cyclic variations of sex hormones experienced by the female athlete during menstruation may significantly influence the mechanical properties of female knee ligaments and predispose females to ligament injury.

Slauterbeck *et al.* (1999) suggest that female athletes are most prone to ACL injuries when participating in running and cutting sports where the constant pressure on the ACL requires the ligament to respond to micro-injuries in its composition by tissue remodeling. The remodeling proteins, matrix metalloproteinases (MMPs), have been found to enhance the degradation of collagen whereas tissue inhibitors of matrix metalloproteinases (TIMPs) counter the effects of MMPs and are vital to reparative tissue processes (Slauterbeck *et al.*, 1999). Together, MMPs and TIMPs regulate tissue remodeling through variations in their respective concentrations. In degradative tissue processes, MMPs are in greater abundance than TIMPs, and in reparative tissue processes, TIMPs are more abundant than MMPs (Edwards *et al.*, 1996). Estrogen and progesterone regulate the production of many TIMPs and MMPs (Matrisian, 1994, Rajabi *et al.*, 1991, Schneikert *et al.*, 1996, Wahl *et al.*, 1977), and the subsequent effect these hormones have on the levels of MMPs and TIMPs in pig pubic ligaments has shown that estrogen aids in MMP production while progesterone aids in MMP degradation (Wahl *et al.*, 1977). The correlation between estrogen promoting MMP production and therefore the degradation of tissue, as well as that found between progesterone and the degradation of MMPs, allowing for TIMPs to have greater influence and therefore stimulate reparative tissue function, suggests that sex hormones in females may play a role in modifying the ability of the body to respond to ACL stress by remodeling tissue (Slauterbeck *et al.*, 1999).

In addition to estrogen and progesterone, testosterone levels vary during the menstrual cycle, albeit in lower concentrations (Rickenlund *et al.*, 2003). Testosterone is released from the adrenal cortex. The binding affinity of testosterone to the rat estrogen

receptor (ER) protein has been verified by Kuiper *et al.*, (1996), who also noted that testosterone and progesterone each have equal affinity for the ER protein. The effect of testosterone on the composition of knee ligaments has not been studied in detail, but some research has shown that administration of testosterone in male rats significantly increased collagen content and fibril diameter. This is the opposite effect observed from estrogen administration, suggesting testosterone is an estrogen antagonist (Hama *et al.*, 1976). Testosterone-treated rats also demonstrated significantly increased tensile strength in components contributing to wound healing (Smith and Allison, 1965). Testosterone has been cited to be physiologically active under many conditions, especially in regards to stimulating protein synthesis during anabolic activity (Smith and Allison, 1965). Tsai *et al.* (1992) researched estrogen's link to knee osteoarthritis in men and women and suggested that the effects of synovial estrogen in men was counteracted by endogenous testosterone. In males, then, lower estrogen/androgen ratios were observed, resulting in lower amounts of unopposed estradiol that would be free to interact with tissues. Postmenopausal females were found to have twice the rate of bilateral knee osteoarthritis, a fact attributed to the large amounts of unopposed estradiol in postmenopausal women due to the absence of testosterone and progesterone, which decline sharply prior to menopause (Tsai *et al.*, 1992). Although these studies offer insight into some effects of testosterone, its effect on estrogenic activity in ligaments is not completely understood, while progesterone's inhibitory effect on estrogen by binding estrogen receptors has been more fully documented (Yu *et al.*, 2001, Kahl, 2000).

In the present study, the effects of female sex hormones and androgens on the medial collateral ligament in prepubescent female rats were examined. The existence of

ACL and MCL biomechanical symmetry has been confirmed by Yiannakopoulos *et al.* (2005), which allows for results gathered from MCL tests to be applied to the performance of ACLs under similar conditions, and vice versa. Kahl (2000) and Kuehn (2005) determined that peak levels of estradiol increased the amount of stretch in prepubescent female rat's MCLs but did not impact the breaking point strength of the ligament.

In order to broaden our understanding of the effects that cyclic female sex hormones have upon knee ligaments, the objective of the present research was to determine the effects that peak levels of testosterone, estrogen, and progesterone have, both solitarily and in combination, on the breaking point strength and elasticity of the MCL in prepubescent female rats. I hypothesized that peak levels of 17- β -estradiol would increase ligament laxity, that progesterone and testosterone would inhibit 17- β -estradiol activity, and that the breaking point strength would not vary between test groups.

Materials and Methods

Animals

Experiments were performed on prepubescent female Wistar rats housed in Carroll College animal facilities on a consistent 12-h light 12-h dark rotating schedule, and sustained on Mazuri Rodent Chow and tap water. Experimental and control groups each contained ten rats which were 25 days old when priming injections were administered, 27 days old when experimental dosages were applied, and 29 days old when terminated via CO₂ euthanasia.

Drug Preparation

The priming dose for the control and experimental groups was 5 µg 17-β-estradiol in 0.1 mL of vehicle (sesame seed oil). Experimental dosages for utilized hormones, based on the studies conducted by Kahl (2000) and Cross and Roselli (1999), were as follows: 100 µg of 17-β-estradiol in 0.1 mL of vehicle, 5,000 µg progesterone in 0.1 mL vehicle, and 200 µg of testosterone in 0.1 mL in vehicle. For combined hormonal groups, the dosages for each hormone were combined in 0.1 mL vehicle. The control group received 0.1 mL of vehicle. Prior to all injections, rats were sedated in a large glass jar with 15 mL of ether soaked into a paper towel.

Experimental Groups

On day one, experimental and control animals were injected subcutaneously with 0.1 mL of the 17-β-estradiol priming dose. Six different protocols were then used for each of the six test groups, each of which consisted of ten rats. Forty hours after the priming injections, each test group was injected subcutaneously with 0.1 mL vehicle containing the experimental dosages of 17-β-estradiol, progesterone, 17-β-estradiol and

progesterone, testosterone, testosterone and 17- β -estradiol in the dosages noted above. The sixth group served as the control and received only 0.1 mL vehicle. Following experimental dosages, rats were terminated within 45 hours. Timing of termination was based on the 36 to 40 hour activation peak of 17- β -estradiol (Samuel *et al.* 1996) and research by Kahl (2000) and Kuehn (2005).

Testing Protocol

Following termination via CO₂, both hind legs were removed, placed in a 0.9% saline solution, and frozen within 20 minutes. Slauterbeck *et al.* (1999) demonstrated that ligaments retain their integrity for further experiments if frozen and thawed. Tests were performed on the right hind leg. The left hind leg was used only if the right leg's femur broke before ligament failure occurred.

Specimens were thawed for no more than 24 hours in a refrigerator at 2.7°C. Once pliable, all muscles, ligaments, and tendons were removed from the knee, except the MCL. The hip joint of the femur and distal end of the tibia/fibula were stripped, dipped in acetone, air-dried, and secured in 19.05mm plastic caps with five-minute epoxy for 30 minutes. The MCL was kept moist throughout via irrigation with 0.9% saline solution. Each plastic cap had a metal eye screw inserted in its base.

The mounted leg was then secured into the testing apparatus (Figure 1). The capped-femur was attached via an S-ring – eye screw connection to a clamp, and the capped-tibia/fibula was attached to a wire via an S-ring – eye screw connection. An ocular lens of 6X magnification was placed over the wire where a hair protruded at a 90° angle so as to accurately measure the stretch of the ligament as additional weight was added. The wire extended over a pulley system attached to a bucket, where incremental

weights were added until ligament failure. Weight increments were determined by Kahl (2000) and Kuehn (2005). The stretch and maximum load were recorded for each ligament. The amount of time from removal of thawed ligament to breakage averaged 50 minutes.

Statistical Analysis

Analysis of variance (ANOVA) followed by TUKEY HSD, were used to determine the effect of experimental hormone treatments on the stretching and breaking point limit of the MCL. The data were log transformed to correct for divergence from normality. All analyses were completed using STATISTICA 4.0.

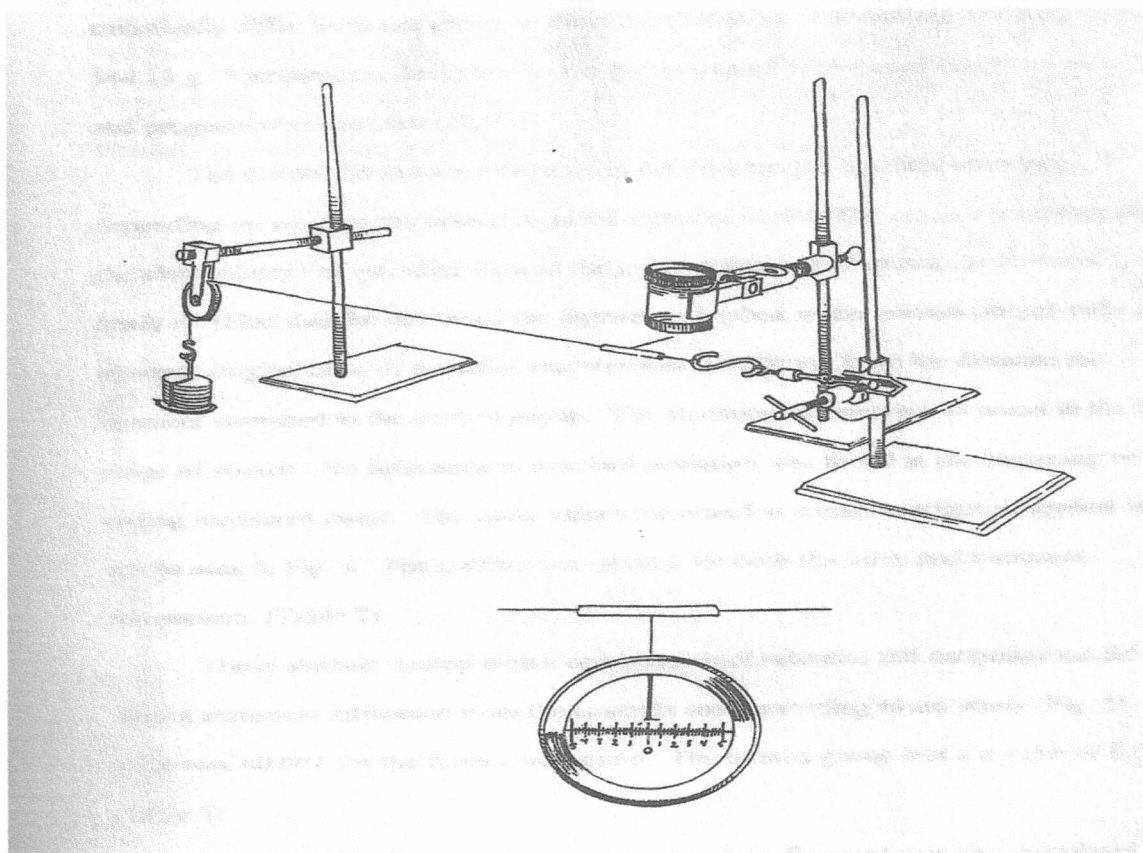


Figure 1. Apparatus and ocular lens for experimentation (Kahl 2000).

Results

The objective of this research was to determine the effects that testosterone, estrogen, and progesterone had, both singularly and in combination, on the breaking point strength and ligament laxity of the MCL in prepubescent female rats. The breaking point strength can be defined as the weight applied to reach ligament failure; ligament laxity can be defined as the amount of stretch observed per incremental weight added. The ligament laxity to 110 grams was also examined to determine if significant variance occurred between treatment groups after the addition of the first 110 grams. One hundred and ten grams was chosen as it was the lowest weight at which ligament failure occurred. Table 1 demonstrates that significant differences were found within various treatment groups regarding ligament breaking point strength, total ligament laxity, and ligament laxity to 110g.

Table 2 and Figure 2 show the average breaking point weights for the control and treatment groups. The treatment groups of progesterone + estrogen and testosterone + estrogen exhibited significantly lower breaking point weights than the control group (Table 3). The control and estrogen, progesterone, and testosterone treatment groups did not vary significantly from one another (Figure 2 and Table 3).

Table 4 and Figure 3 show the average total ligament laxities for the treatment groups. The progesterone + estrogen-treated group exhibited significantly lower total ligament laxity than the control and estrogen- and progesterone-treated groups. The testosterone-treated group exhibited significantly lower total ligament laxity than the control and estrogen, progesterone, and progesterone + estrogen treatment groups. The

testosterone + estrogen-treated group exhibited significantly lower total ligament laxity than the control and estrogen- and progesterone-treated groups (Table 5).

Table 6 and Figure 4 show the average ligament laxities to 110 grams for the control and treatment groups. The testosterone-treated group exhibited significantly lower ligament laxity to 110g than the control and estrogen-, progesterone-, and progesterone + estrogen-treated groups (Table 7). The testosterone + estrogen-treated group did not vary significantly from any group (Figure 4).

Table 1. Results of ANOVA testing for effects of treatment on ligament breaking point strength, total ligament laxity, and ligament laxity to 110g.

Effect	d. f.	F	p-level
Breaking Strength	5, 52	3.824157	0.005
Total Laxity	5, 52	20.69023	<0.001
Laxity to 110g	5, 52	4.946949	<0.001

Table 2. Mean breaking point with standard deviation for the control and each test group.

Treatment	Breaking Point (g)*
Control	418.9 ± 155.4
Estrogen	345.0 ± 108.3
Progesterone	372.2 ± 83.3
Progesterone-Estrogen	259.0 ± 68.4
Testosterone	297.0 ± 127.8
Testosterone-Estrogen	247.0 ± 81.9

*mean value ± STDEV

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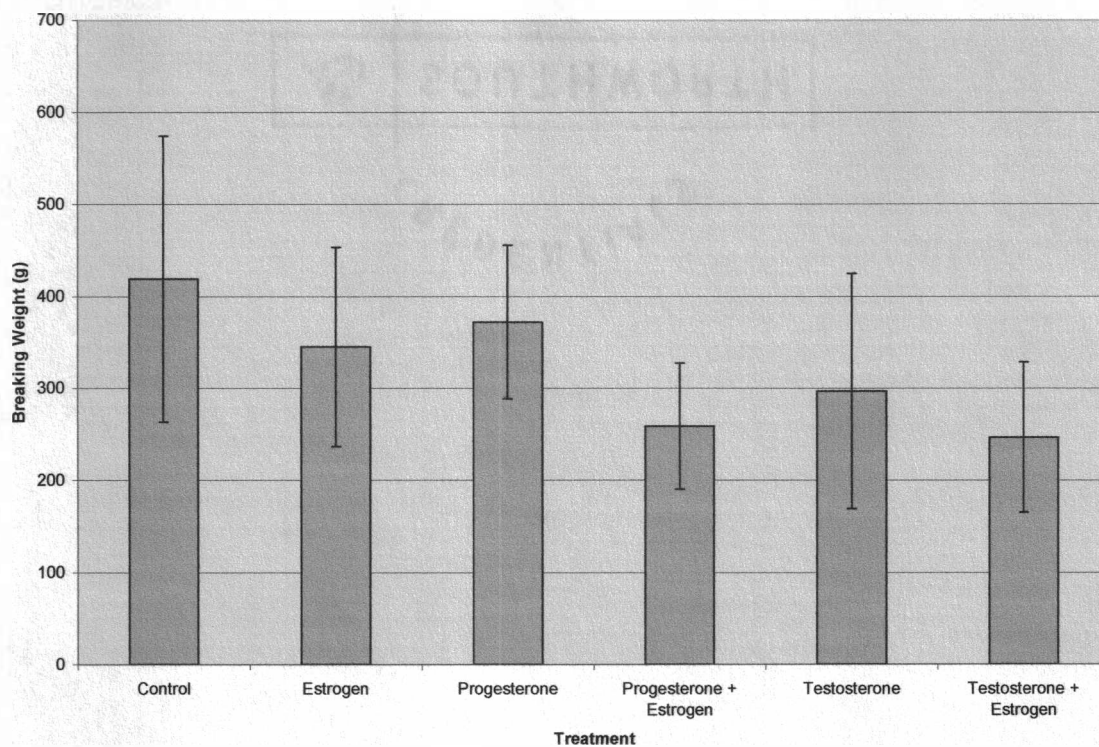


Figure 2. Average breaking weight for the MCL of control and each treatment group. The control group significantly varied from two treatment groups, progesterone + estrogen and testosterone + estrogen. No significant variation occurred between all other treatment groups. N = 10 for estrogen, progesterone + estrogen, testosterone, and testosterone + estrogen treatment groups. N = 9 for the control and progesterone treatment groups. p-values may be found in Table 3.

Table 3. p-values of Tukey HSD tests showing significant variances ($p < 0.05$) between the control and treatment groups for breaking point strength.

	Control
Estrogen	0.849
Progesterone	0.995
Progesterone + Estrogen	0.046
Testosterone	0.156
Testosterone + Estrogen	0.017

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Table 4. Mean joint laxity with standard deviation for the control each test group.

Treatment	Total Stretch(mm) [*]
Control	2.5 ± 0.46
Estrogen	2.7 ± 1.01
Progesterone	2.7 ± 0.52
Progesterone-Estrogen	1.6 ± 0.54
Testosterone	1.1 ± 0.29
Testosterone-Estrogen	1.2 ± 0.34

* mean value ± STDEV

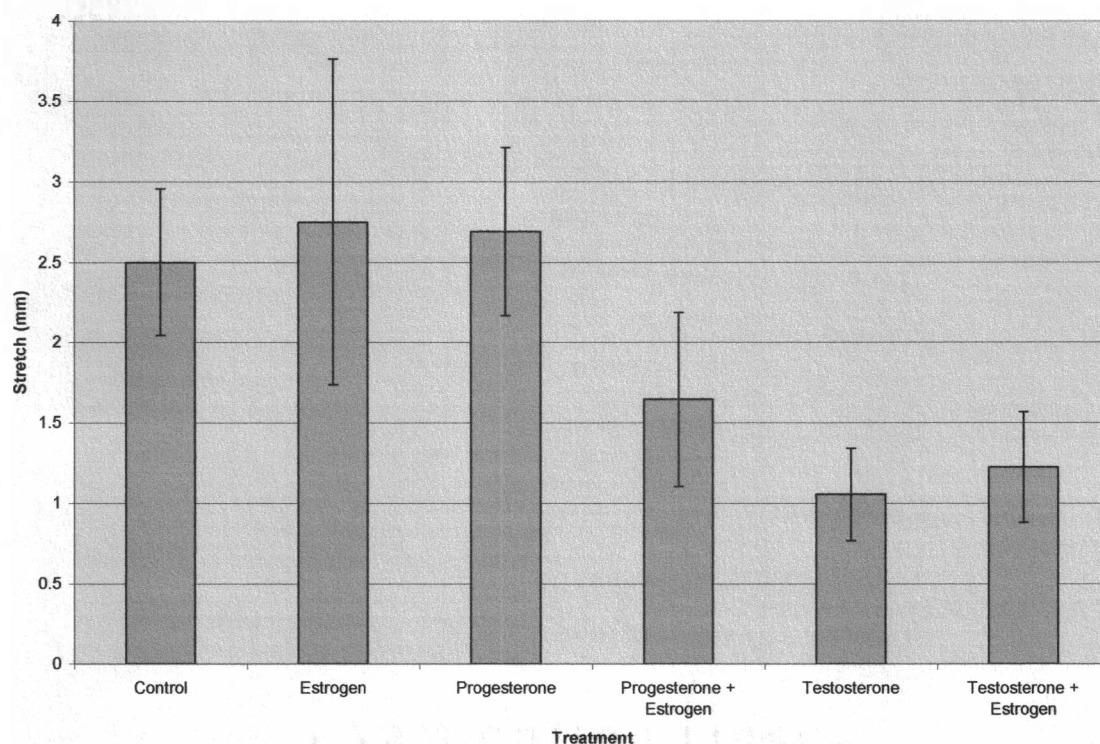


Figure 3. Average total ligament laxity of the MCL for the control and each treatment group. Significant differences were found between the first three treatment groups, which includes the control, and the final three treatment groups. Testosterone-treated ligaments were also found to vary significantly from progesterone + estrogen-treated ligaments. N = 10 for estrogen, progesterone + estrogen, testosterone, and testosterone + estrogen treatment groups. N = 9 for the control and progesterone treatment groups. p-values are found in Table 5.

1950

1951

1952

1953

Table 5. p-values of Tukey HSD tests showing significant variances ($p < 0.05$) between the control and treatment groups for total ligament laxity.

	Control	Estrogen	Progesterone	Testosterone
Progesterone + Estrogen	0.012	0.002	0.002	0.044
Testosterone + Estrogen	<0.001	<0.001	<0.001	0.932
Testosterone	<0.001	<0.001	<0.001	

Table 6. Mean ligament laxity to 110g with standard deviation for the control and each test group.

Treatment	Stretch to 110g (mm)*
Control	0.93 ± 0.31
Estrogen	1.05 ± 0.38
Progesterone	0.96 ± 0.18
Progesterone-Estrogen	0.90 ± 0.34
Testosterone	0.54 ± 0.18
Testosterone-Estrogen	0.73 ± 0.29

* mean value ± STDEV

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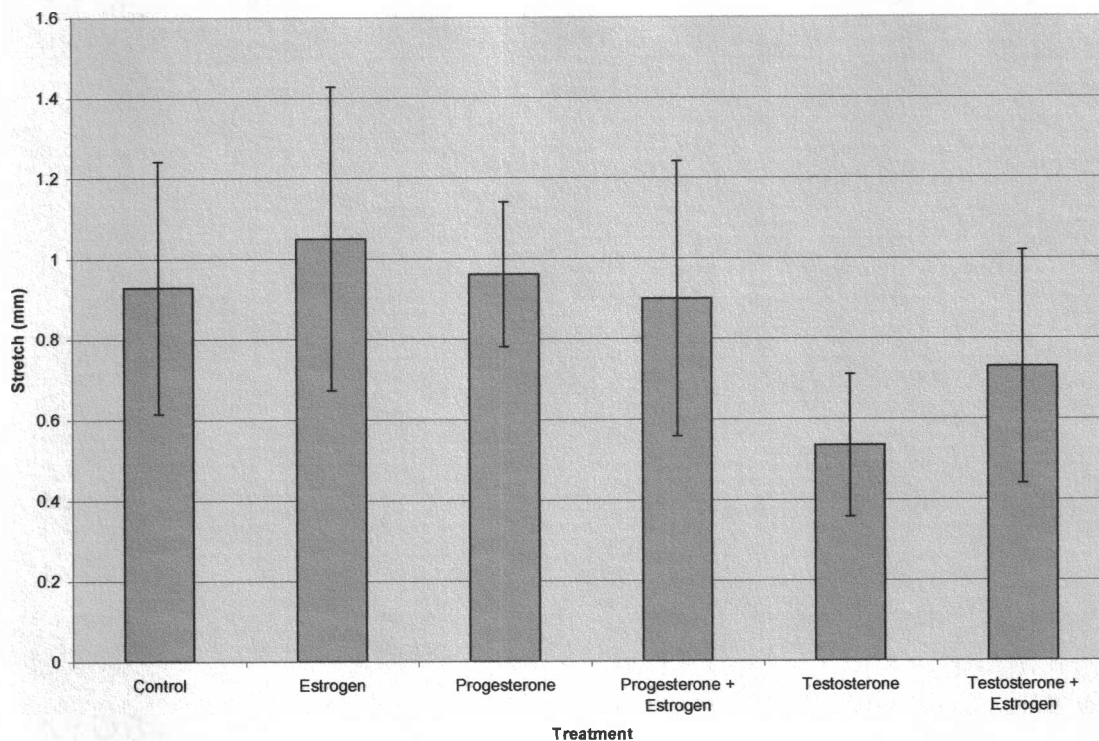


Figure 4. Average stretch of the MCL in the control and treatment groups to 110 grams (the minimum breakage point found in all tests). Testosterone-treated ligaments were found to vary significantly in stretch to 110 g from the control and estrogen-, progesterone-, and progesterone + estrogen-treated groups. Testosterone + estrogen-treated ligaments did not vary significantly from any group. N = 10 for estrogen, progesterone + estrogen, testosterone, and testosterone + estrogen treatment groups. N = 9 for the control and progesterone treatment groups. p-value can be found in Table 7.

Table 7. p-values of Tukey HSD tests showing significant variances ($p < 0.05$) between the control and treatment groups for stretch to 110g.

	Testosterone
Control	0.021
Estrogen	0.001
Progesterone	0.008
Progesterone + Estrogen	0.031
Testosterone + Estrogen	0.526

1881

1882

Discussion

Although not all hypotheses were supported, it was determined that estrogen in combination with testosterone or progesterone and testosterone alone may play significant roles in influencing the breaking point strength and joint laxity of knee ligaments in females.

In rat collagen metabolism research, Samuel *et al.* (1996) observed the anti-estrogenic activity of progesterone, a property supported by Steinetz *et al.* (1965), who found that progesterone specifically blocked ligament growth induced by an estrogen/relaxin combination. The inhibitory effect of progesterone on estrogen occurs through a process known as quenching, where the progesterone receptor complex interferes with the ability of the estrogen receptor to productively utilize the transcriptional machinery, thereby repressing its activity (Kraus *et al.* 1995). However, progesterone has been shown to effectively inhibit estrogen only when its concentration is greater than that of estrogen, since when both hormones are present in equal amounts, estrogen exerts the dominant effect (Yu *et al.* 2001).

The anti-estrogenic property of progesterone may have serious implications regarding the strength of female knee ligaments during the menstrual cycle when peak levels of progesterone and estrogen are present, commonly referred to as the luteal phase. Wojtys *et al.* (2002) measured hormonal levels of injured females within 24 hours of injury, and their data revealed that a lower than expected injury percentage was observed during the luteal phase of the menstrual cycle. Beynon *et al.* (2006) demonstrated this effect through serum analysis of progesterone in injured females, showing that females are three times more likely to tear their ACL in the preovulatory phase (low levels of

progesterone) than the postovulatory *i.e.* luteal phase (high levels of progesterone). Their study also revealed that while serum concentrations of estradiol were found to be similar between the control and ACL-injured women, serum concentrations of progesterone were elevated by a mean of 70% for women in the uninjured control group as compared to those women who sustained an ACL injury.

In my study, analysis of hormonal effects on ligament laxity revealed that the progesterone + estrogen, testosterone + estrogen, and testosterone treatment groups had significantly lower measurements of ligament laxity than the control, estrogen, and progesterone treatment groups. Beynnon *et al.* (2005) support the finding that progesterone and estrogen, at individual peak level concentrations, do not alter ligament laxity as their study reported no relationship between estrogen and progesterone fluctuations and variances in ligament laxity. In a study exploring the effect of estrogen on ovine ACLs, Seneviratne *et al.* (2004) determined through biochemical analysis of mechanical properties that the tensile strength of sheep ACLs did not differ between the control and estrogen-treated specimens.

While Beynnon *et al.* (2005) and Seneviratne *et al.* (2004) suggest that estrogen and progesterone do not significantly alter ligament laxity individually, they do not take into account the combined effect that estrogen and progesterone may have upon ligament laxity. My study determined that the simultaneous administration of estrogen and progesterone resulted in a decrease in ligament laxity as compared to the control and estrogen and progesterone-treated groups. Yu *et al.* (2001) reported that estradiol significantly decreases fibroblast proliferation and procollagen Type I synthesis and further observed this effect to be reduced with the administration of progesterone. In

addition, Yu *et al.* (2001) determined that when levels of progesterone exceeded those of estradiol, fibroblast proliferation and procollagen Type I synthesis increased. Therefore, in the estrogen + progesterone-treated ligament, progesterone's inhibition of estrogen would result in stable and perhaps increased cell proliferation and procollagen synthesis in ACL fibroblasts. This implies that with increased levels of progesterone and base levels of estrogen, ligament collagen content would increase, thereby increasing the tensile strength of a ligament and decreasing its elastic properties. While the estrogen + progesterone-treated group had significantly less laxity than the estrogen-treated group, it would be expected that the estrogen-treated group would demonstrate significantly greater ligament laxity than the control, but no significant difference was found between the estrogen-treated group and the control group. This discrepancy may be accounted for by the small group size used for study (N = 9 and N = 10 for the control and estrogen-treated group, respectively) and the large standard error for the estrogen-treated group. Due to the limited number of animals available for experimental treatment and the wide variation in collected data, the results do not necessarily reveal significant trends.

Testosterone- and testosterone + estrogen-treated groups also showed reduced ligament laxity as compared to the control, estrogen-, and progesterone-treated groups in my study. Testosterone has been linked to increased collagen content in various tissues (Asano *et al.*, 2003) while estrogen decreases Type I collagen synthesis (Yu *et al.*, 2001). While the relationship between simultaneous administration of high levels of testosterone and estrogen has not been well documented, females with higher concentrations of free testosterone near ovulation had greater ACL stiffness (Lovering and Romani 2005). Despite the lack of research pertaining to the effect of testosterone on female knee

ligaments, the presence of androgen receptors on the female ACL (Lovering and Romani 2005) suggests that androgens may play a role in the normal remodeling and tensile strength of knee ligaments.

My results show testosterone alone significantly reduced stretch up to 110 grams when compared to the control, estrogen-, progesterone-, and estrogen + progesterone-treatment groups. This may stem from testosterone's reported ability to increase repair of knee ligaments (Tipton *et al.* 1971), so microinjuries to knee ligaments occurring at the onset of applied stresses are mended at an increased rate, which thereby increases the initial strength of the ligament and its subsequent resistance to stretching.

My results also suggest that high levels of estrogen alone have no significant effect on the breaking point strength or ligamentous laxity in female knee ligaments. Seneviratne *et al.* (2004) support this finding through their research examining the effect estrogen has on sheep ACL fibroblasts, where biochemical analysis of the mechanical properties of sheep ACLs showed no difference between the control and estrogen treated groups in the tensile strength of the ligament. Warden *et al.* (2006) also support the null effect of estrogen on a female knee ligament's mechanical properties. However, research conducted by Kahl (2000) found that estrogen treatments significantly increased ligament laxity in prepubescent female rats, but at the same time found that treatments with progesterone and estrogen did not significantly alter the weight required for ligament failure as compared to the control group.

My study found that the weight applied to reach the breaking point did not statistically differ between the control, estrogen-, progesterone-, and testosterone-treatment groups. This supports research completed by Kahl (2000) and Kuehn (2005)

which found that treatment of female knee ligaments with estrogen or progesterone does not significantly alter the breaking point strength of knee ligaments as compared to the control group.

As previously cited, progesterone has an inhibitory effect upon estrogen; testosterone also has an antagonistic relationship with estrogen, as demonstrated by Lovering and Romani (2005) and Burke and Anderson (1972). Taking into account the formerly discussed effects of estrogen on ligament structure and composition, the treatment groups of progesterone + estrogen and testosterone + estrogen would, then, theoretically result in a normal ligament laxity relative to the control group and the estrogen-treated group would, theoretically, demonstrate a significantly greater ligament laxity than the control, progesterone + estrogen-, and testosterone + estrogen-treated groups. However, the testosterone + estrogen and progesterone + estrogen treatment groups demonstrated that significantly less weight was required on average for ligament failure to occur as compared to the control group. It was also observed, though, that the progesterone + estrogen-treated group did not significantly vary from the estrogen-treated group, and the testosterone + estrogen-treated group did not significantly vary from the testosterone- or estrogen-treated groups. These observations demonstrate that while the progesterone + estrogen- and testosterone + estrogen-treated groups statistically show significant deviation from the control group, these results may not be relevant due to the wide variation found among test groups. Due to limited resources, only ten rats maximum were allotted to each test group, including the control group. Also, precise experimental hormonal injections may have been compromised by inadequate administration of dosages. (Either the injecting apparatus was not fully inserted into the

animal or the dosage was not completely administered before the animal resisted and dislodged the needle.) The wide variation among test groups and the control group, then, may not give adequate reliability from which to obtain pertinent results.

There appears to be an emergent consensus that hormone fluctuations within the menstrual cycle affect a woman's propensity to tear her ACL when all relevant studies are considered. Contradicting theories abound, however, for while various studies cite a significantly greater percentage of female ACL injury occurring in the ovulatory phase (Wojtys *et al.* 2002, Wojtys *et al.* 1998, and Beynon *et al.* 2006), others find the greatest risk associated with the late luteal phase (Myklebust *et al.* 1998), and still more research suggests that hormonal fluctuations caused by the menstrual cycle do not increase a female's risk of ACL injury (Warden *et al.* 2006 and Seneviratne *et al.* 2004).

The significance of my study lies in the information it provides regarding the effects different combinations of sex hormones have upon the properties of knee ligaments. While this study does not show that estrogen affects ligament laxity in females on a mechanical property level, as was suggested by my original hypothesis, it does provide insight into the antagonistic effects of progesterone and testosterone on estrogen, effects that resulted in decreased ligament laxity in female knee ligaments. Further research is invited to more fully understand the effects of the varying hormone levels associated with the menstrual cycle on female knee ligament structure and composition. Only then can a more comprehensive study dealing with environmental, anatomical, and neuromuscular risk factors pertaining to the female's increased propensity to tear her ACL be assessed.

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APPENDIX:**A. Weight increments used measured in grams.**

50
70
90
110
130
150
160
170
180
190
200
210
220
230
240
250
260
270
280
290
300
350
450
550
600
610
620
630
640
650
660
670
680
690
700
710
720
730
740
750

B. Average stretch per increment among control rats

Increments	Controls
50	0.439
20	0.161
20	0.211
20	0.117
20	0.100
20	0.133
10	0.061
10	0.063
10	0.069
10	0.069
10	0.067
10	0.067
10	0.072
10	0.067
10	0.072
10	0.096
10	0.056
10	0.081
10	0.057
10	0.093
10	0.058
50	0.183
100	0.238
100	0.233
50	0.225
10	0.060

C. Average stretch per increment among estradiol-treated rats

Increments	Experimentals
50	0.485
20	0.220
20	0.195
20	0.150
20	0.140
20	0.145
10	0.090
10	0.075
10	0.090
10	0.085
10	0.100
10	0.095
10	0.094
10	0.072
10	0.083
10	0.088
10	0.100
10	0.093
10	0.079
10	0.079
10	0.148
50	0.250
100	0.300
100	0.250

D. Average stretch per increment among progesterone-treated rats

Increments	Experimentals
50	0.472
20	0.167
20	0.189
20	0.133
20	0.133
20	0.150
10	0.067
10	0.083
10	0.100
10	0.067
10	0.072
10	0.083
10	0.083
10	0.072
10	0.106
10	0.069
10	0.079
10	0.100
10	0.057
10	0.057
10	0.064
50	0.207
100	0.463

E. Average stretch per increment among estrogen + progesterone-treated rats

<u>Increments</u>	<u>Experimentals</u>
50	0.500
20	0.175
20	0.120
20	0.105
20	0.090
20	0.085
10	0.055
10	0.065
10	0.050
10	0.060
10	0.056
10	0.056
10	0.060
10	0.050
10	0.025
10	0.050
10	0.050
10	0.050
10	0.050
10	0.050
10	0.067
50	0.200

F. Average stretch per increment among testosterone-treated rats

Increments	Experimentals
50	0.295
20	0.115
20	0.055
20	0.070
20	0.072
20	0.078
10	0.033
10	0.050
10	0.022
10	0.017
10	0.033
10	0.014
10	0.021
10	0.007
10	0.014
10	0.036
10	0.017
10	0.017
10	0.020
10	0.030
10	0.025
50	0.183
100	0.200
100	0.300

G. Average stretch per increment among testosterone + estrogen-treated rats

Increments	Experimentals
50	0.395
20	0.135
20	0.070
20	0.130
20	0.100
20	0.075
10	0.025
10	0.035
10	0.033
10	0.025
10	0.014
10	0.029
10	0.050
10	0.030
10	0.050
10	0.083
10	0.033
10	0.033
10	0.025
10	0.025
10	0.075
50	0.000
100	0.400