ACL rupture rates and disparities: Using dog CCL rupture as a translational medical model for humans

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Knees are the second most physically injured site with anterior cruciate ligament (ACL) ruptures accounting for over 50% of those injuries with an accumulated incidence rate of 68.6 per 100,000 person-years. Men account for most injuries, but women rupture it at a 2-8 times greater rate and the reason for this disparity is not well understood. Comparatively in dogs, cranial cruciate ligament (CCL) (dog's ACL) rupture is the leading canine orthopedic problem and primary cause of stifle lameness. Gonadectomized dogs rupture their CCL significantly more than their intact counterparts and the reason for this disparity is also not well recognized. Nearly 75% of rupture in both humans and dogs are caused by non-traumatic means, suggesting biological factors are a component in rupture susceptibility. This study used dogs as a comparative anatomical model to identify biological variables associated with rupture using databases from the Oklahoma State University Veterinary Hospital and the Morris Animal Foundation Golden Retriever Lifetime Study. Gonadectomy and rupture were significantly associated (chi-square = 21.7, $p < .01$, $n = 57$) with different probabilities of rupture in those gonadectomized at $\leq$6 months and $\geq$13 months (OR = .12, $p < .01$, $n = 3{,}044$). There was a significant association between rupture and height (OR = 1.21, $p = .02$, $n = 3{,}044$) and weight (OR = 1.04, $p < .01$, $n = 3{,}044$) overtime. Females reported more significant and trending results than males overall, including between rupture and other musculoskeletal conditions (OR = 4.67, $p < .01$, $n = 68$) and infectious diseases (OR = .38, $p = .05$, $n = 68$). The age at gonadectomy impacts limb length and ossification rates, modifying

overall limb conformation and ligament properties that become more susceptible to secondary microtraumas, weakening the CCL. The limb is potentially further influenced by additional musculoskeletal conditions and vitamin D absorption. This is more prevalent in large breed dogs because they are gonadectomized at the same age as small breeds despite requiring longer development time. In humans, these results indicate ACL rupture as a multifactorial process predisposed through biological means with hormone levels being an essential component.

Table of contents

List of tables

List of figures

Preface

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Dedicated to those who saw the journey start but never got to see the end.

Love and miss you every day.

William R. Kruse

Alex Toth

Cisco

1.0 Introduction

The knee complex is the largest joint in the human body and includes four primary ligaments that limit flexion and extension, while simultaneously providing robust stabilization. The paired external collateral ligaments (medial and lateral) provide the stabilization and the paired internal cruciate ligaments (anterior and posterior) facilitate proper movement. Knee injuries are the second most commonly injured site after the ankle with anterior cruciate ligament (ACL) ruptures accounting for over 50% of those injuries and an accumulated incidence rate of 68.6 per 100,000 person-years (Joseph et al. 2013; Sanders et al. 2016). Men account for the majority of injuries, but women are more likely to rupture it at a two to eight times greater rate (Carter et al. 2018; Lin et al. 2018). The reasons behind this sex discrepancy rate pattern are not well understood and require further investigation with the simplification of comparative anatomical modeling.

Comparatively in dogs, the homologous cranial cruciate ligament (CCL) structure, sometimes referred to the dog's ACL, is also susceptible to rupture. Cranial cruciate ligament rupture is the leading canine orthopedic problem in the world and the primary cause of stifle lameness (Griffon 2010; Harasen 2003). While common, current overall nationwide incidence rates are not well reported (Cook 2017). However, it has been shown that rupture cases have been increasing progressively over the years and have more than doubled in the last 30 years (Griffon 2010; Witsberger et al. 2008). It is estimated only 20% of rupture cases are directly trauma related (Griffon 2010; Moore & Read 1996). This suggests biological factors are a prominent rupture influence. Animal modeling allows for better understanding of how biological factors directly and indirectly impact the body.

In addition to being a homologous structural model with similar rupture discrepancies, dogs are an exceptional evolutionary model. Dogs are undisputedly the first domesticated species and only species domesticated before the advent of agriculture (Larson et al. 2014). Understanding the genetic and evolutionary history of dogs enhances human evolutionary studies (Vonholdt & Driscoll 2017). Evolution of the expansive breed varieties and formations, temperaments, sizes, and diseases identify them as excellent medical models. Almost every human disease exists in dogs and the physiology, disease symptoms, and clinical phenotypes many times reflect human diseases (Kukekova et al. 2008). Dogs are an exceptional resource for evolutionary anatomical studies and, as such, make them a substantial ACL correlation studies model.

1.1 Research rationale

The main intention of this dissertation is to correlate biological variables and cruciate ligament rupture patterns by using the comparative anatomy of humans and dogs. The predisposition factors for non-contact ACL and CCL rupture are not well understood. The forced twisting motion of this ligament does not produce consistent rupture patterns. Dogs as an anatomical model provide more consistent data reporting, easier to characterize behavior variables, and a more controlled incidence reporting timeline. Most ACL and CCL studies examine one variable, but this dissertation study analyzes multiple variables. Previous single variable studies produced a foundation for using clinical database testing for multivariable analyses to understand the possible relationship between potential biological factors and rupture. This research could help reduce high ACL rupture incidence rates, while mutually benefiting human and animal medicine in further understanding anatomical variation and development.

1.2 Brief background

Anterior cruciate ligament (ACL) and cranial cruciate ligament (CCL) ruptures are continuously being researched due to their high occurrence rate in both humans and dogs [see Figure 1.1 for anatomically labeled knee]. This introductory chapter is designed to highlight the importance and need for continued ACL studies and the impact it can have on both the individual and population levels, in addition to mentioning the methodology used in the three papers and the general theory of dog domestication. Described within these two following subsections are brief backgrounds on human ACL studies and CCL studies. There is substantially more depth reported on human studies here than dog studies as more specific research variables and outcomes regarding dog factors are discussed in the later three paper chapters.

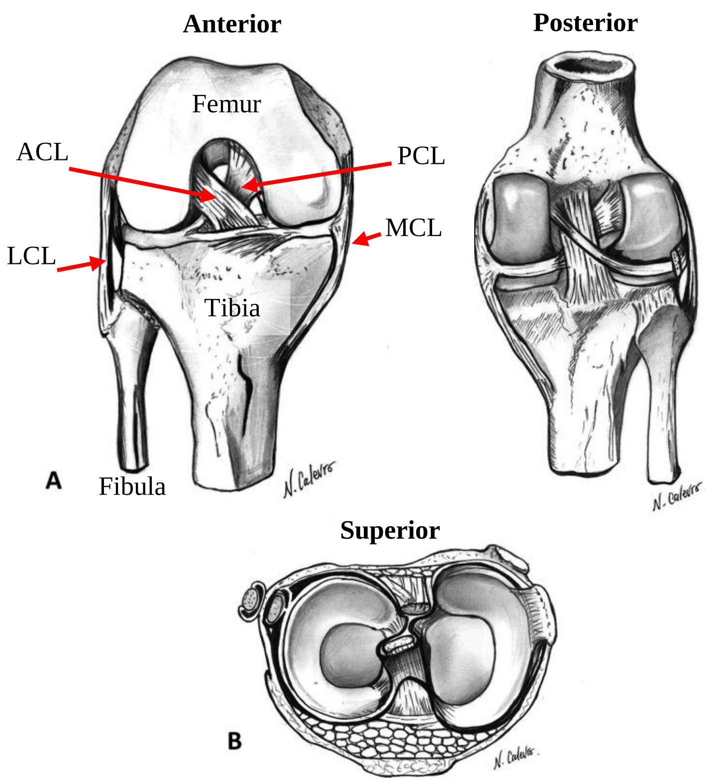

Figure 1-1: "Anatomy of the knee. A; ligaments between femur and tibia. B; menisci (superior view)" by and adapted from Vaienti et al. 2017 BY CC 4.0

1.2.1 Human ACL

The musculoskeletal system is the integration of bones, muscles, ligaments, tendons, and joints to serve three primary bodily functions – (1) enable efficient movement, (2) protect and provide mechanical support for soft tissue, and (3) serve as a calcium reservoir (Gregson 2010; Jenkins, Kemnitz, & Tortora 2007; Lowe & Anderson 2015). Bones act as stiff levers that articulate with other bones through joints, which are connected by ligaments. Muscles act as contractile levers and connect to bone by tendons (Lowe & Anderson 2015). The musculoskeletal system plays important functions in everyday life and conditions that impact this system can cause irreversible disability. There are 1.7 billion people in the world affected by musculoskeletal conditions, which contributes to the nearly 166 million years lived with disability and make up the largest proportion of persistent pain in all geographic regions and age groups (Briggs et al. 2018; Herzog et al. 2017; Tsang et al. 2008; Vos et al. 2012). Musculoskeletal conditions are the leading cause of disability worldwide and reason for early leave or retirement from work, contributing to less financial security (Briggs et al. 2018; World Health Organization 2021b). The lack of physical activity can contribute to declined mental health and other diseases, such as obesity, cardiovascular conditions, and certain cancers (Briggs et al. 2016). A study found less than 0.5% conference abstracts over a five-year span were related to musculoskeletal disorders (Perruccio et al. 2017). There is a need and call for more research into musculoskeletal conditions to alleviate the associated pain, secondary diseases, and high costs (Storheim & Zwart 2014).

In the United States, one in two adults suffer from a musculoskeletal condition (BMUS 2020). In 2010, musculoskeletal problems were the third costliest medical expenses ($170 billion) following circulatory conditions ($234 billion) and preventatives, colds, and other basic care ($207 billion) (Altman 2015). Anterior cruciate ligament (ACL) injuries are one of the most common musculoskeletal conditions and contribute to this large sum (BMUS 2020; Herzog et al. 2017; Joseph et al. 2013). The ACL is one of the four major ligaments of the knee joint and allows for proper gate movement and stability. It is roughly estimated 32,000-320,000 people in the US rupture this ligament yearly, translating into 3-40 tears per hour, with estimates occasionally as high as 400,000 people per year (Many papers cite around 200,000 cases yearly [Griffin et al. 2000; Mall et al. 2014; Paterno et al. 2014; Stanley et al. 2011] but the study referred to was published in 1999 and is outdated [Frank 1999]) (Junkin et al. 2009; Vavken & Murray 2013). These number are consistent across countries (Vavken & Murray 2013).

Surgical reconstruction of the ACL is one of the most performed orthopedic surgeries and comes with expensive surgical costs in addition to extensive post-operative rehabilitation (Herzog et al. 2017; Joseph et al. 2013; Mall et al. 2014; Mather et al. 2013). While surgical intervention and rehabilitation are common practice, only about 50% of people will return to their full pre-injury, competitive state after recovery, which can make an ACL tear a career ending injury (Ardern et al. 2012, 2014; Kvist et al. 2005; Langford et al. 2009; Swart et al. 2014). Injury to the ACL does not usually occur in isolation either. In a sample study in New York, approximately 66% of ACL surgeries performed required concomitant injury repair and 32% required meniscal repair (Vavken & Murray 2013). Overall, meniscal tears are concurrent with ACL rupture 55% of the time (Mansori et al. 2018). This increases rehabilitation, surgical costs, and less chance of full sports reintegration. An ACL tear increases lifelong issues, such as the likelihood of reinjury to the same ligament, contralateral injury, and osteoarthritis later in life (Grindem et al. 2016; Lohmander et al. 2007; Øiestad et al. 2009; Paterno et al. 2012, 2014; Swärd, Kostogiannis, & Roos 2010).

Sports involving cutting and pivoting (e.g., soccer, basketball, football, tennis, and alpine skiing) are most associated with the ACL rupture, and injury occurs more frequently during games than in practice (Arendt & Dick 1995; Beynnon et al. 2005; Bjordal et al. 1997; Hewett et al. 2010; Øiestad et al. 2009; Paterno et al. 2012; Wiggins et al. 2016). Females between 15-19 years old are the most susceptible to ACL rupture and the younger the individual, the higher chance of reinjury (Sanders et al. 2016; Vavken & Murray 2013; Webster & Feller 2016). Female sports participation has increased more than 1000% since the passing of the 1972 Title IX Education Assistance Act that mandates all institutions that receive federal money must offer equal access to curricular and extra-curricular activities for men and women (Mall et al. 2014; Traina &

Broomberg 1997). Nearly 8 million students currently participate in high school sports with continuously rising numbers over the past 29 years and female participation at an all-time high with more than 3.4 million athletes (NFHS 2018).

The increase in female participation suggests ACL studies prior to Title IX were focused mainly on male rupture factors and only in the past 50 years, at most, has the literature and research grown to include female athletes to address their rupture rate. Less than 30 years ago in 1993 the National Institute of Health (NIH) mandated the inclusion of women in NIH-funded clinical trials through the NIH Revitalization Act to better understand sex health disparities (Clayton & Collins 2014). Including females in ACL studies suggests biological factors may be a critical component to rupture susceptibility since women rupture their ACL at a two to eight times greater rate than men and the sex discrepancy in rupture is not substantial until the onset of puberty (Carter et al. 2018; Lin et al. 2018; Voskanian 2013). It also allows for equal sex examination of an orthopedic problem, which is important for representation in the orthopedic field. Orthopedic surgery has the lowest rate of females than any other medical field – only 4% of the members of the American Academy of Orthopedic Surgeons (AAOS) are women (Rohde, Wolf, & Adams 2016; Van Heest & Agel 2012). The inclusion of women in orthopedic research reduces unintentional biases imposed from male-only researchers and can expand the field by being inclusive and accepting of women in orthopedic practices and research.

Studies have examined an assortment of factors that may impact non-contact rupture, ranging from extrinsic factors such as various weather conditions (i.e., icy conditions, high evaporation, low rainfall), playing surface, and footwear choice to internal variables like anatomical variation (i.e., intercondylar notch width, increased generalized laxity, Q angle), genetics, and biomechanical [see Pfeifer et al. 2018 for an extensive citation list of various studied variables] (Di Brezzo & Oliver 2000; T. E. Hewett et al. 2010; Lin et al. 2018; Myer et al. 2011; Myer et al. 2008; Olsen et al. 2003; Orchard, Seward, & McGivern 1999; Paterson et al. 2015; Pfeifer et al. 2018; Ruedl et al. 2011; Silvers & Mandelbaum 2011; van Eck et al. 2010). However, no one factor is consistently shown to be the primary cause of rupture. It has been suggested that it is multifactorial (Griffin et al. 2000; Hewett, Myer, & Ford 2006; Toth & Siegel 2021). Over 70% of ACL injuries are non-contact, further suggesting biological factors may be a central factor in rupture patterns (Boden et al. 2000; Griffin et al. 2000). Single variable studies have produced a strong foundation for this dissertation to use multivariable analyses of anatomical models to examine biological factors that may influence rupture rates and disparities.

The lack of socialized national healthcare databases creates difficulties in obtaining patient ACL data. National registries in other countries connect ACL injury with other patient data, such as age and sex, and are connected to social security numbers. Similar databases have been created in the US but are run by for-profit entities like healthcare companies, which inherently have conflicts of interest in preventative research studies (Vavken & Murray 2013). Vanderbilt University Medical Center has two ACL patient databases – (1) Multicenter Orthopedic Outcomes Network (MOON) and (2) Multicenter ACL Revision Study (MARS). The MOON study focuses on long-term outcomes (mainly osteoarthritis onset) after ACL reconstruction surgery and MARS examines longitudinal outcomes of revision ACL reconstruction (as opposed to primary reconstruction) (MARS Group 2019; MOON Knee Group 2018). These databases are beneficial for learning about the aftermath of injury but not for prophylactic studies. Only 3% of ACL publications pertain to prevention (Griffin et al. 2000; Uhorchak et al. 2003). Therefore, to better understand the proposed multifactorial causation of ACL rupture, longitudinal animal studies provide an excellent, reliable data modeling source and will be the methodology used here to best understand the biological underpinning of ACL rupture.

1.2.2 Canine CCL

The anterior cruciate ligament (ACL) in the human knee is a homologous structure to the cranial cruciate ligament (CCL) in the canine knee (Meeson et al. 2019; Milachowski, Weismeier, & Wirth 1989). The CCL is sometimes referred to the canine ACL and it is susceptible to rupture like in humans (for simplicity, the ACL will refer to humans and the CCL to dogs). Comparable to humans, some dogs rupture this ligament at a greater rate than others (Guthrie et al. 2012). However, a discernable difference between human and dog rupture patterns is that certain breeds and sizes have varying rupture rates, and the sex disparity that is distinctly observed in humans is not consistent in dog studies (Antosh et al. 2018; Biskup et al. 2014; Björnerfeldt, Webster, & Vilà 2006; Hart et al. 2016). The reason for these rupture disparities in dogs is unknown, like in humans. Given the ACL and CCL are homologous structures, they signify potential relationships between rupture factors and can be used to better understand rupture patterns in each other.

There have been many variables studied to better understand the rupture patterns seen in dogs, such as specific breed groups, genetics, hormonal changes (neutering/spaying), age, biomechanics, weight, and conformation variability (Edney & Smith 1986; Goin et al. 2019; Hart et al. 2014, 2020; Mostafa et al. 2010; Taylor-Brown et al. 2015; Torres de la Riva et al. 2013; Wilke et al. 2006; Witsberger et al. 2008; Zeltzman et al. 2005). No one variable can account for the rupture majority; a study examining genetic predisposition in Newfoundlands, a highly susceptible breed to CCL rupture, found only 27% of the phenotypic expression of CCL rupture could be attributed to genetics and the remaining 73% was caused by environmental factors, further supporting the thought that rupture is multifactorial (Griffon 2010; Wilke et al. 2006). A related Newfoundland follow-up study by the same researchers found recessive inheritance with 51% penetrance and four microsatellite markers on four chromosomes that were significantly associated with rupture (Wilke et al. 2009). Whether the genetics are associated with the ligament structural integrity or conformation fault is not evident, but suggests genetics may influence rupture in some breeds and warrants future research (Griffon 2010).

A variable that has been receiving a large portion of research in CCL rupture studies is the examination of early gonadectomy on the influence of rupture and limb conformation. It has been traditionally advised to neuter/spay dogs around six months of age. Many rescue groups and shelters mandate their animals are gonadectomized before adoption as a method of population control (Howe et al. 2001). Veterinarians have historically supported this view because it is estimated humane societies acquire 5-7 million dogs and cats per year and 3-4 million are euthanized (Root Kustritz 2014). However, recent research is indicating dogs gonadectomized before one year of age can cause future illness and injury (Howe 2015).

Studies have shown prepubertal gonadectomy encounters short-term problems, such as increased surgical and anesthetic complications that can occur days or weeks after the procedure (Yates & Leedham 2019). However, there are substantially more long-term negative effects, including increased chance of obesity, certain cancer types (prostatic carcinoma, lymphosarcoma, transitional cell carcinoma, mast cell tumors, hemangiosarcoma, and osteosarcoma), urinary incontinence, and musculoskeletal conditions (hip and elbow dysplasia and CCL rupture). Conversely, there are some long-term positive effects of early gonadectomy besides population control, such as no chance of pregnancy associated complications, tumors in organs that were removed (ovarian, uterine, testicular neoplasia), cryptorchidism, pyometra (life-threatening infection in females), and positive behavioral traits (Root Kustritz 2014; Yates & Leedham 2019). A leading reason for early ovariectomy is the reduced chance of mammary cancer, the most common type of tumor in female dogs with 50% malignancy (relatedly, breast cancer is also the most common cancer type in women worldwide) (Gilbertson et al. 1983; Howe et al. 2001; Philibert et al. 2003; Sorenmo 2003; Sorenmo et al. 2000; World Health Organization 2021a). There are conflicting studies suggesting that the earlier the age of ovariectomy (<2.5 years), the less chance of mammary cancer, but the results are not consistent (Sorenmo, Shofer, & Goldschmidt 2000; Yates & Leedham 2019). The risks and benefits of early gonadectomy are a debated issue, especially regarding CCL rupture. Early gonadectomy may increase the most common cause of lameness in dogs but decrease the foremost cancer in female dogs.

This is of particular concern because spayed female dogs are at the highest risk for CCL rupture, and gonadectomized dogs (both male and female) have significantly higher rupture rates than sexually intact dogs (Hart et al. 2014, 2016; Hart et al. 2020b; Hart et al. 2020a; Reiter, Jagoda, & Capellini 2016; Slauterbeck et al. 2004; Taylor-Brown et al. 2015; Witsberger et al. 2008). Female dogs spayed before one year of age had the highest risk of rupture and the reason for this is unclear (Torres de la Riva et al. 2013). The hormonal changes may impact other variables like patellar luxation, femoral angle, and tibial plateau angle and increase the chance for CCL rupture. Secondary microtraumas are influenced by the increase in the cranial tibial thrust, which weakens the CCL and makes it more prone to rupturing. Early gonadectomy influences the chances of developing excessive tibial plateau angle due to longer limbs, which are then prone to the microtraumas that can eventually rupture the CCL (Ragetly et al. 2011). Altering the tibial angle is the most common form of CCL surgery (TPLO – tibial plateau leveling osteotomy) (Bergh et al. 2014; Christopher, Beetem, & Cook 2013; Shahar & Milgram 2006). The relatedness of these variables reinforces CCL rupture as a multifactorial process.

1.3 Knee structure

Joints are a bodily site where two skeletal elements come together. There are two general joint categories – (1) synovial joints (elements are separated by a cavity) and (2) solid joints (elements are held together by connective tissue and there is no cavity) (Drake, Wayne Vogl, & Mitchell 2010). Synovial joints consist of a synovial capsule with dense fibrous connective tissue that is lined by a synovial membrane that secretes synovial fluid to lubricate the joint. The ends of the bones are covered in articular (hyaline) cartilage (Kardong 2012). Synovial joints can further be classified into seven types – (1) plane joints (e.g., acromioclavicular), (2) hinge joints (e.g., elbow), (3) pivot joint (i.e., head rotation), (4) bicondylar joints (e.g., knee), (5) condylar (ellipsoid) joint (e.g., wrist), (6) saddle joint (e.g., thumb), and (7) ball and socket (e.g., hip). The focus here is on the bicondylar joint in relation to the knee. A bicondylar joint is a joint that allows for movement mainly along one axis with limited rotation around the second axis and is formed by two convex condyles that articulate with flat or concave surfaces (Drake, Wayne Vogl, & Mitchell 2010) . Sometimes bicondylar joints are included with hinge joints, which are joints that have the same convex structure but described as moving along only one axis with one bone in a fixed position (Drake, Wayne Vogl, & Mitchell 2010; Jenkins, Kemnitz, & Tortora 2007).

The knee is the largest and most complex joint in the body and principally allows for flexion and extension. There are three bones that compose the basic knee structure – the (1) femur, (2) tibia, and (3) patella. These three bones create three joints within the synovial capsule – (1)

the lateral tibiofemoral joint, (2) the medial tibiofemoral joint, and (3) the patellofemoral joint. The femur and tibia are weight bearing bones that allow the knee to lock and create a weak articular cavity that is reinforced with tendons and ligaments (Jenkins, Kemnitz, & Tortora 2007). The patella allows for the movement of the quadriceps femoris muscle over the joint by working as a fulcrum in a lever system and does not ossify until about 5 or 6 years old (Fox, Wanivenhaus, & Rodeo 2012).

Two c-shaped fibrocartilaginous menisci adhere to the proximal end of the tibia and accommodate the changing shape of the joint during movement and work as shock absorbers. They may also help lubricate and provide nutrients to the joint, but how it occurs is still uncertain (Fox, Bedi, & Rodeo 2012). The margin of the medial meniscus is attached to the joint capsule and the tibial collateral ligament (extends from the femoral medial condyle to the tibial medial condyle to provide support to the medial surface of the joint) (Drake, Wayne Vogl, & Mitchell 2010; Jenkins, Kemnitz, & Tortora 2007). The lateral meniscus margin is not fully attached to the capsule and does not attached to a ligament, making it more mobile than the medial meniscus (Cox & Hubbard 2020).

The synovial membrane is attached to the articular surfaces and the outer margins of the menisci. It is separated from the anterior patellar ligament by an infrapatellar fat pad. The synovial membrane creates pouches in two areas for the purpose of providing a low friction surface for the tendons (Drake, Wayne Vogl, & Mitchell 2010). The smallest is the subpopliteal recess that separates the tendon of the popliteus muscle from and the lateral meniscus (Woodburne & Burkel 1988). Another expansion is the anterior suprapatellar bursa (recess), which is a fluid-filled sac that reduces friction during movement. There are several other bursae around the joint to aid in friction reduction, but do not directly share the articular cavity (Drake, Wayne Vogl, & Mitchell

2010). The synovial membrane secretes synovial fluid that provides additional low-friction and low-wear properties to the articular surfaces. The fluid is composed of ultra-filtrated blood plasma, proteoglycans, hyaluronan (hyaluronic acid), and phospholipid molecules (Tamer 2013).

The joint is reinforced with a collateral ligament on each side – the medial (tibial) collateral ligament (MCL) and the lateral (fibular) collateral ligament (LCL). These two fibrous connective tissues stabilize the hinge-like knee structure around the fibrous membrane – an extensive membrane that encloses the articular cavity and intercondylar region and is reinforced by tendons and ligaments. The MCL is attached to the fibrous membrane and anchored to the femoral epicondyle and medial meniscus. Due to its attachment, when the MCL is torn the medial meniscus usually sustains damage, along with a cruciate ligament (ACL) (Jenkins, Kemnitz, & Tortora 2007). The LCL extends from the lateral femoral epicondyle to the fibular head and is separated from the fibrous membrane by a bursa. Injury to the LCL is not common and is usually associated with other injuries (Krukhaug, et al. 1998; Majewski, Susanne, & Klaus 2006).

Two ligaments composed of fibrous connective tissue connect the femur and tibia inside the joint and are termed cruciate ligaments. The cruciate ligaments received their name because of the crossed pattern to each other, and the individual ligament is named based on its anatomical position – the anterior cruciate ligament (ACL) and the posterior cruciate ligament (PCL). The ACL restricts anterior displacement of the tibia relative to the femur (hyperextension), while the PCL prevents posterior displacement; an action that is crucial for movements like going down stairs or steep inclines, like Pittsburgh hills (Drake, Wayne Vogl, & Mitchell 2010; Jenkins, Kemnitz, & Tortora 2007). The ACL extends posterior and laterally from its attachment on an anterior aspect of the intercondylar eminence of the tibia to the medial surface of the lateral condyle of the femur. It is the most common ligamentous injury (Evans, Shaginaw, & Bartolozzi 2014).

The PCL extends anteriorly and medially from a posterior aspect of the intercondylar eminence of the tibia to the lateral surface of the medial condyle of the femur (Jenkins, Kemnitz, & Tortora 2007; Woodburne & Burkel 1988). The cruciate ligaments, posterior ligaments, and extensor and flexor muscles work in conjunction to provide antero-posterior stabilization (Guyot 1980).

The joint is reinforced with several other structures around the joint, such as the medial and lateral patellar retinacula, patellar ligament, oblique popliteal ligament, and arcuate popliteal ligament. It receives its blood supply through the genicular anastomoses, which consist of the descending and genicular branches from the femoral, popliteal, and lateral circumflex femoral arteries in the thigh and the circumflex fibular artery and recurrent branches from the anterior tibial artery in the leg. The knee is highly innervated by branches of the obturator, femoral, tibial, and common fibular nerves (Drake, Wayne Vogl, & Mitchell 2010).

1.3.1 ACL structure

The anterior cruciate ligament (ACL) is an intra-articular fibrous connective tissue in the knee joint and is surrounded by synovial fluid. It is considered intra-articular, along with the posterior cruciate ligament (PCL), because it sits inside the articular space and passes through the knee joint, unlike the collateral ligaments that are termed extra-articular because they are on the outer joint surface. Extra-articular are not surrounded by synovial fluid but instead are surrounded by solid tissue with a good blood supply, and include the medial collateral ligament (MCL) and lateral collateral ligament (LCL) (Murray & Fleming 2013). The ACL extends from the anterior portion of the tibia to the posterior aspect of the femur. The main purpose of this ligament is to restrict anterior displacement of the tibia relative to the femur, stabilize the knee during internal-external rotation, and varus-valgus angulation (Beynnon et al. 2005b; Kiapour & Murray 2014;

Murray & Fleming 2013). The ACL is useful in actions that involve planting and pivoting, however high intensity pivoting sports like soccer, skiing, handball, and volleyball also have the highest ACL rupture rates (Majewski et al. 2006; Murray & Fleming 2013).

Ligaments act like springs to aid in proper movement. There is a crimp (waviness) structure that allows them to easily stretch and when stretched, the ligament can hold high loads. This keeps the bones operating at the appropriate motion range by restraining improper movement. For instance, the ACL aids the knee in proper flexion and extension and when the knee starts to go out of range it sends a nerve signal to surrounding muscles, particularly the hamstrings in the posterior thigh. This signal directs the hamstring to move in the same direction to avoid the knee from going in an incorrect direction. However, if the force is too great or too sudden, displacement may occur. The hamstrings typically contract together and stabilize the knee. Yet, the quadriceps in the anterior thigh straightens the knee and pulls on the tibia, which then pulls on the ACL. Strong quadriceps with weak hamstrings and jeopardizes the ACL during a sudden force or motion (Murray & Fleming 2013).

The ACL is formed from two major fiber bundles – the anteromedial (AM) and posterolateral (PL) bundles. Sometimes they can be differentiated into three bundles - the anteromedial (AM), intermediate (IM), and posterolateral (PL) bundles since the bundles are not distinct from the anatomical view (Beynnon et al. 2005b; Otsubo et al. 2012). Others have suggested 6-10 bundles in each ACL (Mommersteeg et al. 1995). However, it is typically reported as the AM and PL bundles (Beynnon et al. 2005b; Hirzinger et al. 2014; Schreiber, van Eck, & Fu 2010; Shirazi & Chrzanowski 2015; Woo et al. 2005; Zantop et al. 2007). The parallel bundles work together to distribute strain placed on the ACL but is not uniform. The AM bundle is the main regulator of anterior-posterior stability and strain on it is greatest in hyperextension and when in full flexion. The PM bundle mainly contributes to rotational stability and has the most strain in hyperextension (Bach et al. 1997; Beynnon et al. 2005b; Schreiber et al. 2010). The bundles become twisted with increased flexion (Otsubo et al. 2012). Single-bundle reconstruction surgery usually only reconstructs the PL bundle, but double-bundle surgery is now becoming more common because it can restore the knee near normal kinematics (Schreiber et al. 2010; Woo et al. 2005). The two bundles may endure different rupture patterns and could be useful in surgery where intact or slightly elongated bundles could be preserved (Zantop et al. 2007).

The fiber bundles are mainly composed of collagen. The insoluble collagen fibers are flexible and have a high tensile strength and are found in most types of connective tissue, like bone and cartilage (Stecco 2014). The polypeptide alpha chains that connect the individual fibrils of the fibers form a helix structure. The various structures of these helixes result in 16-28 different types of known collagen, making it the most abundant protein within the animal kingdom (Kadler et al. 2007; Lodish et al. 2000). Types I, II, and III are the most common in the body, comprising 80-90% of the observed collagen (Lodish et al. 2000). Type I is the most abundant and is found in dermis, bones, ligaments, and other places. Type II is mainly in cartilage. Type III is made of reticular fibers and is in skin, blood vessel walls, and among other places, and is the first secreted during all connective tissue development.

In addition to the collagen fibers, connective tissue is also comprised of cells and ground substance within an extracellular matrix (Barnes 1997; Stecco 2014). The cells provide metabolic properties, the ground substance contributes flexibility, and the fibers give motion. The area directly around the cells, the extracellular matrix, are in both types of connective tissue – general (appears throughout the body, mainly as fibrous tissue that creates tendons and ligaments) and special (includes bone, cartilage, blood and hemopoietic tissue). The type of connective tissue determines the physical properties of the developing tissue, which influences its function. For instance, a hard matrix is found in bone, while a fluid matrix is in blood. This matrix is composed of a protein fiber (collagen, reticular, and elastic) and amorphous gel-like ground substance (Jenkins, Kemnitz, & Tortora et al. 2007; Kardong 2012; Stecco 2014). The ground substance is made of macromolecules (proteoglycans, mainly glycosaminoglycans (GAGs)) that allow the fibers to slide with minimal friction (Jenkins, Kemnitz, & Tortora 2007; Stecco 2014). The ability of the ACL to maintain its matrix is necessary for it to resist external forces and prevent injury by activating matrix-remodeling pathways that maintain its homeostasis (Suijkerbuijk et al. 2019).

Collagen is a matrix molecule that composes 70-80% of the dry ligament weight and is composed of over 90% type I collagen and the remainder being type III (Murray & Fleming 2013). Collagen has a turnover rate of 300-500 day half-life, but the factors regulating turn-over are still not identified (Murray & Fleming 2013; Neuberger & Slack 1953). The remaining dry ligament weight is composed of molecules like proteoglycans (e.g., chondroitin-4 sulfate and dermatan sulfate) (<1%), elastin, fibronectin, and other glycoproteins (e.g., actin and laminin) (Frank 2004; Murray & Fleming 2013). The wet weight is 2/3 water (Frank 2004).

Ligament cells are in line with the collagen fibers where fibroblasts are the most evenly dispersed cell type. There are more cells per unit volume in the ACL than other joints, but still far less than in other cell-dense tissue like organs (Murray & Fleming 2013). The cells are responsible for building and maintaining the tissue. Fibroblasts are derived from multipotent mesenchyme stem cells (i.e., stromal stem cells, stromal fibroblastic stem cells, connective tissue stem cells), meaning they can differentiate into closely related groups of cells (Deng & Liu 2005). They secrete the fibers and ground substance of the extracellular matrix. The average lifespan of a fibroblast is 57 days based on chick embryo observation studies (Stecco 2014).

Collagen molecules are formed when the fetus starts to move and can only be formed with joint movement. If movement ceases, the condensation disappears, and no ligament is formed (Murray & Fleming 2013; Ruano-Gil, Nardi-Vilardaga, & Tejedo-Mateu 1978). Formation of ligaments is not like bone where there can be multiple ossification centers. During embryonic development, ligaments are a condensation of cells between the two bone attachment sites and grow throughout their length, not at discrete centers. The attachment site of the ligament to bone has rapid cell division where collagen becomes incorporated into the bone (Murray & Fleming 2013). The attachment sites at the femur and tibia have four transitional tissue layers – (1) ligament, (2) unmineralized fibrocartilage, (3) mineralized fibrocartilage, and (4) bone (Dai et al. 2015; Mutsuzaki et al. 2019).

There are three cartilage types, (1) hyaline, (2) fibrocartilage, and (3) elastic. Hyaline (i.e., articular cartilage) is the most common type, but also the weakest. It is in the ribs, nose, larynx, and joint ends. It contains very fine, dispersed collagen fibers (type II) that provide flexibility and support (Jenkins, Kemnitz, & Tortora 2007; Pittenger et al. 1999). It also reduces joint friction and absorbs shock. Fibrocartilage is the strongest of the three types and is found in intervertebral discs, symphyses, joint capsules, and ligaments. It is composed of dense collagen fibers (type I) that provide strength and rigidity (Hall, 2005; Jenkins, Kemnitz, & Tortora 2007). The third type, elastic cartilage, has a thread-like network of elastic fibers that give it high flexibility and resilience. This composition helps to maintain shape in structures such as the ear and nose.

Most types of cartilage are covered by perichondrium, but fibrocartilage is classified as a transitional tissue (properties intermediate between dense fibrous tissue and hyaline cartilage that occasionally merge together) and, as such, does not have a perichondrium (Benjamin & Evans 1990). Ligaments, including the ACL, have an epiligament layer that is hardly indistinguishable and merges into the periosteum around the bony attachment site (Frank 2004). The epiligament is rich in type III and V collagen, which are more flexible than type I and potentially aid in ligament recovery (Stamenov et al. 2019). The more cellular structure and extensive branching vascular structure may also aid in ligament healing (Frank 2004; Stamenov et al. 2019). The underlying ligament itself is poor at self-healing, which impacts surgery efforts and options.

The gold standard of ACL rupture surgery is the use of autografts, preferably the hamstrings (semitendinosus and/or gracilis tendon) or patellar tendon, but allografts and synthetics grafts are also used in reconstruction (Sajovic et al. 2011). It had been thought the ACL could not heal and it was irreplaceable, which is why reconstruction became the most common form of surgery (Ferretti 2020; Kiapour & Murray 2014). However, it has been shown there is a possibility it can heal, but it may not be anatomically correct or mechanically stable (Ferretti 2020). Studies have shown suture healing resulted in more than 10 times greater failure rate than reconstruction surgery (Gagliardi et al. 2019).

Ligament and tendon healing is slow and involves three overlapping steps – (1) tissue inflammation, (2) cell proliferation, and (3) remodeling (Hirzinger et al. 2014). These occur over about an eight week period where scar tissue is formed that is biologically and biomechanically inferior to the original tissue (Frank 2004; Hirzinger et al. 2014). This may be because a blood clot is not formed during the first step and does not activate the humoral coagulation system like it does in extra-articular ligaments such as the MCL (Hirzinger et al. 2014; Murray et al. 2007). The lack of vascularization, cellular density, and incasing synovial tissue are cited as the main reasons for poor ACL self-healing (Murray et al. 2000; Shirazi & Chrzanowski 2015). New biologically augmented repairs have been studied to find new approaches to ACL surgery, including cell therapy, gene transfer and gene therapy, growth factors applications, and bioscaffolds (Kiapour & Murray 2014). Ligaments were once thought to be inert structures, but now are known to respond to local and bodily factors, and injuries impact their overall structure and physiology (Frank 2004).

1.4 Animal model methodology

Animal models are an essential preliminary step in scientific research for identifying factors in various biological fields. The emerging field of evolutionary developmental biology (evo-devo) has discovered similar developmental genes across species, such as *Hox* genes, and how genes are impacted by the environment, further supporting translational studies across the animal kingdom (Müller 2007). The anatomical and physiological similarities between humans and animals, especially non-human mammals, are beneficial for both humans and the model animal because many of the same diseases and their mechanisms are observed in various species, including infectious diseases, type I diabetes, hypertension, allergies, cancer, epilepsy, and many more, aiding in better comprehension and potential treatment of the disease (Barré-Sinoussi & Montagutelli 2015). For instance, the anatomical and clinical similarities in the dog and human pancreas aided in the development of insulin to treat diabetes (Adin & Gilor 2017; Barré-Sinoussi & Montagutelli, 2015). Nearly 90% of veterinary drugs are identical or very similar to those used in humans because of the high relatedness of diseases (Barré-Sinoussi & Montagutelli 2015). Together with this, animal model studies follow the Center for Disease Control and Prevention One Health, One Medicine Initiative that "breaks[s] through the species barrier" to link human and animal medicine for the benefit of both the human and animal patient (Christopher 2015; Meeson et al. 2019; Ribitsch et al. 2020). Every animal model has its own advantages and disadvantages, and selection of the model is dependent on the disease being studied (Gregory et al. 2012).

Most of the nearly 400 inherited disorders characterized in dogs are relevant to humans, making the dog an excellent model for numerous human diseases, such as cancer, osteoarthritis, aging, and cruciate ligament rupture (Gregory et al. 2012; Mazzatenta et al. 2017; Rowell, McCarthy, & Alvarez 201; Starkey et al. 2005). Dogs share approximately 80% of diseases with humans (Momozawa 2019; Shearin & Ostrander 2010). Beyond sharing similar diseases with humans, dogs are a useful model due to accessibility, diverse living environments, sharing the same environment with humans (exposure to same bacteria, pollutants, and toxins), exhibiting breed-specific diseases (some that are rare in humans, such as sarcomas), appearance of spontaneous diseases (as opposed to induced, like in mice), and they age five to eight times faster than humans, aiding in a disease timeline, observation of disease onset, and condensed study time (Momozawa 2019; Oláh et al. 2021; Rowell et al. 2011). Dogs are also the most phenotypically diverse species and have the most known naturally occurring diseases of all land mammals after humans (Rowell et al. 2011; Starkey et al. 2005). Dogs are especially useful in cancer studies because they develop the same types and respond in a similar manner to conventional treatments, in addition to tumors being histologically similar and the canine genome being sequenced (Lairmore & Khanna 2014; Rowell et al. 2011). Dogs are considered the closest animal to a gold standard for human osteoarthritis due to the highly similar knee structure (Gregory et al. 2012). The similar knee structure identifies them as an excellent model for other knee diseases, such as anterior cruciate ligament (ACL) rupture in humans.

Diarthrodial joint structures and organization, such as the knee, are conserved across all mammals and the trabecular and subchondral bone are adapted for body mass (Mancini et al.

2019). The entire knee structure is referred to as the stifle in quadrupedal animals. The major difference between the human and dog knee is the increased number of sesamoid bones in the dog's popliteus, gastrocnemius, and extensor tendon (Gregory et al. 2012). The cranial cruciate ligament (CCL) is composed of the same structures, including the two fiber bundles, which are termed as craniomedial and caudolateral (instead of the PM and AM bundles). It originates on the femur in a fan shape and inserts into the tibia like that of humans, which has been beneficial for reconstructive models (Bascuñán et al. 2019). The average length of the CCL is 13.5-18.7 mm (de Rooster & Comerford 2018). Overall, the human ACL is wider and longer than the dog CCL, but not after it is normalized by the tibial plateau (Proffen et al. 2012). The tibial plateau angle in dogs differs from humans due to biomechanics, which is a consequence to some degree of using any quadrupedal animal model. The tibial plateau in humans and dogs both have a greater slope in the lateral than medial compartment but vary in magnitude. The tibial plateau average in humans is 7° $\pm$ 4 and in dogs is 24° $\pm$ 4, with a 76 $\pm$ 5 mm and 36 mm width, respectively (Bascuñán et al. 2019). On the microscopic level, the dog CCL has similar cell number density, SMA expression, vascularity, and cell nuclear morphology (Murray & Fleming 2013). Histological comparison of various animal models found the dog to be the closest to the human ACL (Proffen & Murray 2013). This structural similarity was a prime factor in its selection as the anatomical model for this study.

An animal model is useful in identifying and narrowing potential ACL rupture factors since rupture is thought to be multifactorial and animal models are useful for such research investigations for the previously mentioned reasons, especially condensed timeline. Previous ACL comparative studies have used cow, pig, goat, sheep, rabbit, dog, and monkey (Proffen & Murray 2013; Xerogeanes et al. 1998) [see Figure 1.2]. This study identified dogs as the best suitable model because of the similarities in anatomy, clinical symptoms, rupture rates and disparities, in addition to readily available data. Furthermore, research that utilizes sentient animals is a controversial topic, but because CCL rupture in dogs is naturally occurring, there are less ethical concerns when studying its relation to human medicine (Ribitsch et al. 2020). This research uses databases obtained from a veterinary hospital and a breed-specific longitudinal study to better identify CCL rupture factors that may predispose certain dogs to injury and translates it to human medicine. The comparative anatomy approach of a human and dog knee disease follows the One Health Initiative with the goal to aid in the overall health of both humans and dogs.

A difficulty with using animal models in locomotion studies is using a quadrupedal model for a bipedal subject. Locomotor mechanics are different in animals that have four weight bearing limbs as opposed to two. The weight (Netwons – N or kg m s^{-2}) of a quadruped is dispersed among the four limbs where the force on the forelimbs and hindlimbs is dependent on the distance of the limb to the center of the animal's mass (Kardong 2012). All weight-bearing structures carry or resist the applied forces (Newton's 3^{rd} Law of Motion) and these forces can be categorized in three ways – (1) compression (pressing down on an object), (2) tension (stretching an object), and (3) shear (sliding an object in opposing directions); most material is weaker under tension or shear forces and withstand compression the best (Kardong 2012). Yet, the main function of ligaments and tendons is the transmission of tensile load (Amis 2004). Tendons possess higher tensile stress than ligaments due to their more organized microstructure with a tensile strength cited at 70MPa, while ligaments tensile strength is at 36MPa (Amis 2004; Pring, Amis, & Coombs 1985; Race & Amis 1994).

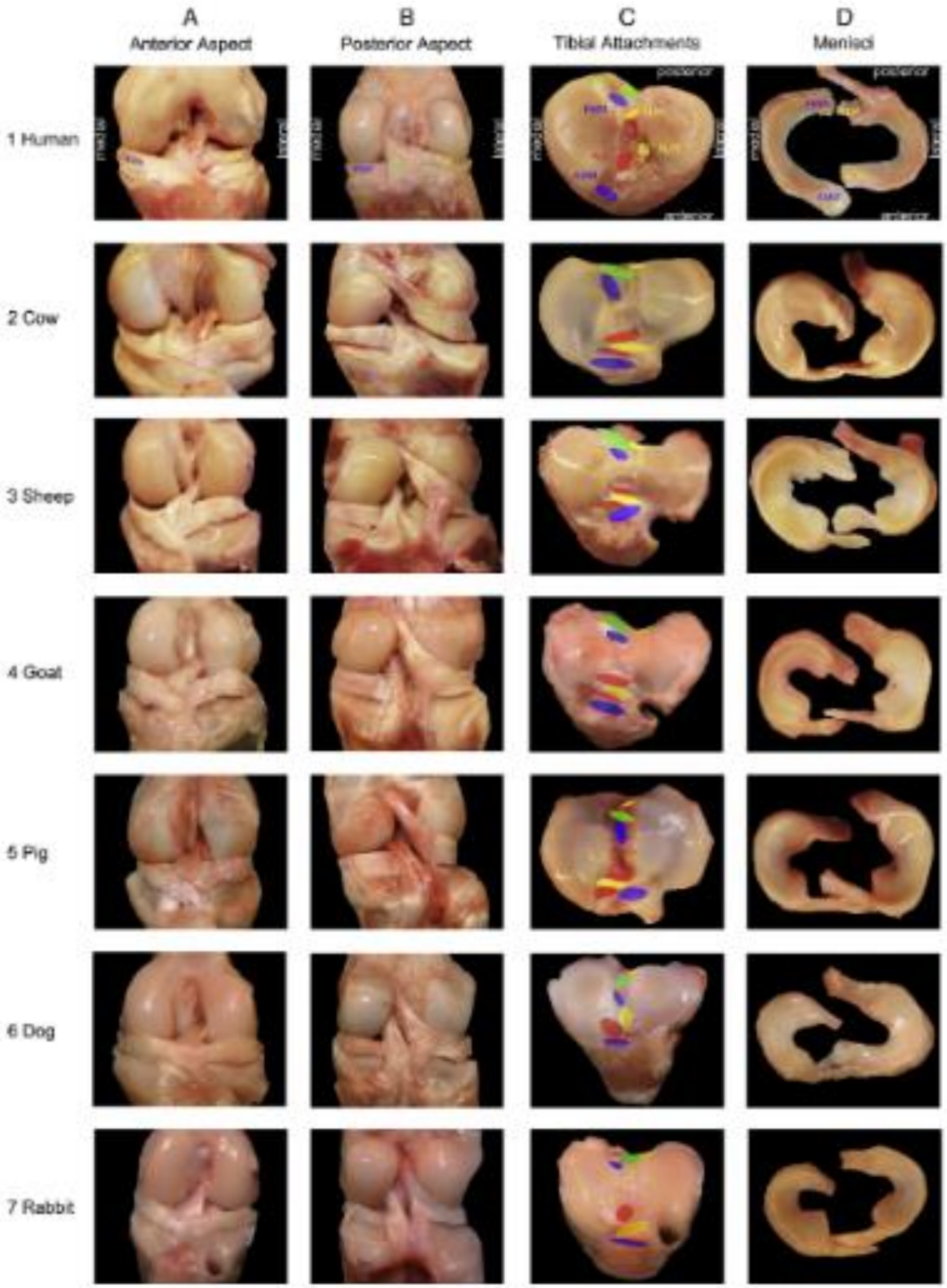

Figure 1-2: Comparison of the knee in 7 species (image from Proffen et al. (2012) with permission from Elsevier)

The breaking strength of the primary stabilizer of the knee in extension and internal rotation in dogs, the cranial cruciate ligament (CCL), is about four times the mass of the dog, and while hyperextension (maximum 150° extension) or acute trauma may lead to rupture, it is suggested those only account for a small number of cases (Fischer & Lilje 2014; Gupta, Brinker, & Subramanian 1969; Johnson & Johnson 1993). Ligaments and other bodily elements are susceptible to fracturing under prolonged or heavy usage due to the formation of microfractures that accumulate over time, comparable to fatigue fracturing in engineering (Kardong 2012). Rupture of the anterior cruciate ligament (ACL) in humans and CCL in dogs is thought to be influenced by microtraumas since rupture mostly occurs under normal loading conditions (Duval, Budsberg, Flo 1999; Griffon 2010; Mostafa et al. 2009; Ragetly et al. 2011; Sumner, Markel, & Muir 2010). Studies have shown in vivo loading in dogs is 10-25% of the ultimate breaking load of biological tissue in normal conditions with the average load of stifle failure at 2449 N and in humans most ligament loading is 2-5% of tensile strength which converts to 500 N for the ACL (Knudson 2007; Patterson et al. 1991). The kinematics of the quadrupedal dog model exemplifies how gait differentiation impacts these microtraumas and utility of the dog model as an overall anatomical model for musculoskeletal diseases.

Biology must follow the laws and limits of mechanics, and biomechanics studies integrate anatomy and physics (Kardong 2012). Studies examine variables such as the force transfer from and to the ground of an object (i.e., a dog) which can be measured using a force plate or treadmill with ground reaction force measurements systems integrated into them. Only one limb used to be able to be measured and technology now allows for the force of all four limbs to be measured at the same time at different gaits and speeds. Ground reaction forces are three directions – (1) vertical (body weight transferred), (2) direction of movement (acceleration, deceleration, or steady state), and (3) mediolateral (forces inwards or outwards perpendicular to the direction of travel). The force is distributed nearly equally across the paw, but not between the forelimbs and hindlimbs. Force is recorded as a percentage of the body mass to account for the different masses of dogs. For instance, in the German Shepherd (a breed susceptible to CCL rupture) when standing distributes 31% of body weight to each forelimb and 19% to each hindlimb (Fischer & Lilje 2014; Hart et al. 2016). Breeds with similar body shapes are almost identical (Fischer & Lilje 2014). When a dog transitions to an upward gait, the forces the limbs apply to the ground need to increase to accelerate the body forward. This results in more than the total body weight endured by a single forelimb at times. For example, over twice the body weight on one forelimb can be observed in the gallop. An exception is for greyhounds where over 10% more weight is in the hindlimbs than forelimbs at the trot (greyhounds are not highly susceptible to CCL rupture) (Fischer & Lilje 2014; Whitehair 1993; Williams et al. 2009).

Measuring the forces exerted by the joints in each limb is invasive and the devices used bias the results greatly, thus the forces on joints are measured through indirect methods, one being inverse dynamics. It involves morphometry (limb measurements), joint angle measurements (kinematics), dynamics (synchronously measuring ground reaction forces and kinematics), and inverse dynamics (calculations of the net forces and net torques from previous measurements) (Fischer & Lilje 2014). A study using this methodology compared Labrador retrievers and greyhounds at the trot and concluded the mechanics in specific dogs, especially those that differ in conformation, should be compared against breed specific patterns as opposed to other breeds as the mechanics in the hindlimb varied, especially in amplitude (maximum amplitude/range of motion is 130°) (Colborne et al. 2005; Fischer & Lilje 2014). More research is needed in this methodology for canine kinematics (Gillette & Angle 2008).

Different methodologies have been used to measure the same variables. For instance, a study using ground reaction forces also concluded the same two breeds (Labrador retrievers and greyhounds) moved differently, but when controlled for body weight the study proposed a conflicting conclusion that the two breeds are dynamically similar at the trot (Bertram et al. 2000). Another study using a pressure walkway and motion capture system found similar stride parameters in German shepherds and Labrador retrievers (after normalization) but different biomechanical parameters, especially in relation to the distribution of forces in the forelimb, center of pressure throughout the body, and hindlimb joint rotations. The two breeds have different conformations and identifying their differences and similarities can help identify potential factors for musculoskeletal disorders such as hip and elbow dysplasia and CCL rupture, which are common in both these breeds (Humphries, Shaheen, & Gómez Álvarez 2020).

In the trotting gait, potential energy transforms into elastic energy rather than kinetic energy that is observed in the gallop. Elastic energy is comparable to a rubber ball bouncing on a concrete surface where energy is stored during ground contact and recovered in the stance phase (Fischer & Lilje 2014). The elastic energy in locomotion is created by the gravity-induced impact of the joints and the resulting stretch of the tendons. The limb acts as a spring in this scenario where it compresses on pressure (touchdown) and then releases (takeoff) (Fischer & Lilje 2014). Gregersen et al. (1998) examined the work performed by every joint in the forelimb and hindlimbs of dogs to identify which muscle-tendon systems worked as a spring and which produced work by actively shortening. Work was calculated using positive and negative values for extension and flexion, respectively. The forelimbs produced 58% of the total positive work compared to the hindlimbs in the trot, but the hindlimbs produced 62% during the gallop (Gregersen, Silverton, & Carrier 1998). Elastic storage in individual joints were calculated with the knee during the trot producing 60% potential recovery and 4.7% potential recovery in the gallop (Gregersen, Silverton, & Carrier 1998). The ankle contributed 98% of the total negative work in the hindlimb through energy transfer between the ankle and knee, which is observed in humans and cats. Overall, most of the work of locomotion is produced by the main six limb joints (wrist, elbow, shoulder, ankle, knee, and hip) and most of the energy absorption occurs at the distal joints (wrist and ankle). These distal joints are driven mainly by a muscle spring system and allow a large amount of energy storage in the muscle-tendon systems where active shortening of muscles is also found to be necessary in locomotion (Gregersen, Silverton, & Carrier 1998).

Extension of the ankle and knee during locomotion occur simultaneously with a 1:0.72 relationship where angular extension of the knee impacts the resulting extension of the ankle and this relationship may contribute up to 27% of the positive work by all six joints produced in the extensor muscles of the knee (gastrocnemius and plantaris) (Gregersen, Silverton, & Carrier 1998). Injury to the knee, including the CCL, produces lameness that can be visually and physically evaluated relatively easily due to its high weight-bearing structure and change in extension and flexion movement. Diagnosis of unilateral and bilateral CCL rupture can usually be observed at the walk, trot, gallop, sit, and rise due to the shifting of weight on the limbs (Canapp 2007). The trot is preferred because it is the only gait where the forelimbs and hindlimbs do not assist the contralateral limb in weight bearing (Carr & Dycus 2016). A non-ruptured CCL is taut in extension and loose in flexion, like in humans (Arnoczky & Marshall 1977). Evaluations of ruptured CCLs show increased flexion throughout the stride at the femorotibial joint as compared to non-ruptured CCLs (DeCamp 1997). Physical examination using the tibial compression test and the cranial drawer test examines stifle instability, localized pain, and identifies inflammation (Canapp 2007).

There is still uncertainty if either biology or biomechanics play a larger role in CCL rupture. Both may be a factor in tibial conformation which contributes heavily to the biomechanics and rupture mechanics of the CCL (Cook 2010). Tibial plateau angle (TPA) is the angle between the tibial plateau and a perpendicular reference line. It has been accepted that dogs without CCL rupture average 22.5° and dogs with CCL rupture average 23.5°-28.5° but as little as 12° and high as 46° have been reported; any value greater than 34° is categorized as excessive tibial plateau angle and a large TPA does not directly equate to rupture (eTPA) (Duerr et al. 2008; Ragetly et al. 2011; Wilke et al. 2002). The importance of the TPA is in relation to the cranial tibial trust. The cranial tibial thrust is the result of ground reaction forces of the hindlimbs being resisted by the muscles and the compression of the femur and tibial plateau which then forms the cranially directed force (thrust) (Canapp 2007). Tibial plateau leveling osteometry (TPLO) is the most common surgery for CCL rupture, changing the TPA to create a more stable joint by reducing the angle to a recommended 6.5° to lessen the cranial tibial trust and minimize strain in the caudal cruciate ligament, however studies have found no post-operative difference in ground reaction forces in TPAs ranging from 0° to 14° (Bergh et al. 2014; Christopher et al. 2013; Conkling, Fagin, & Daye 2010; Robinson et al. 2006; Ron Shahar & Milgram 2006; Warzee et al. 2001).

A recent study measured nearly 4,000 dogs with CCL rupture and found the average TPA among all dogs in the population study was 29°, a higher reported measurement than in previous studies. There was a significant difference in dogs that were gonadectomized compared to their intact counterparts. Gonadectomized dogs averaged 28.87° and intact dogs averaged 26.88° and there was no significant difference in males or females regardless of their gonadectomy status (Fox et al. 2020). It is thought the TPA can accurately be measured after 90 days of age (Odders, Jessen, & Lipowitz 2004). However, the study that published this finding did not report gonadectomy status change throughout the study population. Long-term studies of TPA change have not been conducted (Zeltzman et al. 2005). Tibial proximal closure in dogs is not complete until 393 days (13 months) and early gonadectomy is encouraged around six months of age (Odders, Jessen, & Lipowitz 2004). This could impact skeletal formation before completed plate closure. The development of the TPA is not well understood and TPLO is not recommended before 9 months of age because of the uncertainty on tibial conformation (Odders, Jessen, & Lipowitz 2004). This suggests gonadectomy may impact the development of the hindlimb structure, which then impacts biomechanics and cranial tibial trust, leading to an increased risk of CCL rupture.

It is important to note that a single muscle cannot maximize force output and speed output due to biomechanical constraints, not biology. Therefore, two or more muscles may split the functions (Kardong 2012). The main muscles that contribute to increased velocity are the hip and tarsal extensors and stifle flexors (Colborne et al. 2006). In dogs, the contraction of the quadriceps and gastrocnemius oppose ground reaction forces during weight bearing which predominate over the hamstrings making an ineffective dynamic control against cranial tibial trust. This is offset by the strength of the flexor muscles contractions during the swing phase to reduce cranial tibial translation. This imbalanced dynamic is thought to be similar between CCL predisposed dogs and ACL predisposed humans (Griffon 2010). Humans that utilize their hamstring muscles over their quadriceps muscles are found to have reduced ACL injuries and this is predominantly found in males (Myer et al. 2009). Females are more prone to quadriceps dominance where the quadriceps are primarily used to stabilize the knee (Hewett et al. 2010). Females have increased femoral anteversion, Q angle, tibial torsion, laxity, and foot pronation which may contribute to the quadriceps dominance (Griffin et al. 2000).

A study examined the difference in the pelvic structure of Labrador retrievers with ruptured CCLs, non-ruptured CCLs, and contralateral ruptures to better understand the musculature in dogs with CCL rupture. It concluded the imbalance between the gastrocnemius and active muscle restraints towards cranial tibial thrust may predispose dogs to CCL rupture (Mostafa et al. 2010). The study found a significant difference in the hip and stifle angle at the stand and the hock angle in extension and flexion between the CCL ruptured dogs and non-ruptured dogs. However, there were no significant differences in the hip, stifle, or hock angle or muscle girth with the contralateral group. There also was no significant difference in the dual energy x-ray absorption, which measured the lean content and fat of each muscle (quadriceps, hamstring, gastrocnemius, muscle to bone ratio, and muscle to muscle ratio), but the lean content of the gastrocnemius to hamstring was greater in the CCL ruptured group (p = .007) than non-ruptured group (Mostafa et al. 2010). This indicates how muscle usage variation may be important in CCL rupture and studies that examine the same variables across several breeds are important for identifying different gait patterns across breeds and sizes.

The Jena Study based out of Germany is the largest conducted study in the world to examine dog locomotion among different breeds. The main purpose of the study was to identify the similarities and difference between various dog breeds, particularly because dogs have a 25-fold weight (mass) range and various body shapes that impact locomotor mechanics (Fischer & Lilje 2014). The study used three different high-frequency methods to capture movement – (1) videography, (2) marker-based movement analysis, and (3) biplanar X-ray videography. The videos were shot at 500 frames-per-second and the marker-based analysis at 280 frames-per-second at the walk, trot, and gallop (including the canter, and two rapid gallops – (1) the diagonal gallop and (2) the rotary gallop). Each individual dog varies on its maximum speed in each gait, even in the same breed. Therefore, gaits were recorded when the dog reached a steady, comfortable pace, not a defined speed. Animations were than produced from the images using complex programs combined with CT scans of corpses and macerated skeletons for the most accurate anatomical rendering. Cadaver dissection and photography of the muscles were also performed and resulted in the most precise reproduction of dog muscle arrangement ever attained in a virtual model (Fischer & Lilje 2014).

The Jena Study calculated relative stride lengths to compare stride lengths while controlling for body size. They found the shortest hindlimb stride at the walk was in the Chihuahua, mastiff, and French bulldog, all of which vary greatly in size. It was noted that surprisingly in the walk not all small dogs were grouped together, but in the trot more small breeds were closer in relative length. Additionally, there was no sex difference in limb proportions in most of the breeds (Fischer & Lilje 2014). The large size range of dogs does not impact joint angles or forces as most terrestrial animals are geometrically similar (isometric) and equivalent mechanical constraints apply across the spectrum and has been comparatively tested in mammals 3,000-fold in size (Biewener 1990). This applies across canids where limbs are isometric, meaning they are in the proper ratio to body size across the genus, but this ratio does not hold true for all skeletal structures such as the scapula (Fischer & Lilje 2014). The limbs may be proportional to body size, but stride length and distribution of body weight varies by breed.

The study of human bipedalism and the evolution of the human gait is a principal topic in biological anthropology (e.g., Laetoli tracks). There has been much advancement in the study of biomechanics, yet there is still much that is unknown, especially in humans. Advancements over the years have produced great strides, such as better understanding of the neuronal control mechanisms that may indicate humans use quadrupedal coordination due to the neuronal linkage between the forelimbs and hindlimbs (Dietz 2002; Zehr, Hundza, & Vasudevan 2009). Biomechanical engineering has advanced for accurate comparison across studies through the engineering of a robotic universal force moment senor testing system that allows for the reproduction and controlling of the multiple degrees of freedom of knee motion. It was created at the Department of Bioengineering, Musculoskeletal Research Center at the University of Pittsburgh and has been adapted by many other institutions (Woo et al. 2006). The adaptations in locomotor mechanics are not only useful for musculoskeletal disease studies, but also for other areas of medicine, such as robotic simulations for amputees (Biewener & Patek 2018).

The complexity of locomotion and its relation to injuries is interdisciplinary (Woo et al. 2006). The advancements in neural control and bioengineering have greatly benefitted the field and both have used animal models for better foundational research. For example, rabbits are a common model particularly for understanding ligament injury healing. Other models include dogs, goats, and monkeys which have been used to examine variables like tensile properties of the femur-ACL-tibia complex (FATC). Human cadavers have been used to assess variables like FATC and age, but human models are not always practical (or accessible) for other studies of motion (Woo et al. 1991). The physical studies of analyzing and calculating motion mainly uses makers placed on the skin to extrapolate muscle movement. Other methods exist but most are invasive, require radiation, and impede natural movement. Therefore, animal modeling is a valuable method for better understanding of locomotion and has been a methodology used since the 1600s to infer information that cannot be physically observed (Andriacchi & Alexander 2000). The locomotion of quadrupedal anatomical models may differ from that of human bipedal locomotion but isolates potential factors on skeletal development and muscoskeletal diseases that may not be readily observable on human subjects at this point in time.

1.5 Dog domestication theory

The co-evolution of humans and dogs suggests the same evolutionary selection pressures acted on both species, such as the adaptation to digest starch, and the sequencing of both genomes reveal convergent evolutionary processes (Mazzatenta et al. 2017). It is necessary to mention dog domestication and their evolutionary history here because dog evolution genetically mirrors human evolution, in addition to dogs being highly diverse and adapted to nearly every climate and environment as humans, making it useful for disease research (Barthélémy, Hitte, & Tiret 2019).

Presented here is a comprehensive overview of dog domestication. There are four main subsections, (1) species and domestication definitions, (2) origins, (3) morphology (broken into skull, body size, and teeth), and (4) physiology. It begins with defining the species concept, and domestication. It then progresses to the origin of dog domestication with a focus on when and where it occurred. Then morphological change is described and how domestication impacted the physical canid structure. Lastly, physiological adaptions are discussed, and the impact domestication had on internal functioning. This layout touches upon the major components of dog domestication and how the method of studying dog domestication is needed to better understand them as anatomical models.

1.5.1 Definitions

The words “species” and “domestication" have academic debatable definitions. They are colloquially understood, but specific definitions vary because of the thin line between species and variety. A full speciation event from beginning to end has never been naturally observed (Bush 1975). Charles Darwin and Alfred Russel Wallace proposed discrete definitions of “species,” even considering interbreeding as part of it (Mallet 1995). However, Darwin rejected many variations of his definitions and in his first edition of the *Origin of Species* explained,

> In short, we shall have to treat species in the same manner as those naturalists treat genera, who admit that genera are merely artificial combinations made for convenience. This may not be a cheering prospect; but we shall at least be freed from the vain search for the undiscovered and undiscoverable essence of the term species (Darwin 1859).

The difficulty in separating species and variety was beneficial to Darwin because it supported his evolutionary speciation theory. Sir Edward Bagnall Poulton (evolutionary biologist), Theodosius Grygorovych Dobzhansky (evolutionary biologist and geneticist), Heinrich Ernst Karl Jordan (entomologist), and Ernst Johann Otto Hartert (ornithologist) are some of other scientists that attempted to revise “species” by incorporating specific interbreeding or subspecies into the definition (Mallet 1995).

The synthetic theory of evolution (also known as the modern synthesis or neo-Darwinism) (1930s and 1940s) revised evolutionary change from individualistic to populational based. It focused on altered gene frequencies instead of spontaneous mutations (Campbell & Loy 2000). Dobzhansky and Ernest Mayr, some of the leading modern synthesis researchers, proposed the biological species concept. Species were identified as reproductively isolated, while varieties (e.g., geographic subspecies) were not (Mallet 2010). The concept described species as “concrete particulars… that can be described and delimited but not defined” and hybridization as individualistic, not populational, which did not redefine the entire species group (Wheeler & Meier 2000). Populations were isolated through three *natural* features – (1) morphology, (2) genetic, and (3) behavioral (possibly the most important feature). Behavioral characteristics (e.g., courtship, signaling) emphasized the natural concept (Campbell & Loy 2000).

There are some well recognized issues with the biological species concept – four are mentioned here. First, the concept appears to give clear reproductive boundaries despite hybridization being relatively common in some animals. For example, about 40% of duck and geese have been observed to hybridize (Campbell & Loy 2000). Mayr redefined the concept to acknowledge this gene leakage (gene flow) and explained it as "biological properties of individuals which prevent the interbreeding [fusion] of populations" (Mayr 1996). This also helped with the high amount of observed plant hybridization (Mayr 1996). A second issue is applying it to the fossil record. Behavior cannot be observed, and fossils are usually categorized solely on morphology (Campbell & Loy 2000). This is a concern in cases such as archaeological canid finds. A third issue is prezygotic (fertilization prevented, i.e. mate recognition) and postzygotic (produce fertile offspring) with isolated populations (Groves et al. 2017). Lastly, this concept does not classify asexually reproducing organisms as a species (Coyne, Orr, & Futuyma 1988).

There are several more critiques of the biological species concept and has resulted in several researchers proposing new concept types all together (e.g. phenetic, phylogenetic, ecological) (Donoghue 1985; Ehrlich 1961; Mallet 2010; Groves et al. 2017). Each newly suggested concept still has its limitations. For instance, the Mishler and Donoghue phylogenetic species concept defines species as the least inclusive monophyletic group usually based on synapomorphies, making species the smallest grouping to maintain a lineage (Mishler & Brandon 1987). This concept is argued as the only means of testable species recognition, but is also limited by restricting species first and then proposing their relationships (Groves 2012; Davis & Nixon 1992). This concept could lead to many species being identified as polyphyletic (artificial group that does not share a common ancestor) if there are identical appearing individuals in two separate species (Davis & Nixon 1992; Kardong 2012).

While the biological species concept does have its drawbacks, it is still the most widely accepted "species" definition (Ereshefsky 2007; Thorp, Rogers, & Dimmick 2014; Campbell & Loy 2000). Therefore, this paper uses the term "species" in reference to the biological species concept. Additionally, some argue trinomial taxonomic nomenclature is just a placeholder for subspecies until a better species concept is defined (Groves et al. 2017). Others consider the trinomial system too disorganized and not of much actual use (Gosline 1954). However, the trinomial naming system aligns with the biological species concept since it includes subspecies classifications. This paper will also follow the trinomial nomenclature system unless otherwise noted. For instance, the grey wolf is *Canis lupus* and the domestic dog is *Canis lupus familiaris* (as opposed to *Canis familiaris* indicating a separate species).

The *Canis lupus familiaris* subspecies classification in canid phylogeny is the result of domestication, a word that has also received debatable definitions (Bökönyi 2014; Russell 2002; Clutton-Brock 1992). Domestication definitions incorporate terms like "controlled breeding," "integrated within socio-economic organization," "human-animal symbiosis," and "complete mastery over breeding." Hecker suggested substituting the word "domestication" altogether with "cultural control" (Bökönyi 2014; Russell 2002). Clutton-Brock (1992) describes domestication as complete when the "new population is permanently isolated from the wild species and its breeding, organization of territory, and food supply is under total human control." Despite the various definitions, domestication is accepted as both a biological and cultural process (Clutton-Brock 1992).

The "species" and "domestication" terms have been challenging to explicitly define. Both refer to a gradual morphological change overtime leading to identification issues in the archaeological record. The Clutton-Brock definition of completed domestication partially conflicts with the biological species concept and highlights the use for trinomial nomenclature when describing archaeological finds. Domesticated dogs could be referred to as “permanently isolated” from grey wolves, but without the subspecies name semantics would not be able to differentiate between the wild wolf and the domestic dog which is another reason why this paper will use the trinomial nomenclature system.

It has been suggested an extinct wolf subspecies gave rise to the modern dog lineage, but some argue that subspecies directly self-domesticated into the modern dog (Freedman et al. 2014; Morey & Jeger 2015). The continuing issue of subspecies versus species is prevalent within the domestication definition. There are continuous, rather than dichotomous, traits within the archaeological record and subspecies in general. As a result, this paper uses the conventional term “dog” to refer to any known domesticate and “wolf” to refer to the known non-domesticate. “Canid” will be used as an ambiguous term to mean either dog, wolf, or any other animal in the Canidae family.

Domestication definitions vary since there is no defined point when something is considered domesticated and different authors have varying perspectives and emphases on the human-animal relationship (Bökönyi 2014; Clutton-Brock 1992; Zeder 2015). Domestication can be considered completed when the “population has reached a maximal steady state” (Price 1984). Like “species”, no definition is regarded as far more superior or completely correct. The definition used here is, “that process by which a population of animals becomes adapted to man and to the captive environment by some combination of genetic changes occurring over generations and environmentally induced developmental events reoccurring during each generation” (Price 1984). This brief definition mentions the main aspects of dog domestication – adapted to man, genetic change, and environmental adaptations.

Humans have the selective ability to choose which features they want in a domesticated population. They can manipulate the population to their own benefit. This is a distinct human domestication trait as compared to non-human species domestication. This goal-orientated process selects which animals are best for domestication (Zeder 2015). Animals suited for domestication have predisposed behavioral characteristics proper for human handling and can be grouped into five main traits – (1) social structure, (2) sexual behavior/selection (readily breed in captivity), (3) parent-young interactions, (4) feeding and habitat adaptation (wide home ranges), and (5) human reactivity (short flight distances) (Clutton-Brock 1992; Zeder 2012). These traits have made animals such as dogs, sheep, and goats appropriate for domestication, but not gazelles which Natufians may have attempted to domesticate (Dayan & Simberloff 1995; Zeder 2012, 2015).

1.5.2 Origins

Canids are highly adaptable animals that inhabit every continent besides Antarctica and live in nearly every environment similar to humans (Vonholdt & Driscoll 2017). These adaptation types are beneficial for human domestication and have led canids to be the only large carnivore to be domesticated (Wayne & Vonholdt 2012). Carnivores appeared after the Cretaceous-Tertiary mass extinction (65 million years ago (mya)) and the Carnivora (order) most recent common ancestor appeared around 51.4 mya. This splits the cat-like and dog-like carnivores, Feliformia and Caniformia, respectively. Caniformia (suborder) diverged 44.9 mya and currently has 163 recognized species, including the sea otter, brown bear, and red fox. Canidae (family) diverged 5.62 mya and currently has 34 species (OneZoom Core Team 2019). The Canidae family is the oldest phylogenetic lineage within Caniformia and has over 214 known species with nearly 177 being extinct (Ostrander & Ruvinsky 2012; Wang & Tedford 2008).

There are three recognized subfamilies, (1) Hesperocyoninae, (2) Borophagines, and (3) Caninae. Caninae originated in the Oligoene and the only surviving subfamily with a continued lineage (Wang & Tedford 2008). There are currently four distinct canid groups, (1) North American grey foxes (outgroup), (2) red fox-like canids, (3) South American foxes, and (4) the wolf-like canids (including the grey wolf and the domestic dog). The Canidae family diverged only 10 mya most likely in North America, but the first domestication episodes presumably occurred in the Old World (Ostrander & Ruvinsky 2012).

There are several areas and theories suggested for initial dog domestication. Since evolution is a gradual process and dogs are a subspecies of wolves, there are no definitive characteristics that denote an archaeological find as domestic, feral, or wild canid. Genetics are a new methodology for pinpointing divergence times, locations, continued lineages, and possible physiological changes. Despite techniques such as high-quality genome sequencing, divergent times range widely from 11,000 to 135,000 years ago, with most citing around 15,000 years ago (Chase et al. 2002; Freedman et al. 2014; Vilà et al.1997). Finds dating around 30,000 years ago are controversial, but it is well accepted that dogs were domesticated before the advent of agriculture (Larson et al. 2014). Time and geographic location are the most debated topics because of archaeological and genetic discrepancies (Dobney & Larson 2006).

Genetics have provided a wide range of possible domestication dates and locations. Methods have included many versions of single nucleotide polymorphisms (SNPs) studies, like genotyping, haplotyping, and mtDNA analysis (Boyko et al. 2009; vanHoldt et al. 2010, 2013). SNP databases have been proposed and implemented to facilitate dog genomic studies. For example, DoGSD is an open access resource for dog domestication studies and provides data on SNPs, diseases, genomes, and gene expressions (Bai *et al.* 2015). Some other non-genomic methods include accelerator mass spectrometry (AMS) radiocarbon dating, 3D morphometrics, and archaeological comparisons (Drake, Coquerelle, & Colombeau 2015; Price 1984, 1998; Wayne 2001). Most non-genomic studies are now used for morphological analyses.

SNP analyses are currently the most common method but have variable conclusions. For example, haplotyping sharing has concluded a Middle Eastern domestication origin, while mtDNA has suggested East Asia based on haplotype diversity. A study by vonHoldt et al. 2010 found that haplotype diversity does not correspond with ancestral populations like in humans. They found no consistent pattern of genetic diversity and location. Instead, they found Middle Eastern grey wolves and modern breeds share the most haplotypes as opposed to other wolf groups. This suggests the Middle East as the epicenter with variation introduced from Europe and Asia (Vonholdt et al. 2010). An insulin growth factor 1 (IGF1) study also suggested modern small dogs have the same variation as Middle Eastern wolves and originated in the same area (Gray et al. 2010; Wayne & Vonholdt 2012). Additionally, the earliest archaeological finds suggest a Middle Eastern or European origin (Dayan 1994; Savolainen 2006).

Some researchers suggest the increased shared haplotypes in Middle Eastern grey wolves and dogs are from dog-wolf introgression instead of origination. Asian origin advocates mainly use mtDNA and Y-chromosome DNA, but there are uncertainties about Chinese region (central, southwest, southeast). East Asian wolves have the highest amount of haplotype diversity, which was originally suggested to show the dog domestication source, but the study is thought to have high sample biases (Boyko et al. 2009). Additional studies involving mtDNA and Y-chromosomes has further pinpointed Asia as the prime origin. A study suggested dog genetic diversity is shared nearly 50% in a universal gene pool. Most regions only share this 50%, but Asian dogs have the remaining 50% of genomic diversity present within the region. This suggests the other diversity seen in other regions originated in Asia (Ding et al. 2012).

It is proposed village dogs are geographically and genetically diverse with less chance of admixture with recent breeds and are a good sampling group. One study combined mtDNA, Y-chromosome, and autosomal markers of village dogs. The dogs exhibited a higher diversity of mtDNA and Y-chromosomes and lower linkage disequilibrium (difference between observed and expected allele frequencies due to nonrandom association and reduced recombination resulting in haplotypes not being in the expected frequency) (Goode 2011; Jobling, Hurles, & Tyler-Smith 2004; Shannon et al. 2015). The lower linkage disequilibrium suggests two results – (1) a weak dog domestication bottleneck and a small current, reproductive population size or (2) a strong dog domestication bottleneck and then an expansive population growth. The second result aligns more with archaeology (Shannon et al. 2015). Some Asian archaeological canid remains may have been domesticated from a smaller wolf subspecies, *Canis lupus arabs* which have been difficult to categorize in the osteological record. Since morphological changes are gradual, it is difficult to separate small wolf subspecies, full sized wolves, and dogs (Clutton-Brock 2016).

Europe has the oldest confirmed archaeological dog remain from a mandible that dates to 14,000 years ago in Bonn-Oberkassel, Germany. It was found in a co-burial with two humans (Benecke 1987). Asia and the Middle East do not have finds older than 13,000 years ago. A genetic mtDNA study using maximum likelihood, coalescence, and Bayesian methodology found modern dogs fall into four distinct clades, A to D. The association of the clades with ancient European canid samples suggests a European origin for dogs. They also found modern wolves from Asia and the Middle East were not as close with the dog clades and previous studies supporting non-European origins had limited sampling and weak statistical significance. Bayesian analysis suggests a divergence time with upper and lower limits of 18,800 to 32,100 years ago (Thalmann et al. 2013).

Freedman et al. (2014) sampled three wolf populations, one each from Croatia, Israel, and China, to represent the three possible centers of domestication. Each center showed a high amount of admixture with certain modern breeds, but this may be due to ancient gene flow. They concluded that no one area is more closely related to modern dogs and the sampled wolf populations and modern dog lineages diverged around the same time, about 15,000 years ago. The ancient lineage in which modern dogs arose may have gone extinct and the current sampled wolf populations are a younger lineage. While the results did not signal one specific area for domestication onset, the study concluded dogs still originated from one area of the world and the domestication hypotheses should be re-evaluated (Freedman et al. 2014).

Re-evaluation of the single origin hypothesis by other researchers have proposed a multi-origin hypothesis for dog domestication (Dobney & Larson 2006). It has been suggested dogs were domesticated in several areas of the world from different wolf populations (Frantz et al. 2016; Larson et al. 2012; Skoglund et al. 2015). These regional groups then interbred and possibly replaced other regional groups. Frantz et al. (2016) sampled mtDNA and nuclear genome from Newgrange, Ireland and found haplotype results could not be completely explained by genetic drift but can be explained by physical migration. It was concluded there were at least two independent centers of origin for domestication and the East Eurasian domestic wolf most likely followed humans into Europe and replaced the Western Eurasian domestic wolf population. However, there may not have been a complete replacement as supported by variation not seen in modern dog or wolf populations (Frantz et al. 2013). Modern wolves have been documented following migratory animals over 1,000km making the replacement model plausible (Perri 2016). Additionally, radiocarbon dates have suggested dogs were domesticated in Europe and the Near East around the same time (Protsch & Berger 1973).

Admixture, migration, bottlenecks, and artificial breeding are many of the issues with dog domestication analyses. Both wolves and dogs underwent multiple bottlenecks limiting the genetic variation from ancient to modern samples (Boyko 2011). Dogs are thought to have experienced two populational bottleneck events, when domestication first occurred and again during modern breed formation. Some argue the first event was strong like in *Homo* leaving Africa, while major histocompatibility complex analysis and other studies suggest it may not have been as extreme (Wade et al. 2006; Wayne & Vonholdt 2012; Shannon et al. 2015; Wang et al. 2013; Gray et al. 2009; Gross 2010). It has been estimated the first bottleneck produced a 5% nucleotide diversity reduction and the breed formation bottleneck resulted in a 35% reduction (Gray et al. 2009). Another study suggested a 16-fold reduction (as opposed to the 3 or 4-fold decrease in other studies) in the domestication bottleneck but did not provide an estimate for the second event. Additionally, wolves may have underwent a 3-fold reduction shortly after the first dog bottleneck (Freedman et al. 2014).

Modern breed formation has also created genomic issues in dog domestication studies because it is an artificial selection process contrary to wolf natural selection. Contemporary dog breeding practices started about 150 years during the Victorian Era (Vonholdt et al. 2010). Single nucleotide polymorphism data have shown there are 16 breeds recognized as ancient or "basal" breeds due to their older genomes and lack of recent admixture from physical and cultural isolations (see Table 1.1 for basal breed list) (Larson et al. 2012b).

Table 1.1: Basal breeds

Afghan Hound	Eurasier
Akita	Finnish Spitz
Alaskan Malamute	New Guinea singing dog

American Eskimo	Saluki
Basenji	Samoyed
Canaan	Shar-Pei
Chow Chow	Shiba Inu
Dingo	Siberian Husky

These breeds are still more closely related to modern dogs than wolves and three (basenji, bingo and New Guinea singing dog) do not come from natural grey wolf territory. The basenji is regarded as the oldest extant breed based on genomic trees (Vonholdt et al. 2010). All but one (Alaskan Malamutes) are Old World derived and none come from central Europe despite some of the oldest excavated canid archaeological remains being found there (Larson et al. 2012).

Taphonomic processes also bias interpretations. Specific areas of the world have better preservation conditions. Areas with high humidity do not generally preserve organic material well resulting in more canid remains found in certain contexts. Dog burials increased preservation, but it may not have been a common practice during the early domestication stages. Additionally, world wars have influenced dog genomes. Some breeds went extinct, while others were recreated, such as the Italian Greyhound and Bernese Mountain Dog. Various breeds were crossed to restore the lost breeds, resulting in original genetic breed signature loss. The Finnish Spitz almost went extinct until one breeder traveled to remote villages and collected dogs least likely to have bred with other breeds. The breed still has a basal lineage proving the practice successful (Larson et al. 2012).

Grey wolf variation and possible lineage extinction could be mistaken for early domestic dogs and influence domestication dating. The wolf population size reduction and bottleneck indicate that some groups may have gone extinct resulting in specific finds not being related to the modern grey wolf or dog. Dietary isotopic analyses at Předmostí, Czech Republic found two wolf groups with varying diets, one reindeer and the other mammoth and large herbivore. It was originally concluded the reindeer focused wolves were provisioned by humans because reindeer did not compose a large portion of the human diet. However, it has also been suggested there were different niche groups. One group was migratory following reindeer, and the other group was scavenging mammoths and targeting other large herbivores. This calls into question the canid finds older than 16,000 years, such as the 33,000 year old Siberian canid-like remain (Perri 2016). Those finds have now been regarded as failed domestication attempts or now-extinct wolf populations (Freedman et al. 2014; Morey & Jeger 2015; Perri 2016).

1.5.3 Morphology

Animal domestication influences endocrinology, docility, reproduction, morphology, and overall increased populational variation (Larson et al. 2014; Trut, Oskina, & Kharlamova 2009). Morphological domestication characteristics are typically coat color and texture change, floppy ears, facial neoteny, curled tails, and reduced body size (Boyko et al. 2010; Dobney & Larson 2006; Kukekova et al. 2008; Wayne & Vonholdt 2012). The exact mechanisms for specific morphological traits coinciding with domestication are still not certain. Domesticated dogs are the most phenotypically diverse mammal and their morphological changes have been extensively researched (Frynta et al. 2012; Schoenebeck & Ostrander 2013). It has been suggested as selection became more relaxed over time, mtDNA nonsynonymous mutations were able to accumulate and contribute to the high variation observed today in modern dogs (Björnerfeldt, Webster, & Vilà 2006).

The famous Russian silver fox (*Vulpes vulpes*) farm experiment at the Institute of Cytology and Genetics (ICG) has provided research data into domestic traits. For example, they found coat color change is the first trait to appear around the 8^{th}-10^{th} selected generation. Piebald *Star* spotting and brown mottling are the most common patterns observed. Researchers found the white spotting is due to incomplete dominant autosomal *Star* mutation, but the brown mottling is still uncertain. In domestic dogs the mutation occurs at the *Agouti* locus. As generations were further selected for tameness, skeletal modifications appeared, including shortened legs, tail, rostrum, and skull shape (Trut et al. 2009).

The wide range of dog sizes and the variation from wolf ancestors proposes the change is due to heterochronic or allometric principles. Heterochrony is the ontogenetic shift in the onset or timing of a skeletal feature appearance from a descendant species compared to the ancestor species. It is a relative basis where descendants are compared to ancestors, or ingroup compared to outgroup. Heterochrony breaks down into paedomorphosis and peramorphosis. Paedomorphosis is descendants retaining juvenile ancestral traits. For example, the human face is thought to be a paedomorphic feature from our primate ancestors. Adult primates have muzzles while young primates are flat faced. Peramorphosis is where descendants have increased or exaggerated features of their adult ancestors. For instance, humans have longer limbs than adult primates. Paedomorphosis and peramorphosis both further breakdown into three subcategories dealing with specific feature timing (Kardong 2012).

Allometry is the change in body proportions during growth and development and can be studied during ontogeny and phylogeny. This is a principle also reliant on comparison, except it is only within the same physical body. For example, children are not proportionally mini adults. They have larger heads and shorter limbs, which gives them the bobblehead-looking effect. Allometry is summarized by the equation $y=bx^a$, where y and x are two relative size body parts and a and b are constants. Features may be positive, negative, or isometric. Positive means the growing feature develops faster than the reference part, while negative means it develops slower than the reference part. Isometric is when proportions stay constant (Kardong 2012). Allometric data can be measured in three ways, longitudinal, cross-sectional, and static. Longitudinal are multiple features observed on individuals over time, cross-sectional is one feature measured on several individuals at different developmental stages, and static measures individuals during one developmental stage, usually adult. Static provides morphological variation, but not developmental variation (Morey 1992).

Dog and wolf archaeological distinctions are problematic because of researcher subjectivity, canid variation, and gradual evolutionary change. Morphological differences are the main identification and classification source of canid remains, but trait variation is highly debated and potentially lead to misdating dog domestication (Morey 2014; Tchernov & Valla 1997; Morey & Jeger 2015). There are three main identified traits used to differentiate between canid remains that are unlikely from human selection – (1) body size, (2) facial shortening, and (3) tooth crowding (in earlier finds). These three traits are important for two reasons. First, it shows a general canid evolutionary change regardless of cultural context in which they were reared. Second, other domestic animals tend to show the same modifications (Morey 1992).

1.5.3.1 Skull

The skull is regarded as the best representation of morphological change. It has the most distinctive, consistent measurable features and can also be used to indicate size (Alpak, Mutuş, & Onar 2004; Morey 1992; Morey & Jeger 2015; Wayne 1986) In dogs, the skull shape is regarded as a modern breed defining trait (Schoenebeck & Ostrander 2013). While domesticated dogs have an increased brain-to-body size ratio compared to wolves, dogs have a relative brain size about 30% smaller. This reduced brain size is a common domestication trait. Herbivore brains are reduced about 14% to 24% and rodent brains are only slightly reduced. In general, the larger the progenitor brain size and degree of folding, the greater the domesticate brain size. This rule holds even for birds and captive bred fish, and it seems to be a permanent evolutionary change (i.e. feral dogs do not regain wolf brain size). There is also no relation between domestication time and brain size (Zeder 2012).

Brain reduction may be a domestication trait, but the specific reduced brain features seem to be species orientated. For example, pigs and sheep have less reduced olfactory and auditory functions than visual or motor functions, and rats and mink show the reverse. The brain area most commonly effected by domestication is the limbic system, which includes the hippocampus, hypothalamus, pituitary gland, and amygdala. In dogs, pigs, and sheep, this portion is reduced greater than 40% from wild animals to their domesticated relative. This brain structure is responsible for endocrine function and autonomic nervous system. It influences behaviors such as aggression, suspicion, and environmental stress (Zeder 2012). The reduced brain area lowers the flight or fight response. This decreased reactivity is a domestication hallmark and enables human interaction tolerance (Schoenebeck & Ostrander 2013; Zeder 2012).

The developmental biology of the canid skull is still not well understood despite skull and brain shape changes as the most prominent domestication feature. It took about 70 years for a confirmed predication, through genome-wide association studies (GWAS), that modern breed defining skull shapes do not adhere to simple Mendalian genetics. Domesticated dog skulls can be described like human cranial birth deformations, such as brachycephalic (e.g., bulldog, pug, and Boston terrier), dolichocephalic (e.g., saluki, borzoi, and collie), klinorhynchy (e.g., bull terriers), and hydrocephalus (e.g., Chihuahua) (Schoenebeck & Ostrander 2013). Brachycephaly receives the most research attention and has been used in association with human brachycephaly, a condition distinguishing several deadly syndromes (e.g., Apert's, Crouzon's, and Pfeiffer's syndromes) (Schoenebeck & Ostrander 2013).

The dog skull is debated as a paedomorphic feature (Morey 1992). The retained juvenile features are thought to be a domestication property (Boyko et al. 2010; Dobney & Larson 2006; Gray et al. 2010; Morey 1992; Roberts, McGreevy, & Valenzuela 2010; Schweber 1977; Tchernov & Valla 1997; Wayne 1986). It is hypothesized it is more attractive to humans and facilitated the co-evolutionary bond (Waller et al. 2013). The generally smaller heads and rostrums of dogs compared to adult wolves suggests dogs have retained wolf-like cranial structures. Some suggest this resulted as a by-product of biomechanical constraints, especially in small breed dogs (Morey 1992). However, other studies have shown dog skull shape may be a neomorphic feature (a new structure in a derived species with no equal evolutionary antecedent) because the palate angle and neurocranium development in dogs differs considerably from wolves (Schoenebeck and Ostrander 2013; Kardong 2012; Drake 2011; Schoenebeck & Ostrander 2013). Drake (2011) found 33.8% of the dog skull can be contributed to allometric patterning, but the remaining is due to *de novo* skull orientation. Drake (2011) suggested the differing conclusions deal with the methodology.

Various researchers have used different methodology to assess skull measurements and evolutionary development. Most have used calipers and calculated various cranial points. The most common measurement is condylobasal length (CL), which measures skull length based on stable anatomical points (Morey & Jeger 2015). Statistical calculations used CL as the independent variable when compared against other cranial measurements. It implies overall skull and body size but cannot not be used as a complete body size substitute. Cranial lengths and widths need to be used together to assess an entire canid skull. Length measurements alone may suggest canid size change is an allometric by-product result. When width is incorporated, it reveals patterns not predicted by allometric scaling, such as wider palates. Instead, heterochrony can be suggested to be the developmental patterning. Overall, these measurements show similar sized dogs and wolves have different cranial patterns (Morey 1992).

Newer methods use 3D geometric morphometric analysis to measure and compare canid skulls. This new technique is more exact than calipers and can measure more points accurately in less time. Drake (2011) argues the new methodology is a better tool because it plots data using multivariate plots instead of bivariate plots. This provides greater depth and insight about the data. This new method concluded modern dogs did not develop through heterochronic means, but neomorphic, and more studies are needed to better understand canid skull development. Dogs were stated as not paedomorphic wolves, no matter the skull shape of the modern dog. Geiger et al. (2017) also used 3D morphometrics and concluded dogs display both neomorphic and paedomorphic features but appear at different ontogeny stages. This is not a result of modern breeding, but possibly from relaxed natural or artificial selection in the beginning of domestication (Geiger et al. 2017).

Calipers and 3D measurements provide different results for dog skull development, but both methods have been used to contradict canid remains. Some canid genetics sequencing have suggested dog domestication started around 30,000 years ago and a few studies exceed 100,000 years ago (Chase et al. 2002). Carbon dating and other genetic studies suggest most archaeological finds are around 15,000 years old (Morey 2014). Goyet Cave in Belgium was thought to be the oldest paleolithic dog evidence and dated around 36,000 years ago (Morey 2014). One of the remains had a dog-like skull morphology and the original author cited new haplotypes suggesting high variation in one site (Perri 2016; Thalmann et al. 2013). Morey (2014) argues the author used skull shape as an independent variable from body size, which goes against allometric principles.

Drake (2015) used 3D morphometrics to account for measurement overlap that may have incorrectly correlated data. The 3D data also concluded the canid remains were wolves and not domesticated dogs. (Drake et al. 2015). Additional mtDNA has placed the remains as an ancient sister group to modern dogs (Perri 2016; Thalmann et al. 2013).

1.5.3.2 Body size

Allometric principles state that different body parts develop at different times and heterochronic principles assess the changeover evolutionary time. Dog domestication is evidenced within postcranial canid remains but are not as reliable as skull features and as a result, little is published on postcranial remains. Modern dogs and wolves are instead analyzed more extensively to understand different morphological features in various dog breeds compared to wolves. Modern dogs have a 40-fold body size range with over a 12-fold leg length variation (e.g., Pekingese versus Scottish Deerhound) (Ostrander & Bustamante 2012). In general, dogs are smaller in size than wolves with a few artificially selected exceptions (e.g., Irish wolfhound). The overall size of the modern dog falls between the juvenile and adult wolf (Schoenebeck & Ostrander 2013). The smaller size is indicative of domestication, but the allometric scaling down is not fully understood.

Casinos et al. (1986) compared the long bones of 63 modern breeds to wolves. The allometric equation and regression lines were used to assess the measurements. Result showed the ulna and tibia are relatively stable between dogs and wolves, but the femur and humerus differ with the femur being statistically significant. Overall, the long bones between modern dogs and wolves do not significantly differ. These results align with geometric theory and has been used to explain limb proportions in many groups of animals, except Bovidae (Casinos et al. 1986). Geometric growth is exponential where a feature is multiplied by a constant. Arithmetic growth is at specific intervals where a feature is added by a constant. For example, the large claw of a lobster would be too large for a young lobster to carry. After the body size increases, the claw grows at an exponential rate (Kardong 2012).

Other studies have examined Rensch's rule in relation to dog domestication. It is an allometric rule involving sexual dimorphism and states that size is related to sexual dimorphism. The larger the animal, the larger difference between males and females. For instance, female hippopotamuses are less than half the size of males, but there is little difference between male and female mice. Frynta (2012) found modern dog breeds followed Rensch's rule despite rapid size variation from wolf divergence and breed formation. The domestication process did not seem to have a large effect on breeds. The wolf is the largest Canidae species and displays the largest sexual dimorphism between male and female body masses at a 1.28 ratio. Dogs of similar size exhibit similar ratios, such as the Beauceron at 1.25, giant schnauzer at 1.22, and the Leonberger at 1.21 (Frynta et al. 2012).

Sutter et al. (2008) also examined Rensch's rule in relation to modern breeds and found dogs do not follow the pattern. Small and large breed dogs mainly exhibit the same sexual dimorphic ratio, which means Rensch's rule does not hold because smaller breeds should display less sexual dimorphism. The only exception to the rule may be Portuguese water dogs because American Kennel Club (AKC) standards allow almost a three-fold difference in body mass between dogs, unlike other breeds which only permit 10% variation (Ostrander & Bustamante 2012; Sutter et al. 2008). The studied measured 27 body parts from 1,155 purebred dogs and the results are robust enough to classify an individual dog into the correct breed category with high accuracy. Discriminant function analysis can use nineteen of these measurement and result in 72.4% accuracy of correctly classifying a dog. This may help to lessen the measurements needed for morphology-based genetic studies, especially in breed studies that show to be under high genetic control (Sutter et al. 2008).

Genetic studies have started to identify various growth factors within dogs. Sutter et al. (2007) found that insulin-like growth factor 1 (IGF-1) is common in all small breeds and almost completely absent from large and giant breeds. Insulin-like growth factor 1 haplotypes groups were found to vary by size. Ninety-six percent of the Portuguese water dogs examined in this study only had two haplotypes, B and I. Dogs homozygous for type B were smaller than group I. Overall, 15% of skeletal size variation in Portuguese water dogs is explained by the IGF-1 haplotype. This gene is a size regulator in most mammals. Genetically IGF-1 deficient mice are 60% the normal birth weight and humans with homozygous partial deletion are, on average, 3.9 standard deviations below normal length (Sutter et al. 2007). Other genes that may influence dog growth are SMAD-2, NPR2, HMGA2, SOCS2, and IGF2Bp2 (Jones et al. 2008). HMGA2 is known to effect body size in both humans and mice (Ostrander & Bustamante 2012).

1.5.3.3 Teeth

The last feature used to discriminate dogs from wolves is tooth crowding. However, this feature is only reliable in early finds because of allometric principles. It was originally questioned whether teeth decreased, or the skull increased, suggesting size-related or size-required change. It was found the muzzle became shorter and wider and resulted in crowded teeth; body size changed before teeth (Morey 1992). The increased sexual maturity of domesticated animals also produces faster bone closer. The basicranium closes faster in dogs, which also contributes to crowded teeth (Elia 2013). Teeth decreased at a slower rate than the skull and resulted with smaller dogs having relatively large teeth and large dogs having relatively small teeth. All dog breeds have smaller teeth than wolves, even giant breeds like Great Danes (Clutton-Brock 2016). Modern dog teeth will not reach the full size of an adult wolf due to artificial selection. For example, a Saint Bernard will most likely never develop a wolf-like jaw despite its size (Morey 1992).

Both premolars (P3 and P4) in domesticate dogs have negative allometry in relation to skull length, while wild canids show positive allometry (Morey 1992). Individual teeth and entire dentitions have been used in archaeological sites as evidence for potential domestication, especially since premolars are usually stable in lineages (Napierala & Uerpmann 2012; Ovodov et al. 2011; Wang & Tedford 2008). Canine teeth are slightly larger, by 3% to 6%, in males than females in all canids (Wang & Tedford 2008). In general, dogs and wolves both have the same dental formula, 3:1:4:2/3:1:4:3, resulting in 42 adult teeth (Evans & de Lahunta 2013; Lazar et al. 2009). However, modern breeds may vary in the number of teeth, such as Xoloitzcuintlis (Mexican hairless dogs) which are permitted to have missing teeth per AKC standards (American Kennel Club 2009).

Teeth may be used as an identifying feature in canid remains and even in modern dog breeds, but alone they are not as reliable as skull measurements. Skull measurements are the main source of canid identification, but differentiation between canid remains are still not discrete or certain with these measuring methods. Since evolution is a slow and gradual process it is difficult to differentiate between canids. *Canis* inhabits every continent besides Antarctica, making extant and extinct similar-sized canid species an issue when examining remains. Foxes, maned wolves, dholes, jackals, and village dogs are some canids that may erroneously be identified and dated as domesticated dog remains (Larson et al. 2012). A 1977 study examining wolf populations in four national parks in central Canada (Jasper, Wood Buffalo, Prince Albert, and Riding Mountain) found feature measurements could classify *Canis* at the genus level, but could not differentiate at the species level (Skeel & Carbyn 1977). Canid morphological distinctions still necessitate better identification methods.

1.5.3.4 Physiology

Physiological effects due to diet, behavior, and hormones changes are also part of the domestication process but are less discernible. Domesticated dogs appearing before the advent of agriculture may have prompted a dietary shift over time and produced physiological changes that are still reflected in modern breeds. Additionally, other physiological changes, such as hormones, were modified by behavior selection. The wide range of modern breeds exhibit markedly different behaviors, diseases, and morphological variations. The anatomical and physiological changes during the domestication process and within artificially selected dog breeds provide excellent models for human medical studies. Almost every trait combination has been created in dog breeds and make for exceptional models for human studies (Young & Bannasch 2006).

The appearance of domesticated dogs before agriculture influenced ancestral and modern dog diets. Studies have examined the amount of amylase in various dog breeds and other canids. Dogs only express amylase in their pancreas, unlike humans that express it in the pancreas and saliva. Starch breakdown occurs in three stages and selection played on all three stages for more efficient digestion. In addition, three genes have been identified that effect starch digestion, AMY2B, SGLT1, and MGAM. AMY2B produces enzymes to break down starch and is found to have a 7-fold increase in dogs compared to wolves and increased amylase activity by 28-fold. SGLT1 also aids in starch breakdown and was found to also increase in dogs. MGAM was noted to have an amino acid substitution, isoleucine appears in wolves and valine in dogs. This may suggest different diets since isoleucine is associated with omnivorous rats and insectivores like hedgehogs and short-tailed opossums (Axelsson et al. 2013).

AMY2B varies not only between dog and wolves, but also in modern dog breeds. The variation is significant between breeds with at least 50% copy number variability based on breed origin. The bottleneck dogs went through during breed formation may have influenced this variation (Arendt et al. 2014). It is also suggested breed formation and origin specifically influenced the copy number. For example, Siberian huskies only average three to four copies and salukis average 29 copies. Huskies were bred by Artic nomadic hunter-gatherers, while salukis were raised in the Fertile Crescent, where agriculture originated (Freedman et al. 2014). This parallels human populations with starch-rich diets. In general humans have a 3-fold increase in AMY1 (salivary amylase) from chimpanzees with copy numbering varying based on diet origin. Populations with higher starch dependence have relatively higher AMY1 copies, such as American Europeans and Japanese groups (Arendt et al. 2014; Pääbo 2014; Richerson, Boyd, & Henrich 2010). Fermented alcohol may have also led to an increased AMY1 selection in humans (Peng et al. 2010).

A few years ago (June 2019) the United States Food and Drug Administration (FDA) released a report stating the connection between grain-free dog foods and dilated cardiomyopathy (DCM) cases in dogs. There has been an increase in DCM cases, especially in dogs not conventionally predisposed to the condition, which are normally large and giant breed dogs, like Great Danes. This disease can lead to death but can also be reversed if treated in time. It has been suggested the increased trend in grain-free foods has significant negative health impacts on dogs. The first 10 listed ingredients in foods labeled as grain-free were found to contain high amounts of peas, lentils, pulses, and/or potatoes (FDA 2009). Some dogs had the condition reversed when switched back to grain-based food with taurine supplements (deficiency may also lead to DCM) and a normal protein source (e.g., chicken or beef) (Kaplan et al. 2018). Research is still ongoing, but it suggests domesticated dogs require grains, particularly since they produce the correct dietary enzymes.

Organs that regulate behavior may have also been under selection in domesticated dogs. The hypothalamic–pituitary–adrenal (HPA) axis has been proposed as a domestication regulator because of its stress response control (Kukekova et al. 2008; Trut et al. 2009; Wilkins, Wrangham, & Tecumseh Fitch 2014; Da SilvaVasconcellos et al. 2016; Trut, Oskina, & Kharlamova 2012; Fischer & Lilje 2011). The HPA is the core of endocrinology and works as a negative feedback loop meaning hormones are released from an endocrine gland in response to other hormones released by a different endocrine gland. Hormones from the hypothalamus control hormones from the pituitary gland, which then regulates other endocrine organs (thyroid, adrenal cortex, and gonads). As the targeted tissue blood hormone level rises, the pituitary gland is inhibited and creates a feedback loop back to the other organs. The nervous system stimulates this system to maintain homeostasis (Marieb & Hoehn 2013).

The increased docility in domesticated animals is thought to be regulated by the HPA axis. Selecting for tameness and docility resulted in observed morphological and physiological changes. For instance, the adrenal gland has decreased in size in domesticated animals (Wilkins, Wrangham, & Tecumseh Fitch 2014). The HPA has also been observed to decrease in function at all levels with continued generations in the fox farm experiment. Measurements started at generation 10 and all tame foxes displayed lower levels of plasma glucocorticoids (stress response hormones) during every season when compared to other foxes. Basal cortisol levels were reduced almost 2-fold and stress-induced levels were about 30% lower in generation 20. Generation 45 exhibited a

3-fold decrease in basal levels and a 5-fold in stress-induced levels (Trut et al. 2009). Significantly higher serotonin and tryptophan hydroxylase levels were higher in the hypothalamus and midbrain of the tamed foxes (Kukekova et al. 2008).

The thyroid, another endocrine organ, has been suggested to be an important feature highly impacted by domestication (Crockford 2006; Dobney & Larson 2006; Morey & Jeger 2015). The thyroid gland is the largest, pure endocrine organ and produces the major metabolic hormone, thyroid hormone (TH). Thyroid hormone is composed of two iodine bonding hormones, T4 (thyroxine) and T3 (triiodothyronine). T4 is mostly secreted by the thyroid and T3 is usually in tissues and converted from T4. Thyroid hormone affects nearly every cell in the body and effects metabolism, body heat, blood pressure; and tissue growth and development (Marieb & Hoehn 2013). It also influences behavior by regulating adrenal stress hormones and may influence social and dominate behavior since thyroid hormones can rise and fall quickly (Crockford 2006).

Hypothyroidism affects hair/fur, stature, diet, brain and metabolic functions, and many areas of the body. Many of these changes are also associated with domestication, such as shorter stature, fur color, and reduced stress response. Studies have shown lab rats have smaller thyroids than wild rats. This reduction is linked to reduced quick escape response and distance. Lab rat adult sizes and in utero are smaller and display floppy ears instead of cropped ears like their wild counterparts. Since the thyroid is a crucial part for normal functioning, it is predicted thyroid hormone production change is not the result of functional change in hormones genes, but from gene expression. This would allow the hormones to function normally but vary the concentrations across the body and still effect behavior and morphology. Gene expression compared in dogs, wolves, and coyotes have been shown to vary in the hypothalamus suggesting domestication effects gene regulations (Dobney & Larson 2006).

The increased selection for tameness in the farm fox experiment and other domestication studies demonstrate how a simple change can have large physiological, morphological, and behavioral changes within a population. The artificial selection of modern dog breeds reflects the co-evolutionary bond between humans and dogs. The symbiotic relationship benefits both humans and dogs. Humans created different breeds to serve various purposes and dogs benefited from human care (Coppinger & Coppinger 1998). There are over 400 breeds worldwide, aiding in mammalian development studies, including humans (Boyko 2011; Boyko et al. 2009; Schoenebeck & Ostrander 2013; Shannon et al. 2015; VonHoldt et al. 2013; Giger, Sargan, & McNiel 2006).

1.6 Research questions and hypotheses

The main goal of this dissertation is to identify the biological variables that most predispose individuals to anterior cruciate ligament (ACL) rupture by using dog cranial cruciate ligament data (CCL) as an anatomical model. Participating in the same sport and performing the same motions does not consistently result in rupture across all individuals or dogs. Studies show rupture is only caused by direct contact 20-30% of the time in both species, thus suggesting the remaining non-contact rupture cases have an underlying predisposition (Boden et al. 2000; Griffin et al. 2000; Griffon 2010; Moore & Read 1996). This can be broken down into three research questions and are examined throughout these following three papers.

1.6.1 First question and hypothesis: Overarching question

Is there a singular cause (e.g., landing stance) for why some individuals, especially adult women, rupture their ACL at a greater rate than others?

Hypothesis: It is hypothesized that the cause is multifactorial, with a large emphasis on hormonal levels. Since the discrepancy between men and women does not appear until puberty is reached, it suggests hormones play an important part in rupture rate. It is hypothesized that the hormone levels influence the tibial plateau angle or the femoral Q-angle, placing excessive strain on the ACL and when positioned incorrectly during cutting or pivoting movement, it ruptures.

Null hypothesis: There is no association between variables and no significant difference between males and females. This is measured using dog models as a proxy for human hormones and the data presents no significant relation between variables, indicating that one variable is the cause of rupture or produces most cases. This suggests males and females have equal rupture susceptibility rates at the biological level and that external forces (i.e., landing stance) has a greater influence on rupture patterns.

1.6.2 Second question and hypothesis: Animal models

Using dogs as an anatomical model for human ACL rupture, which variables are associated with CCL rupture in dogs?

Hypothesis: Previous studies have shown there is a difference in rupture rate between intact and gonadectomized dogs. This suggests reproductive hormones are essential in rupture patterns. Large dogs also rupture their CCL at a greater rate than small dogs. It is hypothesized the increased long bone lengths from early gonadectomy overextends the CCL and eventually causes biomechanical failure during an abrupt movement.

Null hypothesis: There is no significant association between variables, especially regarding hormones. Sexually altered dogs do not have significantly higher rupture rates than their intact counterparts. All dog sizes have no significant rupture disparities between them regardless of the time they were gonadectomized. This suggests gonadectomies at any age for any size breed does not significantly influence the chance of CCL rupture.

1.6.3 Third question and hypothesis: Genetics

Is there a genetic predisposition to rupture as observed through greater breed prevalence in rupture data, or are other biological factors more prevalent?

Hypothesis: The high rupture rate observed in specific dog breeds suggests a potential genetic predisposition to CCL rupture. This indicates there is a genetic predisposition for CCL rupture. It is hypothesized that there is not strong enough evidence to claim full genetic predisposition of rupture and that other forces (i.e., early gonadectomy) is a larger influence on CCL rupture susceptibility.

Null Hypothesis: There is a greater portion of specific breeds with rupture within a multi-breed sample or single-breed studies show larger than average rupture rates than other breeds, suggesting there is a genetic component to CCL injury. This indicates other biological forces, such as early gonadectomy, does not critically influence the development and rupture of the CCL. Previously cited variables (i.e., tibial plateau angle) are then contingent upon the genetic predisposition of the dog to produce rupture.

1.7 Broader impacts

This section outlines major broader impacts of this research and its importance outside of academia. Since anterior cruciate ligament (ACL) rupture is a common injury within the population, it can have profound impacts on society and the individual. It is also important for research to be beneficial for the public in the goal to expand human knowledge, innovation, and future endeavors. The broader impacts discussed here in relation to ACL rupture is broken down into four discrete sections – (1) the high costs associated with treatment in humans, (2) treatment cost in dogs, (3) the specific impact it has the United States military, and (4) the potential for secondary disease and injury.

1.7.1 Human cost

It is well known the United States spends more on healthcare than any other country (Papanicolas, Woskie, & Jha 2018). It was predicted that in 2020 the United States healthcare system would surpass the entire German national economy, making it the fourth largest economy in the world (Emanuel 2016). The National Center for Health Statistics, part of the Center for Disease Control and Prevention (CDC), calculated the total 2017 national health expenditure at $3.5 trillion, comprising 17.9% of the gross domestic product (GDP) (CDC/National Center for Health Statistics 2020). It is predicted the GDP percentage will increase to 20.1% by 2025 (Branning & Vater 2016). Orthopedic problems are one of the largest contributors of this total. In 2010, musculoskeletal problems were the third costliest medical expense ($170 billion) and totaled 18,623,616 physician visits for symptoms and complaints (Altman 2015; Department of Research & Scientific Affairs 2013). This number has continued to rise over the past decade.

A 2020 study examined the breakdown of disease costs and payment type (private insurance, public insurance, and out-of-pocket) between 1996-2016 (Dieleman et al. 2020). It examined the total cost associated with 14 aggregated health categories and 154 conditions, which were broken down by age group, sex, and type of care (e.g., ambulatory care, inpatient care, nursing home facility). Anterior cruciate ligament rupture can be categorized as musculoskeletal or injury since it is orthopedically known as a musculoskeletal injury (to note, The World Health Organization [WHO] groups musculoskeletal injuries under musculoskeletal conditions) (World Helath Organization 2021b). The discrepancy regarding ACL categorization is mostly likely based on insurer payment type (e.g., an ACL injury covered by school insurance). When comparing the 2016 aggregated health categories, musculoskeletal disorders had the highest cost at $380.9 billion (95% CI, $360.0 – $405.4), surpassing the second-place category of diabetes, urogenital, blood, and endocrine disorders by $71.8 billion. The $71.8 billion difference is the second largest gap between disorders after the last two ranking categories of treatment of risk factors ($117.0, 95% CI, $109.3 – $125.7) and cirrhosis ($32.5, 95% CI, 27.0 – 40.4) with a $85 billion difference. Injuries ranked sixth at $231.1 billion (95% CI, $211.7 – $250.7) (Dieleman et al. 2020).

Dieleman et al. (2020) broke down each disease category into three age ranges (based in years) – less than 20, 20-64, and 65 and over. Musculoskeletal disorders were most common in the 20-64-year age range, comprising 61.3%. Three musculoskeletal conditions were ranked in the top 10 of the top 100 most expensive conditions of the 154 conditions analyzed – (1) low back and neck pain, (2) other musculoskeletal disorders, and (3) osteoarthritis. Low back and neck pain was ranked the most expensive health condition at $134.5 billion (95% CI, $122.4 – $146.9) followed by other musculoskeletal disorders (joints, muscular, and connective tissue, including myalgia and osteoporosis) at $129.8 billion (95% CI, $116.3 – $149.7). Osteoarthritis ranked eight at \$80.0 billion (95% CI, \$72.2 – \$86.1), which is a secondary disease to ACL injury (Lohmander et al. 2007). Additional musculoskeletal disorders in the top 100 included rheumatoid arthritis (#26) and gout (#92) (Dieleman et al. 2020).

According to Dieleman et al. (2020), the aggregated injuries category was the sixth costliest classification at \$231.1 billion (95% CI, \$211.7 – \$250.7) with the 20-64 age group also comprising the majority at 57.8%. When categorized into the top 10 most expensive conditions, falls ranked fifth at \$87.4 (95% CI, \$75 – \$100.1). Other injuries in the top 100 included road injuries (#16), other intentional injuries (#31), injuries due to mechanical forces (falling objects, being struck by an object, cutes, or being crushed) (#32), interpersonal violence (#59), poisoning (#81), animal contact (#91), foreign body (#96), and self-harm (#97). Both musculoskeletal and injury categories had equally rounded numbers in each payer type category – public insurance (37%), private insurance (54%), and out-of-pocket payments (9%) (Dieleman et al. 2020). Musculoskeletal diseases and injuries are a large portion of the healthcare market and more knowledge about conditions, like ACL rupture, is necessary to reduce their large national costs.

The International Classification and Diseases (ICD) is the system published by the WHO for purposes of detecting health trends and statistics worldwide, as well as being the clinical and research classification standard (World Health Organization 2021). The US had been using ICD-9-CM (clinical modifications) since 1979; however, it is no longer considered accurate for reasons including not being clinically accurate, limited data of patient medical conditions and procedures, inadequate number of codes, and a restrictive coding structure (Center for Disease Control and Prevention 2015). The US transitioned to the ICD-10 in October 2015 and the ICD-11 is set to release in January 2022 (Center for Disease Control and Prevention 2015, World Health Organization 2021).

The ICD-9-CM had 3,824 procedure codes and 14,025 diagnosis codes, while the ICD-10 has 71,924 procedure codes and 69,825 codes – almost 19 times more procedure codes and five times more diagnosis codes (Center for Disease Control and Prevention 2015). This change is necessary to note because the US was the only industrialized nation still using ICD-9 while other nations were using ICD-10 and did not allow for international comparisons. The ICD-10 also modifies how conditions are grouped, thus the reporting system of some conditions across analyses has changed and there are systems in place to translate from ICD-9 to ICD-10. The change in the coding system allows for improved healthcare utilization analyses and patient care (Center for Disease Control and Prevention 2015). Nonetheless, the numbers reported here are based on ICD-9 codes.

The Burden of Musculoskeletal Disease in the United States: Prevalence, Societal and Economic Costs (BMUS), addresses musculoskeletal issues, advocates for more research and education, and is in its 4th edition. It is produced by United States Bone and Joint Initiative (USBJI), which is the U.S. national action network of the Global Alliance for Musculoskeletal Health (G-MUSC), an international partnership sanctioned by the United Nations/World Health Organization (BMUS 2020). Burden of Musculoskeletal Disease analyzed total cost by medical codes and found the total aggregate direct costs for musculoskeletal injury was $214 billion in 2012-2014, a 117% rise from 1996-1998 (BMUS 2020). These number have continued to rise over the years and illustrates a substantial economic burden to the US healthcare system and the individual payer.

Obtaining current overall national cost of ACL surgery is limited due to the US not having a national ACL database like other countries (e.g., Sweden, Denmark, and Norway), making most epidemiological studies rely on private insurance databases (Mall et al. 2014; Renstrom et al.

2008). As such, it is estimated that the cost of ACL reconstructive surgery is nearly $13,000 with a lifetime societal cost (e.g., disability, decreased productivity) of more than $38,000 per person (Mather et al. 2013; Pfeifer et al. 2018). Total national surgery cost was cited in 1999 as costing over $2 billion dollars and has only continued to increase (Gottlob et al. 1999).

The high costs have incurred debate on the most cost-effective means to address the injury. Various treatment approaches, including conservative and surgical reconstruction, have been analyzed for cost-effectiveness. Nearly 90% of overall ACL injuries eventually undergo surgery with reconstructive surgery being the most common with more than 100,00 performed per year (Afzali et al. 2018; Beynnon et al. 2005; Buller et al. 2015; Farshad et al. 2011; Lubowitz & Appleby 2011). These costs also do not include concurrent injuries, such as meniscal or other ligament tears, that are common with ACL rupture (Herzog et al. 2017). Anterior cruciate ligament rupture rarely occurs in isolation, increasing treatment costs and overall outcome (Beynnon et al. 2005).

Despite surgery having the high rehabilitation rate, patients are still at risk for reinjury, contralateral injury, chronic knee instability, and menisci and chondral surface damage (Daniel et al. 1994; Finsterbush et al. 1990; Stanley et al. 2011; Webster & Feller 2016). Wiggins et al. (2016) found the reinjury chance in all previously injured ACL age groups is 15%, and those younger than 25-year-old had a 21% chance. Young athletes that return to sport after injury are 30 to 40 times more likely to reinjure it when compared to uninjured athletes. It was also calculated that contralateral injury increased by 8% in all age groups. Overall, it was found that athletes 25 years and younger who previously ruptured their ACL and returned to sport had a 23% chance of a secondary ACL injury (Wiggins et al. 2016). The potential for reinjury creates additional treatment costs, in addition to prospective lifetime costs for secondary diseases, like osteoarthritis.

These costs do not account for rehab that lasts an average of 6 months, missed work or sports activities, scholarship loses, or impaired mobility problems (Herzog et al. 2017; Hewett et al. 2010). Total healthcare spending in the United States in 1999 was $1.4 trillion, 13% of the GDP, and increased to $3.1 trillion, 18% of the GDP, in 2016, and it has since continued to grow (Dieleman et al. 2020). The average treatment cost per person has almost doubled in that same time frame, where it was $5,259 in 1999 to $9,655 in 2016 (Dieleman et al. 2020). These prices are predicted to continue increasing with the expected growth in the overall national healthcare expenditure. Musculoskeletal conditions comprise a large portion of this total. The high costs, immobility, increased surgery risk, and pain emphasize the need for more research into musculoskeletal conditions, yet musculoskeletal research only constitute less than 2% of the National Institute of Health (NIH) budget (Herzog et al. 2017). Anterior cruciate ligament rupture is one of the most common forms of musculoskeletal injury and increased research on its predisposition can help reduce incidence rates and associated costs.

1.7.2 Canine cost

Pet ownership is cited as having several benefits for both physical and mental health. A brief list of benefits includes increasing mental health (especially in the elderly), stress relief, providing a greater sense of purpose (shown to reduce frailty, disability, and strokes), improving physical activity, establishing a structured lifestyle, offering feelings of protection, building social networks, and reducing depression (Enders-Slegers & Hediger 2019; Hui et al. 2020). Studies have also shown clinical improvement in cardiovascular disease, coronary artery disease, quality of life for breast cancer patients, human gut microbial diversity, and childhood asthma and allergens (Binotto et al. 2017; Collin et al. 2015; Kates et al. 2020; Schreiner 2016; Xie et al. 2017).

There are countless other benefits of pet ownership, such as the positive impact of service dogs, but goes beyond the scoop of this research.

It is estimated between 57-67% of United States households own at least one pet with dogs being the most common at an estimated 38-48 million owners (percentage discrepancy based on survey methodology) (Applebaum, Peek, & Zsembik 2020). A main reason for people who do not engage in pet ownership is due to the cost associated with taking care of the pet (American Humane Association 2013). A study found people who previously owned a dog but did not get another one cite veterinary cost (30%) and general cost (29%) as the main reasons for not owning another dog (American Humane Association 2013). People who have never owned a pet have three main reasons for not owning a dog – (1) lifestyle (30%), (2) cleaning up after them (30%), and (3) general maintenance expenses (29%) (American Humane Association 2013). The overall lifetime cost of owning a dog is an estimated $22,000 over the average 12-year lifespan; comparatively, lifetime cost of a cat is $17,000 for their higher average 15-year lifespan (Greenfield 2011).

The American Pet Products Association (APPA) has released information on the collective costs of pet ownership, practices, and products since 1988. Their reports show the total US pet industry expenditures have continued to rise over the years. The 2018 US market actual sales total for pets was $90.5 billion (American Pet Products Association [APPA] 2020). This grew to $95.7 billion in 2019 and an estimated $99.0 billion for 2020 (APPA 2020). The data is broken down into four main categories – (1) pet food and treats, (2) supplies, live animals, over the counter medicine, (3) vet care and product sales, and (4) other services (boarding, grooming, insurance, training, pet sitting and walking, and all other services outside veterinary care) (APPA 2020). Pet food and treats is the costliest at $36.9 billion (2019) (estimated 2020 total of $38.4 billion). The second most expensive category is vet care and product sales at $29.3 billion (2019) (estimated

2020 total of $30.2 billion) (APPA 2020). This category includes all routine and surgical care, including cranial cruciate ligament (CCL) surgery.

Veterinary surgical costs for CCL rupture costs an average of $5,000, but varies substantially by location (Trupanion 2019). There are some non-surgical options, but studies have shown surgery results in a better prognosis, especially for large dogs (Christopher et al. 2013; Kim et al. 2010). Pet insurance has become more popular over the years with annual premiums reaching $1 billion in 2017 (McGinty 2018). These insurance companies usually have caveats regarding CCL surgery and treatment. All pet insurance companies do not cover pre-existing conditions. Therefore, if a pet ruptures their CCL prior to enrollment, there is no chance of coverage by a pet insurance company. Even if enrolled, most pet insurance companies avoid covering it through extensive waiting periods or bilateral condition clauses. For example, Nationwide Pet Insurance will not cover "cruciate ligament or meniscal damage or rupture" during the first year of the policy (Nationwide 2021). Pets Best has a six-month waiting policy for "any cruciate ligament issues and any related conditions" (Pets Best 2020). ASPCA has an allowance for accepting pre-existing conditions except for "knee and ligament conditions" (ASPCA Pet Health Insurance 2020). Healthy Paws will not cover future treatment for "a leg if cruciate ligament problems to any other leg" exist prior to the policy effective date (Healthy Paws 2020). Similarly, Trupanion, which has been touted as the best pet insurance company, does not cover bilateral conditions "such as luxating patella or anterior cruciate ligament (ACL) weakness" (Trupanion 2020). The exclusion of CCL treatment in pet insurance policies emphasizes the high cost of treatment.

The average American family cannot cover a multiple-thousand-dollar expense for their pet. The Federal Reserve found that nearly 40% of families could not cover a hypothetical, unexpected $400 expense, such as a broken appliance or car repair (Federal Reserve 2019). The high cost of CCL rupture can place a financial burden on families and internet discussion boards debate euthanizing a dog due to CCL surgery cost despite a very high surgery and rehabilitation success rate. Many news outlets have covered people incurring debt for their pet, especially among the millennial generation. Nearly 36% of pet-owners have been in debt for a pet and is highest for the millennial generation (42%) despite the generation barring the biggest burden of the $1.6 trillion national student debt (Delfino 2019; Friedman 2020). Despite the high cost and daily demands of owning a dog, many believe the cost is well worth the price because as mentioned previously, dogs provide several mental and physical benefits to humans and regarding them as a family member has also shown to improve health and wellbeing (McConnell, Paige Lloyd, & Humphrey 2019). Examining CCL rupture factors can alleviate some of the financial costs associated with dog ownership, increasing the pet ownership experience.

1.7.3 Military studies

The anterior cruciate ligament (ACL) rupture rate is high in the civilian population, and is 10 times higher in the United States Armed Forces (Owens et al. 2007). The return to service/sport rate for service members is lower than for the general population, most likely due to the higher demands of the job as compared to sports. More than 50% of active duty members that receive ACL surgery are permanently restricted from activities or removed from active duty all together (Antosh et al. 2018). A study evaluating potential risk factors faced in military populations such as younger age, lower rank, smoking, concomitant meniscal or chondral injury, allograft use, and smaller graft did not produce significance in any one variable, further supporting the prediction that ACL rupture is multifactorial (Antosh et al. 2018).

The lower rank variable is of interest because it is a proxy for stress and cortisol levels, a variable that is difficult to study in civilian populations (Sherman et al. 2012). Lower rank may not be a variable for rupture but can impact return to duty rates. Higher ranking and seniority service members that do rupture their ACL (or multiple ligaments) are found to have a greater chance of returning to duty than low ranking members, which is also observed with other injuries (Islinger, Kuklo, & Polly Jr 1998; Kuklo et al. 1997; Richards & Dickens 2019; Ross et al. 2009). There are four proposed reasons for this – (1) senior rank has more office duty than physical duty, (2) having seniority may allow for modifying the environment and duty service, (3) the military has invested enough resources in their senior ranking officials, and it is in their best interest to allow them to return, and (4) senior ranks may endure it more because they are closer to retirement and their lifetime military pension (Richards & Dickens 2019; Ross et al. 2009).

In both the civilian and military populations, females are disproportionately affected by ACL injuries. Antosh et al. (2018) found only 34% of women return to full duty, without restrictions, after rupture. However, previous studies that were conducted before women were permitted in combat have conflicting evidence regarding the sex discrepancy in the military. One study found that women at the US Naval Academy were at an increased rupture risk, but another study examining rupture across all branches found no discrepancy (Gwinn et al. 2000; Owens et al. 2007). Another study found that women do rupture at a greater rate and are associated with more potential factors. Uhorchak et al. 2003 found female West Point cadets with a higher body-mass-index (BMI) and increased knee laxity were significantly at a greater risk. Narrow tibial notch width combined with higher BMI may also increase rupture risk (Evans et al. 2012; Uhorchak et al. 2003). The increased rate of women rupturing their ACL in the general population is impactful, but in the military population it sets women back even more, especially since women were not permitted to fight in combat until six years ago in 2015 and still face constant cases for withholding them from earning higher rank (Kamarck 2016). Arguments that women have increased musculoskeletal injuries rates have been used to sexually discriminate against women in the military and have re-opened the question for rescinding the 2015 women in combat policy (Nindl et al. 2016). This reinforces the need to increase research in this area.

The high risk of ACL rupture in the civilian population has many broad impacts and can be viewed as even greater in the military, particularly if it occurs on the front lines. Combat military members are thought to have less marketable and transferable skills into the civilian workforce than noncombat members (Antosh et al. 2018). Muscoskeletal problems can lead to increased drug abuse, especially with comorbid mental health diagnoses (Spelman et al. 2012). Studies of combat veterans have found a prevalence of mental health diagnosis at nearly 37%, in addition to 22% diagnosed with PTSD and 17% with depression (Spelman et al. 2012). Injury to the ACL is devasting for the average person but can be substantially worse for military members. There is a need provide more research in combat and training for musculoskeletal injuries, particularly the ACL.

1.7.4 Secondary disease and reinjury

Anterior cruciate ligament (ACL) injury comes with many complications, such as surgery, rehabilitation, and associated costs. Years after surgery there is still a high potential for secondary diseases and injuries, including osteoarthritis, reinjury, and contralateral injury. Osteoarthritis (OA) is a predominant concern with the annual cost of treatment in the United States at $80.0 billion (95% CI, $72.2 – $86.1), making it the eight costliest expense overall and the third costliest musculoskeletal condition in the US healthcare system (Dieleman et al. 2020). It is the most common form of arthritis (Barbour et al. 2017). The condition is clinically characterized by joint pain, tenderness, limited movement, crepitus, occasional effusion, and inflammation (Woolf & Pfleger 2003). It is a degenerative joint disease that is anatomically distinguished by articular cartilage loss within the joint, bone hypertrophy, eburnation, osteophytosis, and capsule thickening (Drake, Wayne Vogl, & Mitchell 2010; Woolf & Pfleger 2003). Osteoarthritis can occur in any joint, but it is most common in the hands, wrist, hip, and knee (Drake, Wayne Vogl, & Mitchell 2010).

Osteoarthritis is common in the older population, but it is not caused by aging; the etiology is uncertain (Drake, Wayne Vogl, & Mitchell 2010). It has been debated if OA is a single disease or many that result in a common pathway. Potential increased risk factors include hormonal status and bone density, nutrition, genetics, biochemical markers of cartilage, obesity, mechanical environment of the joint, articular cartilage loading, occupational factors, and sports participation (Felson 2000). Sports with repetitive impact, such as soccer, have increased early onset OA rates (Kujala et al. 1995). Athletes have a higher risk of early onset OA than the non-athletic population (Takeda et al. 2011). Sports injuries, particularly ACL injuries, increases early onset risk considerably regardless of treatment type (Friel & Chu 2013; Nebelung & Wuschech 2005; Von Porat, Roos, & Roos 2004). Injury to the meniscus in association with ACL injury is cited as the most important factor in OA risk (Aït Si Selmi, Fithian, & Neyret 2006; Meunier, Odensten, & Good 2006).

When total US spending is broken down into three age categories (in years) – <20, 20-64, and ≥65 – there is a zero reported rate for the <20 age group and nearly an equal split between the other two groups with 49.9% in the 20-64 group and 49.9% in the ≥65 group (Dieleman et al. 2020). An ACL injury is thought to age the knee by 30 years (Friel & Chu 2013; Lohmander et al. 2004). There have been studies that estimate as low as 10% will develop OA and as high as 90% will result in OA after ACL injury (Cohen et al. 2007; Gillquist & Messner 1999; Lohmander et al. 2007; Øiestad et al. 2009). There is no precise percentage, but it is frequently cited, on average, 50% of previous ACL or meniscus injuries will result in an OA diagnoses 10-20 years after the initial injury and creates a young person with an old knee (Lohmander et al. 2007; Øiestad et al. 2009). This young population accounts for a large portion of the overall OA total.

As with ACL ruptures, females are at a greater risk for OA, but not until they reach 50 years old, and the sex disparity flips. Before 50, OA is most common in men and after 50, it becomes more pronounced in women (Felson 2000; Srikanth et al. 2005). However, some studies have shown that women are at higher risk in all age groups and have higher distress levels after their diagnosis (Ackerman et al. 2015; Ackerman et al. 2017; Barbour et al. 2017). Overall, three in five people with diagnosed arthritis are younger than 65 years old in the US (Barbour et al. 2017; Perruccio et al. 2017). Treatment options can be more difficult in younger patients because there is an effort to reduce the need for major surgery and the associated complications that come with it in this population (Feeley et al. 2010; Lützner et al. 2009).

Avoiding OA through reduced sports injuries is crucial for the individual and the healthcare system. It is a progressive disease where symptoms can be relieved for improved function, but it cannot be reversed or completely prevented (Woolf & Pfleger 2003). Osteoarthritis is the most common reason for total hip and total knee replacement surgery, which comes with its own risks and complications, including infection and failure (Drake, Wayne Vogl, & Mitchell 2010; Felson 2000). The reduced physical activity that results from arthritis and musculoskeletal disorder is the most prevalent risk factor for functional decline and increases risks for other serious health conditions, like heart disease (the number one cause of death worldwide), especially in the older population (Briggs et al. 2016; Perruccio et al. 2017; World Health Organization 2020). There is an increased chance of OA with reinjury to the ligament and contralateral injury (Grindem et al. 2016; Swärd et al. 2010).

There is an increased likelihood of reinjury to the same knee or contralateral injury after ACL reconstruction surgery. The chance for graft rupture in the previously injured knee and contralateral injury are nearly equal (Paterno et al. 2014). There is a 35% overall rate of reinjury, with the chance higher in younger individuals, especially within two years after initial surgery (Paterno et al. 2014; Webster & Feller 2016). Nearly one in four young individuals that return to sport will have a reinjury (Wiggins et al. 2016). Graft choice or sex does not seem to impact these rates (Dunn et al. 2004; Leys et al. 2012). The increased rate in younger individuals may be due to their higher return rate to more strenuous activities and pivoting sports (Webster & Feller 2016; Wiggins et al. 2016). A study found that 88% of patients under 20 years old returned to high activity levels as compared to older individuals where only 53% returned (Webster et al. 2014). Reinjury rates are over four times higher in a two-year period in ACL reconstruction patients that returned to level I sports, with meniscus injuries having the highest re-rupture rate, increasing OA risk (Grindem et al. 2016). Grindem et al. (2016) found all individuals that returned before five months had a reinjury and decreased to 19% after nine months, but time alone was not sufficient for determining reinjury rate. The initial ACL rupture increases lifelong risks of reinjury, contralateral injury, and OA. Identifying variables that predispose individuals to the initial rupture is critical for prolonged health in the population.

Athletic participation comes with many risks, primarily injury, such as ACL rupture. Sports-related injuries are the leading cause of injury in children (Bijur et al. 1995; Luke et al. 2011). There is potentially a higher number of risks than benefits in very young children (6-12 years) (Wiersma 2000). Over 212 million individuals worldwide participate in professional or recreational sports (Pfeifer et al. 2018). While participating in physical activities comes with inherent risks, it also has many benefits. There is a decrease of depression and anxiety symptoms in individuals who engage in regular physical activity (Paluska & Schwenk 2000). Sports participation is also well known to aid in physical activity and promoting a healthy lifestyle.

Childhood obesity has been declared an epidemic and a worldwide issue (Wang & Lim 2012). Obesity in children increases the risk of obesity in adulthood and future health issues, including diabetes, heart disease, and cancer (Herman et al. 2009; Kissebah, Freedman, & Peiris 1989). Children that are categorized as obese also face increased health problems at a young age, like gallbladder problems, hypertension, and diabetes (Wang & Lim 2012). A study found that decreasing sedentary lifestyles in children significantly decreased being overweight, body fat, and improved aerobic fitness (Epstein et al. 2000). Physical activity-friendly school environments are also cited as having a lower risk of obesity (Ip et al. 2017). Being physically activity is well known to be important for maintaining a healthy body and weight in both children and adults.

There is a risk to physical activity and ACL injuries can be a lifetime, costly issue, but being physically active and participating in team sports is well researched to show many physical, psychological, and social benefits in both professional and recreational sports. The reduced activity level after ACL injury can increase sedentary behavior and contribute to weight gain and more substantial health issues. Injury to the ACL has many drawbacks and not much is known on how to best prevent it. This research is aimed to better understand the biological variables that may predispose individuals to rupture with the intent to target them in future research focused on how to reduce their associated rupture risk. Physical activity is necessary for a healthy lifestyle and should not be restrained or terminated due to a knee injury.

1.8 Three paper format

This dissertation is composed of one previously published paper and two unpublished papers with the central theme to improve the understanding of cranial cruciate ligament (CCL) rupture in dogs and its translation to anterior cruciate ligament (ACL) injury in humans. All three papers use dogs an anatomical, comparative model for human health to identify factors that predispose certain dogs to CCL rupture and reconnect it to human ACL rupture disparities. The three-paper format is laid out in a logical order that will be beneficial for later publication and broke down the overall study into smaller, more detailed papers that focus on specific topics. Each paper becomes more detailed in its aim to identify potential variables for CCL rupture. The first paper was a preliminary study to assess previously published variables, the second expands upon this to include a broader range of detailed variables not previously published, and the third paper narrows in on the reproductive history of each dog and its relation to rupture. Discussed in the following paragraphs is a brief overview of each paper and its main goal.

The first paper is a previously published paper in a special edition of *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology* (1.634 impact factor, December 2021). It is a preliminary study using specific variables identified in the literature to assess their accuracy by comparing a CCL ruptured and non-CCL ruptured group. It uses random samples obtained from the Oklahoma State University Veterinary Medical Hospital. The variables evaluated are patellar luxation, size (weight), sex, gonadectomy status, and tibial plateau angle. Two other variables mentioned but not quantified are angle of inclination and femoral anteversion angle. Chi-square and t-tests were performed. The results found a greater proportion of large dogs in the CCL group and the most abundant breed was "mixed." The surgical group had more gonadectomized dogs than the control group. Sex was not statistically significant between the two groups. Tibial plateau angles were only reported for the CCL group and the values fell in line with the published literature on the angle degree and rupture susceptibility. This study supports multiple variables identified in the literature as CCL rupture factors.

The second paper expands on the previous paper by examining more broad variables and is prepared for publication in the *Journal of Anatomy* (2.013 impact factor, December 2021). The goal of this study is to identify broader categories associated with rupture by using a control and sample group with more defined parameters and a higher degree of variables than examined in paper one. It uses data obtained from the Morris Animal Foundation Golden Retriever Lifetime Study (GRLS), one of the largest longitudinal breed studies conducted. Unlike the first paper that uses a mixed breed sample, this data set only uses a single breed that is cited as having high rupture susceptibility. This isolates other potential variables as breed acts as a controlled factor. It compares a CCL ruptured group and non-CCL ruptured group paired on age, sex, enrollment date, and gonadectomy status to control for basic variables. They are then compared in several medical categories (e.g., cardiac, endocrine, and gastrointestinal disorders) through unadjusted analysis, analyses stratified by sex, and tests for moderation and calculated Mantel-Haenszel combined odds ratios that controlled for sex. Females had more significant and trending results between CCL rupture and other diseases than males. The reason could be due to estrogen levels and low vitamin D levels. Dogs obtain vitamin D strictly through their diet and it is essential in ligament health, in addition to overall health. Studies have shown many humans, including children, are low in vitamin D due to modern lifestyles and this may impact skeletal health and increase ACL rupture susceptibility.

Lastly, the third and final paper uses longitudinal data to assess the relationship of CCL rupture and reproductive variables overtime and is prepared for publication in *Developmental*

Dynamics (3.78 impact factor, December 2021) with co-author Dr. Alicia Grosso. This study takes a closer look at the reproductive variables of dogs with CCL rupture and compares them to dogs without rupture since hormone levels were identified as a prominent variable for CCL rupture susceptibility in the literature and in the first and second papers. The objective was to identify more specific reproductive variables associated with CCL rupture. This study uses the same GRLS dataset used in the second paper and unlike the second paper, the entire population is assessed, in addition to males and females being analyzed separately. Height (controlling for weight), weight (controlling for height), body condition score, gonadectomy age, and gonadectomy age range are used in each study category (total population, male, and female). Additional variables are measured in females including pregnancy status, being in heat the previous year, and being spayed while in heat. There are no additional variables for males. Mixed-effects linear regression and general linear regression are used in each category. Height, weight, gonadectomy age, and specific gonadectomy age ranges were associated with CCL rupture. Rupture is a multifactorial process and is showing to be highly impacted by early gonadectomy impacting ossification rates and subsequent limb and ligament development. Estrogen levels in dogs and humans influences muscle stability and may be the reason for the associated rupture disparities.

The last section of this dissertation is the conclusion and summarizes the major findings of each paper and how they are connected. It intertwines the three papers to provide a cohesive analysis of CCL rupture and its relation to ACL rupture. Future research directions are also briefly mentioned.

2.0 Paper 1: Canine cruciate ligament ruptures: Implications for financial costs and human health

Toth, SA & Siegel, MI. Canine cruciate ligament ruptures: Implications for financial costs and human health. Anat Rec. 2021; 304: 222–230. https://doiorg.pitt.idm.oclc.org/10.1002/ar.24460 Reproduced with Permission © 2020 American Association for Anatomy.

Note: “Mixed breed” in this sample population refers to non-purebred dogs as opposed to various sampled breeds.

2.1 Overview

The cranial cruciate ligament (CCL) in dogs is homologous to the anterior cruciate ligament (ACL) in humans. Factors that place an individual at-risk for noncontact ruptures are not clearly defined in humans or dogs. Cyclic variation in human females as well as early spay/neuter in canines has frequently implicated hormonal variation, however these factors do not fully explain the human dimorphic or canine breed rupture rates. The present study examined dogs as a proxy model for humans to better understand the covariance. A random clinical data sample from the Oklahoma State University Veterinary Hospital was obtained on (n = 29) CCL surgical cases and nonsurgical (n = 28) controls. A statistical test for association of spay/neuter with CCL rupture was significant (chi-square = 21.7, p < .01). Sex balance between the groups was not significantly

($p > .01$) different. Data on other variables related to morphometric variability such as the tibial plateau angle was not available on the nonsurgical sample and comparisons could only be made to values from the literature. Though there may have been sample bias, this preliminary study found that more large than small dogs were represented in the surgical sample. Our results also support the claim that spayed/neutered dogs are more likely to rupture their CCL than intact dogs. Given the high costs of surgical repair, both for canines and humans, we argue for multivariate studies that investigate the interaction of variables in a larger subject sample which can provide comparable data on all parameters.

Keywords: anterior cruciate ligament, cranial cruciate ligament, hormones, patellar luxation, tibial plateau angle

2.2 Introduction

Anterior cruciate ligament (ACL) injuries are not uncommon in professional or recreational sports and can be a career ending injury with only about 50% of people fully returning to their pre-injury, competitive level after surgery (Ardern et al. 2012, 2014; Kvist et al. 2005; Langford et al. 2009; Swart et al. 2014). It is well cited that women are more likely than men to rupture this knee stabilizing ligament (Arendt & Dick 1995; Haida et al. 2016; Hewett et al. 2006, 2010; Ruedl et al. 2011; van Diek et al. 2014). In the United States in 2010, musculoskeletal problems were the third costliest medical expense ($170 billion) behind circulatory conditions ($234 billion) and preventatives, colds, and other basic care ($207 billion) (Altman 2015). Wilke (2005) found an average of 250,000 people ruptured their ACL annually, costing nearly $2 billion in surgery costs.

Like humans, dogs are susceptible to rupturing this ligament. The cranial cruciate ligament (CCL), sometimes referred to as the dog's ACL, is a homologous structure that provides the same function for a dog that the ACL provides for humans. Certain breeds and sizes seem to rupture their CCL more than others. The reasons behind these rupture discrepancies are not well understood. Previous studies have examined potential factors for noncontact rupture in humans and dogs, but no single variable fully accounts for rupture and suggests it is multifactorial (Jerram & Walker 2003; Uhorchak et al. 2003). Therefore, there are presumably interrelated variables responsible for noncontact ruptures. Seemingly, the at-risk factors that predispose rupture are not well defined. As is the case for humans, CCL surgery is costly, totaling $1.32 billion annually (Wilke et al. 2005). The total expense of CCL surgery has increased greatly over the years. In 2005, the average cost of one CCL surgery ranged from $898.00 to $1,840.50 and now costs about $5,000 but varies substantially by geographic location (Trupanion 2019; Wilke et al. 2005). There are some nonsurgical options available for CCL repair, but data have shown that CCL rupture primarily affects large dogs and surgery results in a better prognosis (Christopher et al. 2013; Kim et al. 2010; see Table 2.1).

Table 2.1: Current issues and aspects of ACL and CCL injuries in humans and dogs

	Humans	Dogs	Sources
Increased post-rupture risks	Reinjury Contralateral injury Osteoarthritis	Reinjury Contralateral injury Osteoarthritis	Crovace et al. (2006); Goranov (2011); Paterno et al. (2014); Rayward et al. (2004); Swart et al. (2014)
Most effected	Females College aged	Large breeds Early spayed and neutered	Arendt and Dick (1995); Haida et al. (2016); Hart et al. (2014, 2016); Hewett et al. (2006, 2010); LaBelle et al. (2014); Ruedl et al. (2011); Torres de la Riva et al. (2013); van Diek et al. (2014)
Annual and surgery costs (2005)	$2 billion $3,679 – $17,000	$1.32 billion $898.00 – $1,840.50	Wilke et al. (2005)
Increased average surgery costs	$11,431.57	$5,000	Herzog et al. (2017); Trupanion (2019)

Note: The use of animal models may isolate the "at-risk" variables and help reduce the high incidence rates and costs, while mutually benefitting human and animal medicine in further understanding anatomical variation. Increased risks and costs associated with ACL and CCL rupture.

Findings from early spay and neuter studies suggests hormonal changes as a prominent factor in CCL rupture, which can be associated with women and their higher ACL rupture rates (Hart et al. 2014, 2016; Torres de la Riva et al. 2013a). The removal of reproductive organs during dog spaying/neutering influences hormone production and may correlate with reproductive aged women rupturing their ACL more than their male counterparts. The relationship between hormones and other variables may predispose dogs, and humans, to rupture and this preliminary study hypothesized that rupture is a multivariable process with interdependent variables.

This study is a first step towards multivariable studies to isolate potential dependent variables and will provide an understanding of variable interrelatedness regarding rupture incidence rates in dogs and humans. Here we assess variables relating to patellar luxation, tibial plateau angle, spay/neuter data, and other variables that may influence CCL rupture incidence. Some multivariate studies have been previously done in humans and a few in dogs, but to our knowledge we are the first to assess these specific variables in one study (Griffon et al. 2017; Ragetly et al. 2011; Smith et al. 2012a, 2012b).

2.3 Materials and methods

2.3.1 Sample composition

A random sample (n = 29) of canine CCL surgical cases was selected from the clinical records of the Oklahoma State University (OSU) Veterinary Medical Hospital and additionally, a random sample of nonsurgical records (n = 28). The random data were drawn from a large database where 29 arbitrary CCL ruptured cases were selected and compared against 28 arbitrary non-ruptured cases. Data were coded and recorded for breed, sex, spayed/neutered/intact, age, weight, and patellar luxation

2.3.2 Measurements and clinical variables

The veterinary hospital provided the osteometric measurements of clinically recorded variables and radiographic assessments (see Table 2.2 for abbreviations). Clinical variables include patellar luxation (PL), a condition where the patella is displaced from the trochlear groove, and early spay and neuter (ESN), which we characterized as reproductive altering before one-year of age.

Table 2.2: Abbreviations

ACL	Anterior cruciate ligament
CCL	Cranial cruciate ligament
PL	Patellar luxation
MPL	Medial patellar luxation
AOI	Angle of inclination
FAA	Femoral anteversion angle
ESN	Early spay/neuter
TPA	Tibial plateau angle

Osteometric data (Figure 2.1) such as the angle of inclination (AOI), the angle of the femoral head into the acetabulum in reference to the femur shaft, and the femoral anteversion angle (FAA), the angle of the femoral neck relative to the mediolateral angle of the condyles were available in veterinary based, and can be obtained from radiographic images. Radiographic images also measure the tibial plateau angle (TPA), the angular measurement between the tibial plateau and a perpendicular reference line. Dogs without CCL rupture average 22.5 (Wilke et al. 2002). Dogs with CCL rupture tend to have a TPA ranging from 23.5 to 28.5, but instances of little as 12 and high as 46 have been reported. Excessive tibial plateau angle (eTPA) is categorized as anything greater than 34 (Duerr et al. 2008) (Figure 2.2).

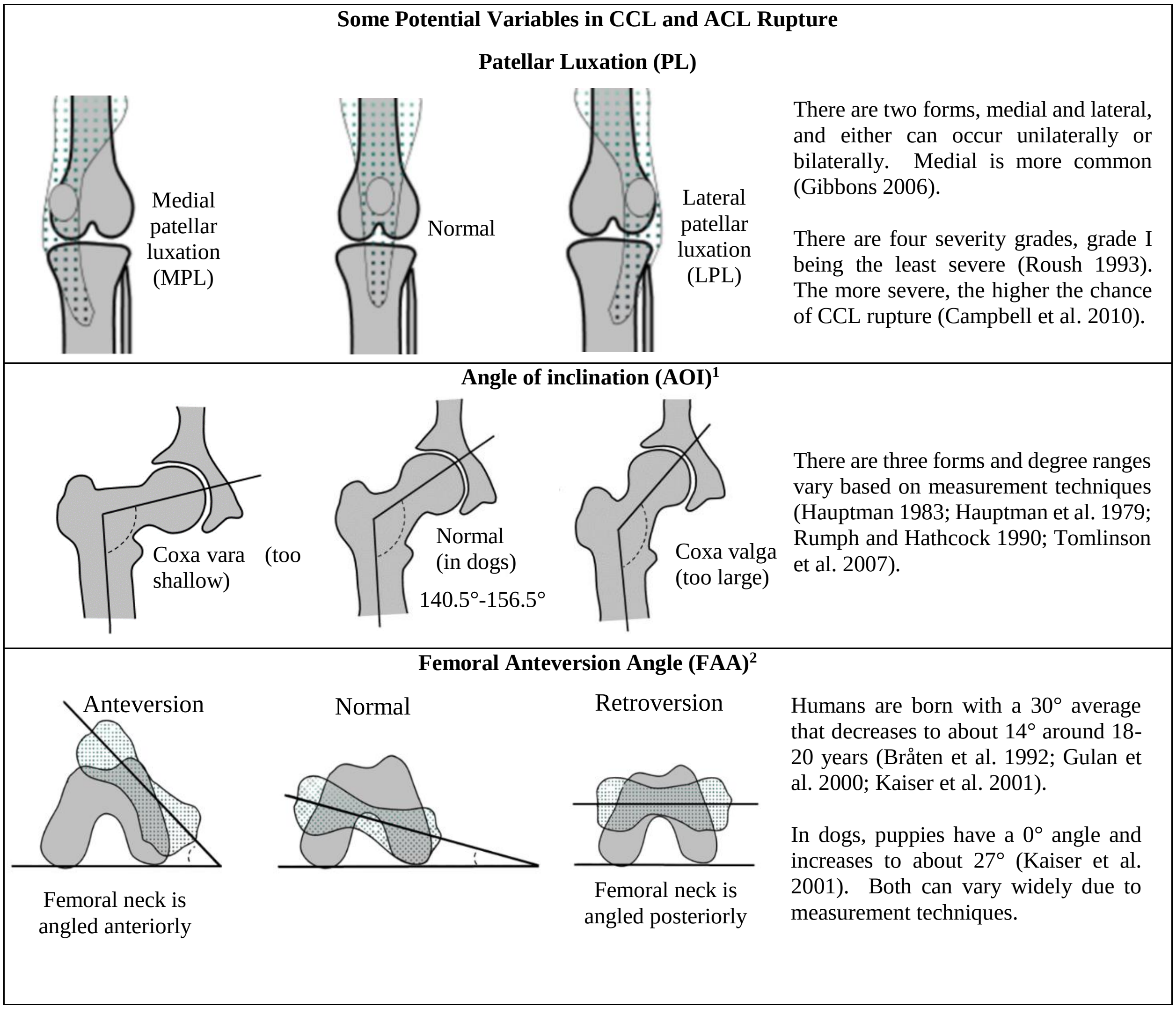

Figure 2-1: Three factors that may influence CCL rupture in dogs and subsequently ACL rupture in humans. Patellar luxation (PL) is a condition which can affect joint stability when the patella is displaced from the trochlear groove. Angle of inclination (AOI) is the angle of the femoral head into the acetabulum in reference to the femur shaft and the in dogs is 142.2° (Bound et al. 2009; Hauptman et al. 1979; Mortari et al. 2009). Femoral anteversion angle (FAA) is the angle of the femoral neck relative to the mediolateral angle of the condyles. [1] Image adapted from Neumann (2010) [2] Image adapted from Cibulka (2004). Description references: (Bråten, Terjesen, & Rossvoll 1992; Campbell et al. 2010; Gibbons 2006; Gulan et al. 2000; Hauptman 1983; Kaiser et al. 2001; Rumph & Hathcock 1990; Tomlinson, Cook, & Keller 2007)

Considering the previously mentioned implications the ideal study would require data on the AOI and FAA. As with the TPA, AOI and FAA angles are not generally available on unoperated animals and therefore can only been coded for surgical cases. This study obtained surgical case data on TPA only. These measures need to be assessed and “control” data obtained to determine if it has been an at-risk factor for the surgical subjects.

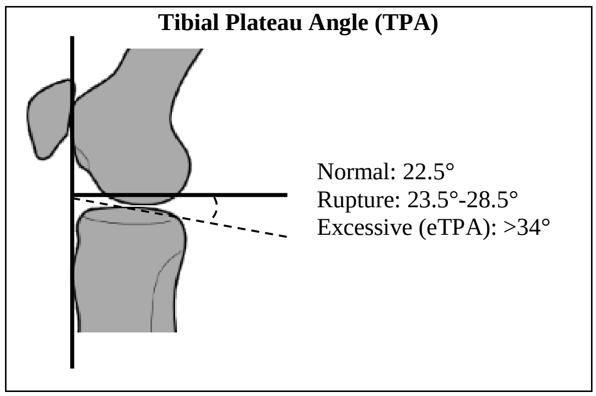

Figure 2-2: Tibial plateau angle (TPA) is the angular measurement between the tibial plateau and a perpendicular reference line. Tibial plateau leveling osteotomy (TPLO) is the most common CCL surgery and changes the TPA to produce a more stable joint (Bergh et al. 2014; Christopher et al. 2013; Shahar & Milgram 2006). Image adapted from Stanford MSK MRI Atlas (2019)

2.3.3 Statistical analysis

Means and standard variations were calculated for age and weight and were compared between groups by Student's *t* test. Nonparametric chi-square was used to test for the association of sex and spay/neuter. Significance was set at $p < .01$.

2.4 Results

Surgical group (results listed in Table 2.3): The chi-square statistic is 1.7079. The *p*-value is .191254. The result is not significant at $p < .01$ (male/female vs. altered/intact). Nonsurgical group: The chi-square statistic is 0.0546. The *p*-value is .815276. The result is not significant at $p < .01$. The surgical group had almost equal numbers of males and females (sex ratio (SR = males/females) = 0.81), while the nonsurgical group had twice as many females as males (SR = 0.47); the mean ages were 5.7 and 4.6 years, respectively. In the two groups there was only one PL and that was in the nonsurgical group. The mean weights were 33 kg for the surgical cases and 19.8 kg for the nonsurgical group. Among the surgical cases only 4 of 29 were intact while for the nonsurgical cases 21 of 28 were intact (chi-square = 21.7, $p < .01$).

Table 2.3: Age and weight results, means, and standard

	Surgical cases (n = 29)	Nonsurgical cases (n = 28)
Age mean	68.62 months (5.72 years)	57.18 months (4.77 years)
Age *SD*	29.82 months (2.49 years)	43.64 months (3.64 years)
Weight mean	33.02 kg	19.80 kg
Weight *SD*	10.43 kg	13.57 kg
		***t*-test**
Age mean		0.26
Weight mean		0.00016

Both the surgical and nonsurgical groups were coded for size based on American Kennel Club (AKC) standards. The three size categories were small, medium, and large. Data were then recoded based on the weights (kg) with large dogs categorized as weighing more than 32 kg, medium as 31.9–18 kg, and small below 18 kg. Weights ranged from 2 to 63 kg. In the surgical group, large was the most frequent category (n = 17), followed by medium (n = 9), and then by small (n = 3). In the nonsurgical group, medium was the most frequent category (n = 13), followed by small (n = 12), and then by large (n = 3). Throughout the entire study, the most abundant "breed" was "mixed" (n = 13). For the mixed breeds, the sizes ranked from highest to lowest in prevalence of CCL rupture in the following order: small (n = 5), medium (n = 4), and large (n = 3).

The results for the surgical group showed that large dogs had 16 spayed/neutered and one intact dog. Medium dogs had seven spayed/neutered and two intact dogs. Small dogs had two spayed/neutered and one intact dog. The nonsurgical group was nearly opposite with large dogs having two spayed/neutered and one intact, medium dogs with five spayed/neutered and eight intact, and small dogs with one spayed/neutered and 11 intact. Overall, spayed/neutered were more prevalent in the surgical group than intact dogs, while the nonsurgical had more intact than spayed/neutered dogs. This finding is consistent with previous research showing that spayed/ neutered dogs have higher incidence rates than intact dogs (Hart et al. 2014, 2016; Torres de la Riva et al. 2013).

In the surgical group there were 16 females and 13 males and in the nonsurgical group there were 19 females and 9 males. The difference in females and males between the surgical and nonsurgical groups was not statistically significant (chi-square = 0.97, $p > .01$). This supports previous research where no difference in sex was found (Duval et al. 1999; Hart et al. 2014).

The TPA was only coded for the surgical group and available for 25 of the 29 cases. The mean value was 25.9. This is consistent with previous research showing that dogs with CCL rupture tend have a TPA in the range of 23.5–28.5 (Duerr et al. 2008). However, it was not possible to calculate this value for the non-ruptured group and an overall conclusion comparing surgical to nonsurgical TPA cannot be made in this population.

2.5 Discussion

Our observations align with previous research findings showing spayed/neutered dogs are more likely to rupture their CCL than their intact counterparts. Large dogs had the highest frequency in the surgical group, which is also consistent with previous research showing large dogs tend to rupture their CCL more than any other size category (Hart et al. 2014, 2016; Torres de la Riva et al. 2013; Whitehair 1993). The nonsurgical group had a greater number of medium and small dogs. The cases that were provided were selected randomly from the surgical and nonsurgical records and not matched for any of the parameters of weight or sex. If the premise is correct, that larger dogs are more likely candidates for surgery and referred to a teaching hospital, it would explain the significantly greater mean weight in the surgical group.

In humans, it is thought increased ACL laxity may be a rupture factor, which is influenced by hormone levels. The primary role of the ligament is to prevent anterior tibial movement and increased laxity may reduce its stability (Pollard, Braun, & Hamill 2006). The ACL has estrogen receptors and estrogen influences type I collagen production by downregulating proliferation, resulting in less collagen in the ACL (Liu et al. 1996; Pollard et al. 2006; Romani et al. 2003). After puberty, laxity increases in females and decreases in males (Giugliano & Solomon 2007). The estrogen increase in reproductive aged women may account for the increased laxity in the ligament and higher rupture rate. Oral contraceptives studies have suggested up to an 20% reduced risk of a ruptured ACL with estrogen reducing pills, but some studies suggest there is no difference and longer-term studies are needed for a more conclusive connection between birth control and rupture rate (Arendt, Bershadsky, & Agel 2002; Herzberg et al. 2017).

The increased laxity from decreased collagen content may account for why spayed/neutered dogs have increased CCL rupture rates. Unlike humans when they reach puberty and estrogen rises, fully matured dogs have relatively low estrogen levels throughout the year except during their bi-annual estrus (Turner 2001). An underdeveloped ligament from early collagen deficiency may suggest why intact dogs have lower rupture rates than early spayed/neutered dogs. However, in both humans and dogs this is thought to be a multifactorial process and hormones are only one potential factor.

Medial patellar luxation (MPL) is the most prevalent PL form and primarily congenital with trauma a rarer occurrence (Alam et al. 2007; Bound et al. 2009; Gibbons et al. 2006; Hayes, Boudrieau, & Hungerford 1994). Dogs with PL subsequently have a higher chance of CCL rupture (Alam et al. 2007; Griffon 2010; Langenbach & Marcellin-Little 2010; Roush 1993). Therefore, it is speculated that PL, mainly MPL, predisposes a dog to CCL rupture (Willauer & Vasseur 1987). The change in hormones might affect AOI, FAA, and the quadricep muscles in which the patella is embedded since the femur is typically distorted in dogs with PL (Tomlinson et al. 2007).

Coxa vara of the femur and decreased FAA are typically associated with MPL (Pinna & Romagnoli 2017). There are two proposed theories for this relationship. The first is that coxa vara results in displacement of the quadricep muscles, which impairs growth on the medial side and rapid growth on the lateral side of the distal femur (Harasen 2006). This affects femur conformation and usually results in genu varum (bowed legs) (Bound et al. 2009). The second theory is based on hormones and argues that estradiol levels create shallow trochlear grooves that easily dislocate the patella and muscles (Roush 1993). The potential relationships among these variables provide evidence that rupture may be multifactorial and prompts a need for multivariable analyses, rather than separate analyses.

Based on our preliminary findings, we hypothesize that hormonal changes from ESN increases TPA due to the removal of essential bone growth hormones. A study followed a dog cohort through their completed development and the results revealed extended growth in the radius and ulna of ESN dogs compared to the intact dogs. The rate of growth was the same in all groups, but in the ESN groups plate closure time was delayed and varied due to the absence of gonadal hormones (Salmeri et al. 1991). We hypothesize large dogs are affected more due to their longer growth period and are spayed/neutered around the same time as small dogs even though they are not as close to their full development. This changes the TPA. These abnormal angles may overextend the ligaments and place increased force on them and result in rupture.

Certain limitations affected our results and will likely present challenges for future studies. Biases in using data from university veterinary hospitals (in addition to questionnaire surveys and pet insurance data) have been noted in the literature and a more inclusive database, such as patient distance relative to the hospital, is beneficial for future epidemiological studies (Adams et al. 2010; Bartlett et al. 2010; Egenvall et al. 2009; O'Neill et al. 2014). Small dogs with ruptured CCLs may be less present in this study due to referral biases and may have been treated by their general practitioner since small dogs have more nonsurgical options than large dogs.

Additionally, TPA was not available for non-ruptured cases and could not be directly compared to ruptured cases. Data were not available and could not be obtained for the age at which each dog was spayed/neutered and spay/neuter studies have been controversial, especially with respect to rupture incidence rate (Hart et al. 2014, 2016; Salmeri et al. 1991; Slauterbeck et al. 2004; Torres de la Riva et al. 2013; Whitehair 1993; Witsberger et al. 2008). Many of the important metrics are not regularly available on nonsurgical animals. To be able to assess the relative importance of the literature suggested variables non-invasive data acquisition on an appropriate normal sample is essential.

2.6 Conclusion

Our study provides a template for future studies with a goal for deciphering the most influential factors affecting ligament injuries, both as a model to understand injury risks in ourselves and in dogs, an important companion animal. The combinations of PL, FAA and AOI, ESN, and TPA may suggest a possible rupture codependence among variables. Secondary microtraumas are influenced by the increase in the cranial tibial thrust, which weakens the CCL and makes it more prone to rupturing. Early spay and neuter influences the chances of developing eTPA due to longer limbs, which are then prone to the microtraumas that can eventually rupture the CCL (Ragetly et al. 2011). The distorted FAA and AOI angles are thought to be a factor for PL. These distorted angles may also influence the TPA and increase strain on the CCL.

Apart from spay/neuter timing and an assessment of PL status, data from CT or MRI sources on the variables of TPA, FAA, and AOI would be critical for any conclusions based on biomechanics. Going forward, multivariable studies are needed to assess all variables, including age at spay/neuter, in a larger sample size. This can further test models related to the question regarding biomechanical or genetic factors influencing ligamentous rupture in dogs and humans.

The increasing costs associated with ACL surgery does not account for expenses associated with missed work, sports participation, scholarship loses, or decreased mobility problems (Hewett et al. 2010). Pets, especially dogs, are our companions and these same inconveniences and financial burdens occur when they become sick and we care for them. Each year there is an increase in overall pet spending and more people have purchased pet health insurance than in the past, which reached $1 billion in annual premiums in 2017 (McGinty 2018). In 2018, $72.56 billion was spent on pet-related expenses with $18.11 billion spent on veterinary costs (APPA 2019). Dogs are the most common household pet and it is estimated a large dog costs an average of $22,000 during its lifespan (12 years) as compared to cats averaging $17,000 during its longer lifespan (15 years) (APPA 2019; Greenfield 2011). The immense costs we dedicate to our canine companions illustrates the strong human-dog relationship and the extent to which we are willing to provide for their health and wellbeing.

3.0 Paper 2: Disease associations with cruciate ligament rupture: Translational medicine model using dog CCL data for human ACL rupture

Toth, SA. Disease associations with cruciate ligament rupture: Translational medicine model using dog CCL data for human ACL rupture. Manuscript prepared for submission to the *Journal of Anatomy*.

3.1 Overview

Eighty percent of anterior cruciate ligament (ACL) rupture in humans and 70% of cranial cruciate ligament rupture (CCL) in dogs are caused by non-traumatic means, suggesting biological factors are a component in rupture susceptibility. Previous studies have examined a wide range of variables, in both humans and dogs, for better identifying rupture causation. However, no one variable is identified as the prominent source of rupture, further supporting it is multifactorial in origin. This study used dogs as an anatomical model for humans to measure the association of rupture with other diseases to observe potential disease linkage that may make an individual susceptible to rupture. This study used the Golden Retriever Lifetime Study database to calculate odds ratios between rupture and conditions through a non-ruptured group (n = 54) and ruptured group (n = 54). Females had more significant and trending results than males, primarily between rupture and other musculoskeletal conditions (OR = 4.67, p = .01) and infectious diseases (OR = .38, p = .05). Males reported gastrointestinal conditions related with rupture (OR = 3.5, p = .06) and there was a significant difference between gastrointestinal values in females and males (p =

.04). These results support a potential biological causation of CCL rupture, and translationally, ACL rupture. Vitamin D and its influence on estrogen levels regulate skeletal and muscular development and maintenance, and impact infection immune response and gastrointestinal conditions. This suggest continued research in vitamin D is needed, in addition to longitudinal studies to identify specific condition timelines.

Keywords: anterior cruciate ligament, cranial cruciate ligament, translational medicine, vitamin D, estrogen

3.2 Introduction

Human knee injuries are the second most common injury site after the ankle with anterior cruciate ligament (ACL) ruptures accounting for over 50% of those injuries and an accumulated incidence rate of 68.6 per 100,000 person-years (Joseph et al. 2013; Sanders et al. 2016). Men account for the majority of injuries, but women rupture it at a two to eight times greater rate (Carter et al. 2018; Lin et al. 2018). Comparatively, dog cranial cruciate ligament (CCL) (anatomically the dog ACL) rupture is the leading canine orthopedic problem and primary cause of stifle lameness with doubled incidence rates in the past 30 years (Griffon 2010; Harasen 2003; Witsberger et al. 2008). Spayed female dogs are at the highest risk for CCL rupture, and sexually altered dogs (both male and female) have significantly higher rupture rates than sexually intact dogs (Hart et al. 2014, 2016; Hart et al. 2020b; Hart et al. 2020a; Reiter, Jagoda, & Capellini 2016; Slauterbeck et al. 2004; Taylor-Brown et al. 2015; Witsberger et al. 2008). The reasons for these rupture discrepancies are unknown and are suggested to be multifactorial (Griffin et al. 2000;

Hewett, Myer, & Ford 2006; Toth & Siegel 2021). An estimated 80% of CCL and 70% of ACL ruptures are due to non-traumatic injuries, suggesting there are biological factors or concomitant diseases causing rupture susceptibility and that is further examined here (Boden et al. 2000; Griffin et al. 2000; Griffon 2010; Moore & Read 1996).

Previous ACL studies have examined an assortment of factors that may impact non-contact rupture ranging from extrinsic factors such as various weather conditions (i.e., icy conditions, high evaporation, low rainfall), playing surface, and footwear choice to internal variables like anatomical variation (i.e., intercondylar notch width, increased generalized laxity, Q angle), genetics, and biomechanical (Di Brezzo & Oliver 2000; T. E. Hewett et al. 2010; Lin et al. 2018; Myer et al. 2011; Myer et al. 2008; Olsen et al. 2003; Orchard, Seward, & McGivern 1999; Paterson et al. 2015; Pfeifer et al. 2018; Ruedl et al. 2011; Silvers & Mandelbaum 2011; van Eck et al. 2010). Dog CCL studies have examined several variables, such as specific breed groups, genetics, hormonal changes (gonadectomy), age, biomechanics, weight, and conformation variability (Edney & Smith 1986; Goin et al. 2019; Hart et al. 2014, 2020; Mostafa et al. 2010; Taylor-Brown et al. 2015; Torres de la Riva et al. 2013; Wilke et al. 2006; Witsberger et al. 2008; Zeltzman et al. 2005). No one variable is associated with rupture. This study examined disease association to identify correlations between specific disease conditions and CCL rupture to recognize potential biological etiologies and better identify where to concentrate future research.

This study used dogs as a translational medicine model following the One Health, One Medicine Initiative, which “breaks[s] through the species barrier” to link human and animal medicine for the benefit of both species (Christopher 2015; Meeson et al. 2019; Ribitsch et al. 2020). Dogs are the most phenotypically diverse species and have the most naturally occurring diseases of all land mammals after humans (Rowell et al. 2011; Starkey et al. 2005). Dogs share approximately 80% of diseases with humans totaling nearly 400 inherited disorders relevant to humans, which makes the dog an excellent model for numerous human diseases, such as cancer, aging, and osteoarthritis (Gregory et al. 2012; Mazzatenta et al. 2017; Momozawa 2019; Rowell, McCarthy, & Alvarez 2011; Shearin & Ostrander 2010). Dogs are the closest animal to a gold standard for human osteoarthritis due to the highly similar knee structures (Gregory et al. 2012). These similar structures identify dogs as an excellent model for other knee diseases, including ACL rupture, and reduces anatomical differences attributed to sexual dimorphism or muscle dominance stance. Histological comparisons found the dog CCL to be the closest to the human ACL (Proffen & Murray 2013).

Dogs were selected as the optimal model for this study because of the similarities in anatomy and rupture rates and disparities, in addition to readily available data. The Golden Retriever Lifetime Study (GRLS) was used because it is one of the most comprehensive longitudinal dog health studies currently available, providing information for dog health and translational human medicine.

3.3 Methods

3.3.1 Sample population

This study used data collected for the Golden Retriever Lifetime Study (GRLS). Articles outlining the structure, collection procedure, and goals of the study have been previously published and a brief synopsis is mentioned here (Guy et al. 2015; Simpson et al. 2017). The GRLS is a longitudinal study with the primary goal to understand developmental factors in four prominent cancer types in dogs – (1) high-grade mast cell tumors, (2) osteosarcoma, (3) hemangiosarcoma, and (4) lymphoma (Simpson et al. 2017). All 3,044 GRLS enrolled dogs are purebred with at least two prior generations of pedigree proof; 95% of the enrolled dogs were from a breeder and no more than two littermates per household were enrolled (Guy et al. 2015; Simpson et al. 2017).

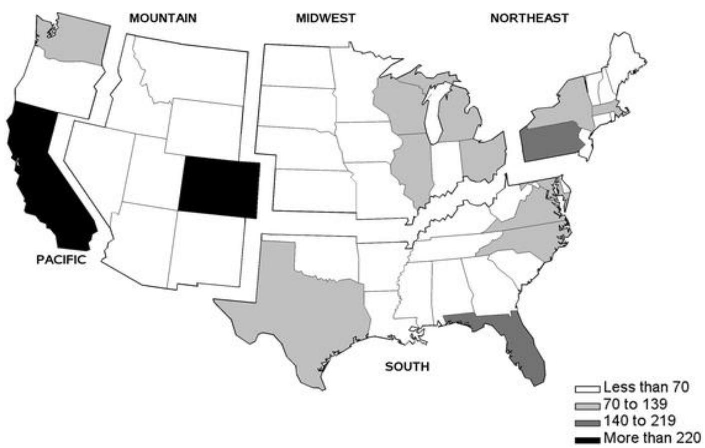

Figure 3-1: "Recruitment regions and number of dogs enrolled by state in the Golden Retriever Lifetime Study" by [Simpson et al. 2017](#) [CC BY 4.0](#)

Golden retrievers were selected because of their overall popularity as being one of the most prevalent breeds in the United States based on American Kennel Club (AKC) registrations, allowing sufficient enrollment numbers and a high diversity of geographical environments and ownership, reducing selection biases in the study population [Figure 3.1] (Guy et al. 2015; Reisen 2021). While cancer is the leading cause of morbidity and mortality in all dogs, golden retrievers are noted of being at a greater risk resulting in them being used as a translational model for human cancer studies (Guy et al. 2015; Kent et al. 2018; Schiffman & Breen 2015). This breed is also noted to be susceptible to other diseases, including muscular dystrophy, sensory ataxic neuropathy, and hip dysplasia, while being low in cases of diabetes mellitus, all of which have been used as translational models for the comparable human disease (Barthélémy, Hitte, & Tiret 2019; Gaiad et al. 2011; Pascual-Garrido et al. 2018; Yoon et al. 2020). Presented in this study is the use of golden retriever cruciate ligament rupture as a model for human ACL rupture.

3.3.2 Study structure

Guy (2015) outlined the specific criteria fulfilled by the owner, dog, and participating veterinarian for partaking in the GRLS [Table 3.1]. A fundamental aspect of the study data collection is the annual physical examination, biological sample collection, and completed online study questionnaire by the veterinarian and owner. A study reported only a 6.3% non-compliance rate in the first year and identified the dog sleeping location variable (garage or in the bedroom) as being the prominent indicator of non-compliance (Ruple et al. 2020).

The GRLS will continue until nearly 500 cases of each of the four studied cancers (high-grade mast cell tumors, osteosarcoma, hemangiosarcoma, and lymphoma) are reached (Guy et al. 2015). The primary endpoint of the GRLS is to determine the incidence, genetic, and environmental and lifestyle factors of the four cancer types, in addition to establishing an extensive database with biological samples for future studies related to golden retriever health. The secondary endpoints are to explore other cancer types and to provide incidence rate and risk factors in other diseases common in golden retrievers (Guy et al. 2015). This database provides substantial information for analyzing cruciate ligament rupture and its correlation to other conditions.

The broad variable categories examined in the GRLS are listed in Table 3.2. Each broad category is subdivided into specific groupings. Table 3.3 lists the broad conditions category and its subdivided groups. The database allows subdivided groups to be analyzed for the current enrolled dog, sire (father), dam (mother), and litter mates. This study focused on the currently enrolled dog to examine which other conditions are associated with rupture.

Table 3.1: "Inclusion and exclusion criteria for the GRLS enrollment" by and adapted from Guy et al. 2015 CC BY 4.0

OWNER INCLUSION CRITERIA	
—	Must be 18 years of age or older, reside in the contiguous USA, be willing to provide registration and identification information for enrolled dog and dam and sire
—	Permit study team to access appropriate registry to retrieve full pedigree
—	Complete online questionnaires successfully prior to enrolment
—	Sign informed consent to participate with study requirements including annual veterinary visits, laboratory evaluations and sample procurement for biorepository storage
—	Consent to have samples of tumor tissue and normal tissue sampled and stored at the time of biopsy or surgery
—	Consent to relinquish rights to biological samples from their pet
—	Consider necropsy (post-mortem) evaluation at the time of death owner exclusion criteria
—	Inability to complete pre-enrolment online survey/questionnaire successfully Golden
GOLDEN RETRIEVER INCLUSION CRITERIA	
—	American Kennel Club, United Kennel Club, other kennel club registration or service dog organization registration as a Golden Retriever with three generations of pedigree documentation
—	Less than two years of age at the time of study application
—	No more than two littermates may be enrolled from the same household
—	Microchip in place or owner willing to allow microchip or alternate permanent identification such as a tattoo
GOLDEN RETRIEVER EXCLUSION CRITERIA	
—	Prior malignancy (benign lipomas or papillomas are allowed)
—	Prior diagnosis with a life-threatening condition that may substantially shorten expected lifespan
—	Inability to demonstrate a verified three-generation pedigree
VETERINARIAN INCLUSION CRITERIA	
—	Licensed in the contiguous United States to practice in their State and in good standing
—	Agree to care for Golden Retriever enrolled in the GRLS including commitment to complete study questionnaires online, biological sample collections and physical examinations
—	Provide medical records at the request of the owner to the owner or the GRLS study team
—	Perform and document diagnostic sample collection procedures, as appropriate, to confirm a diagnosis of cancer or refer dog to a specialty center for collection of tumor tissue
—	Participate with the GRLS Team regarding education of client and hospital staff about the importance of this study to future canine health by providing counselling, in-service training and dissemination of GRLS communications

Table 3.2: Broad categories examined in the GRLS database

Broad Categories
Behavior
Conditions
Diet
Environmental Conditions
Washing and Grooming
Reproductive History
Physical Exam
Dental Care

The database provides a conditions summary in addition to the subdivided groupings listed in Table 3.3 outlining the disease-related conditions included in the GRLS. The conditions summary indicates if a particular dog has one of the diseases listed under the broader group (e.g., if a dog has cataracts, there would be an indicator in the "eye" column). Conditions summary is beneficial because it allows for comparative studies across condition groups. This study used the conditions summary to analyze condition groups against cruciate ligament rupture to identify other ailments that may be prevalent with rupture.

3.3.3 Statistical analyses and control group

Condition totals were calculated for the entire GRLS population to assess total disease presence. The total population was then analyzed by sex (female, male) and sex status (female spayed, female intact, male neutered, and male intact), as CCL rupture varies by these factors. Splitting by these variables was critical in identify the reasoning behind low or non-existent diseases in calculations. Numbers were later found to be non-reported in some cases due to the low overall number observed in the total population.

At the time of this study, there were only 54 cruciate ligaments rupture cases reported in the population. A control group (non-ruptured cruciate ligaments) was created from the larger data set and compared against the ruptured group. A control dog had not sustained a cruciate ligament tear and was selected to pair with a cruciate ligament ruptured dog and was matched based on same sex, sex status (spayed, neutered, intact), date of birth, and enrollment date. There were eight pairs that were not exact matches, but only differed based on enrollment date with seven months being the largest gap. The average enrollment date difference was 2.25 months. All other criteria were matched for the control and ruptured groups.

Condition totals were calculated for each sex status group in the entire GRLS population. Then odds ratios of the condition summaries were calculated between the control and rupture group through unadjusted analysis, analyses stratified by sex, and tests for moderation and Mantel-Haenszel combined odds ratios that controlled for sex. *P*-values, confidence intervals, and proportions exposed are reported. Significance was set at $p < .05$.

3.4 Results

3.4.1 Total population

The total population of the GRLS is 3,044 dogs. The study target goal was 3,000 dogs and the additional 44 were included because they met eligibility and applied for the study prior to the closing date (Simpson et al. 2017). There is nearly an equal number of females (n = 1,504) and males (n = 1,540) (sex ratio = .98).

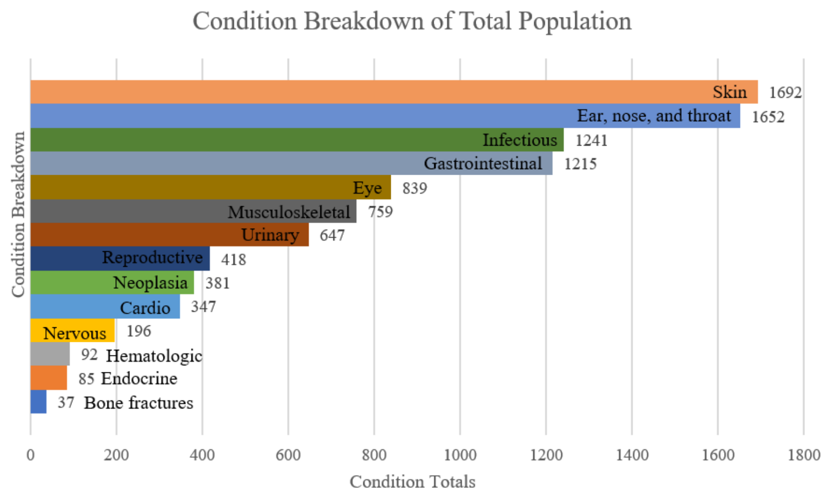

Figure 3-2: Total of each condition category for the entire GRLS population

Table 3.3: All diease-related conditions examined by the GRLS

<table>
<tr>
<td><u>Cardiac</u>
Arrhythmia
Cardiomyopathy
Congestive heart failure
Cough
Heartworm infection
Heart murmur
Pneumonia
Pulmonic stenosis
Subaortic stenosis
Other</td>
<td><u>Ear, Nose, and Throat</u>
Aural hematoma
Epistaxis
Otitis externa
Pharyngitis
Rhinitis
Tonsilitis
Upper respiratory infection
Other</td>
<td><u>Endocrine</u>
Addison's disease
Cushing's disease
Diabetes insipidus
Diabetes mellitus
Hypercalcemia
Hypothyroidism
Pancreatic insufficiency
Other</td>
<td><u>Hematologic</u>
Anemia
Anemia hemolytic
Thrombocytopenia
Von Willebrand's disease
Other</td>
<td rowspan="3"><u>Neoplasia</u>
Adrenal tumor
Basal cell tumor
Bile duct tumor
Bladder tumor
Brain spinal cord tumor
Breast or mammary cancer
Epidermoid cyst
Eye tumor
Hair matrix tumor
Heart tumor
Hemangioma
Hemangiosarcoma
Histiocytic sarcoma
Histiocytoma
Kidney tumor
Leukemia
Leukemia specify
Lipoma
Liver tumor
Lung tumor
Lymphoma
Mast cell tumor
Melanoma
Multiple myeloma
Nasal tumor
Osteosarcoma
Pancreatic tumor
Papilloma
Perianal adenocarcinoma
Perianal adenoma
Pituitary tumor
Plasmacytoma
Prostate tumor
Sebaceous adenoma
Soft tissue sarcoma
Squamous cell carcinoma
Stomach intestinal tumor
Testicular tumor
Thymoma
Thyroid tumor
Unknown</td>
</tr>
<tr>
<td><u>Gastrointestinal</u>
Bloat with torsion GDV
Bloat without torsion
Chronic colitis
Diarrhea
Food sensitivity
Gastritis
Gastrointestinal foreign body
Malabsorptive disorder
Megaesophagus
Pancreatitis
Vomiting
Other</td>
<td><u>Eye</u>
Cataracts
Conjunctivitis
Corneal ulcer
Distichiasis
Ectropion
Glaucoma
Imperforate lacrimal punctum
Iris cyst
Keratoconjunctivitis sicca
Pigmentary uveitis
Progressive retinal atrophy
Third eyelid tear gland prolapse
Trauma injury
Uveitis
Other</td>
<td><u>Urinary</u>
Bladder infection cystitis
Bladder stones
Crystalluria
Ectopic ureter
Incontinence
Kidney failure
Kidney infection
Kidney stones
Proteinuria
Other</td>
<td><u>Nervous</u>
Cauda equina syndrome
Dementia senility
Horner's syndrome
Laryngeal paralysis
Limb paralysis
Myasthenia gravis
Seizure's epilepsy
Trauma injury
Wobbler syndrome
Other</td>
</tr>
<tr>
<td><u>Skin</u>
Atopy
Dermatitis bacterial
Dermatitis contact
Demodectic mange
Dermatophytosis
Dry skin
Dermatitis flea allergy
Dermatitis food allergy
Hot spots
Ichthyosis
Lick granuloma
Dermatitis nonspecific
Papilloma
Dermatitis perianal
Dermatitis perivulvar
Dermatitis pododermatitis
Pruritis
Sarcoptic mange
Seasonal allergy
Sebaceous cyst
Unknown</td>
<td><u>Reproductive</u>
Cryptorchid
Cryptorchid unilateral
Cryptorchid bilateral
Dystocia
Mastitis
Papilloma genital warts
Pregnancy
Preputial infection
Prostate abscess
Prostate enlargement benign
Prostatitis
Pyometra
Recessed vulva
Vaginitis
Other</td>
<td><u>Infectious</u>
Anaplasma
Babesia
Coccidia
Eimeria
Ehrlichia
Fleas
Fungal infection
Fungal infection specify
Giardia
Granuloma
Hookworms
Influenza
Isospora
Lyme disease
Parvovirus
Rocky mountain spotted fever
Roundworms
Tapeworms
Ticks
Tracheobronchitis
Whipworms
Unknown</td>
<td><u>Musculoskeletal</u>
Bone fractures
Cruciate ligament rupture
Elbow dysplasia
Growth deformity
Hip dysplasia
Intervertebral disc disease
Lameness
Osteoarthritis
Osteochondritis dessicans
Panosteitis
Patellar luxation
Rheumatoid arthritis
Spondylosis
Trauma injury
Other</td>
</tr>
</table>

Figure 3.2 displays the condition summary totals for the entire GRLS population. The top three condition groups were skin (*n* = 1,692), ears, nose, and throat (ENT) (*n* = 1,652), and infectious diseases (*n* = 1,241). Musculoskeletal ranked sixth out of the 14 condition groups (*n* = 759).

Figures 3.3 and 3.4 show the population condition summary total broken into female and male, with a further sex status (spayed/neutered/intact) breakdown. The three highest conditions for females (*n* = 1,504) are the same for the total population with skin (*n* = 808), ear, nose, and throat (*n* = 784), and infectious disease (*n* = 602). Musculoskeletal (*n* = 357) ranked seventh as opposed to fifth in the general population. The top three conditions for males (*n* = 1,540) were the same with skin (*n* = 884), ear, nose, and throat (*n* = 868), and infectious disease (*n* = 639). Gastrointestinal was nearly equal with infectious disease (*n* = 637). Musculoskeletal (*n* = 402) ranked sixth for males.

The top three conditions were the same for intact females (*n* = 326), but in a different order and differed only by one case each. Ear, nose, and throat had the most cases (*n* = 113), followed by skin (*n* = 112), and then infectious disease (*n* = 111). Musculoskeletal conditions ranked eight (*n* = 45). The top three conditions in spayed females (*n* = 1,178) were skin (*n* = 696), ear, nose, and throat (*n* = 671), and gastrointestinal (*n* = 512). Musculoskeletal conditions ranked seventh (*n* = 312).

Similar numbers were reported for intact males (*n* = 488). The three most common conditions were skin (*n* = 247), ear, nose, and throat (*n* = 205), and infectious disease (*n* =182). Musculoskeletal conditions were sixth (*n* = 108). Neutered males (*n* = 1,052) reported ear, nose, and throat (*n* = 663), skin (*n* = 637), and gastrointestinal (*n* = 493), respectively. Musculoskeletal conditions ranked sixth (*n* = 294).

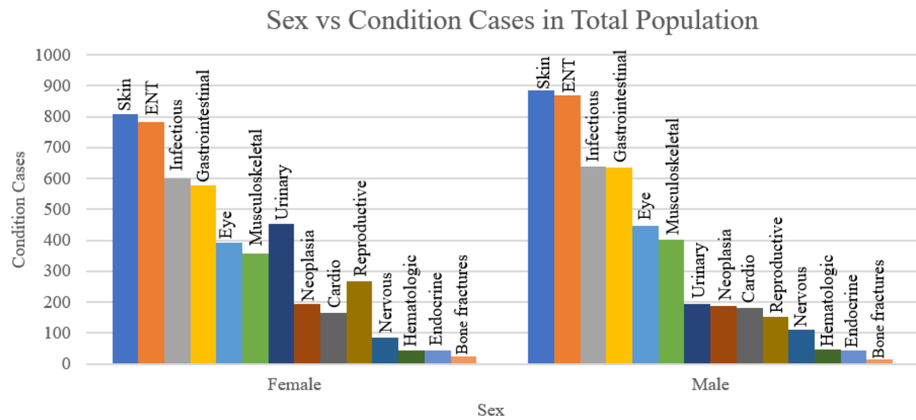

Figure 3-3: Breakdown of the total population into female and male.

Skin, ear, nose, and throat, and infectious disease were the most common conditions in both groups.

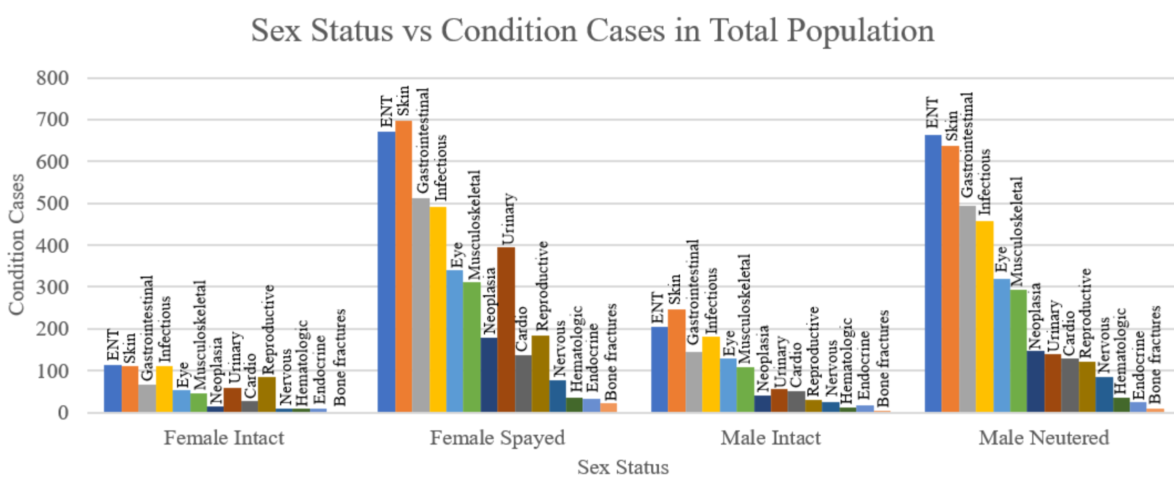

Figure 3-4: Breakdown of the total GRLS population into sex status.

Skin, ear, nose, and throat, infectious disease, and gastroinetsinal conditions were the most common in the four groups.

Condition totals, means and standard deviations are reported in Table 3.4 for sex and sex status groups. All significance was set at $p < .05$ for analyses. Males had the highest gross number of reported conditions compared to females but were not significantly different ($p = .99$). When separated into sex status groups, spayed females had the highest conditions total and was significantly different from intact females ($p < .01$) and intact males ($p < .01$), but not neutered males ($p = .67$). Neutered males were significantly different than intact males ($p = .02$) and intact females ($p < .01$). There is no significant difference between intact females and intact males (p = .12).

Table 3.4: The combined conditions totals with the means and standard deviations for the entire GRLS popualtion.

	Conditions Total	Mean	SD
Female	4,795	342.5	271.1
Female Spayed	4,082	29.6	41.4
Female Intact	713	50.9	41.4
Male	4,806	343.3	304.5
Male Neutered	3,555	253.9	226.1
Male Intact	1,251	89.4	79.7

3.4.2 Disease associations

The odds ratios (OR) measuring the relationship between conditions and rupture in the control group (non-ruptured group) and the ruptured group were recorded. Table 3.5 provides summary statistics and Table 3.6 shows the unadjusted rupture odds ratios for all disease condition groups in the control and rupture groups. There were not enough cases to calculate the values for endocrine conditions. All significance was set at $p < .05$.

Table 3.5: Total breakdown of control and ruptured population used for disease association tests

Average Date of Birth	Average Enrollment Date	Female	Female Spayed	Female Intact	Male	Male Neutered	Male Intact
7/27/2012	8/11/2013	68	66	2	40	32	8

Table 3.6: Unadjusted odds ratios for CCL rupture and disease condition groups for ruptured and controlled cases

	Rupture OR	P-Value	CI	Proportion Exposed (Cases: Control)
Cardio	.394	.077	[.11, 1.25]	11% : 24%
Ear, nose, and throat	1.195	.673	[.48, 2.97]	72% : 69%
Endocrine	[redacted]			
Eye	1.313	.523	[.52, 3.31]	31% : 26%
Gastrointestinal	1.16	.700	[.51, 2.65]	54% : 50%
Hematologic	1	1.000	[.07, 14.28]	4% : 4%
Infectious	.549	.123	[.24, 1.26]	39% : 54%
Neoplasia	1.395	.481	[.5, 3.97]	24% : 19%
Nervous	.856	.781	[.24, 2.96]	13% : 15%
Other	2.038	.558	[.1, 122.48]	4% : 1.9%
Reproductive	1.15	.792	[.36, 3.76]	17% : 15%
Skin	.923	.841	[.39, 2.18]	63% : 65%
Urinary	.753	.515	[2.91, 1.93]	24% : 30%
Bone fractures	1	1.000	[.01, 79.96]	1.9% : 1.9%
Other Musculoskeletal	3.52	.004	[1.37, 9.41]	44% : 19%

A significant association was shown between cruciate ligament rupture and other musculoskeletal conditions (conditions besides cruciate ligament rupture) (OR = 3.52, $p < .01$). Of those with a ruptured cruciate ligament, 44% had at least one other musculoskeletal condition. Trending results were observed in the cardio analysis (OR = .394, $p = .08$) and of those with rupture, 11% had a cardiac condition, whereas having a cardiac condition was protective of rupture in 24%.

Table 3.7: Ruptured and controlled cases odds ratios for diseases condition groups for all females

	Rupture OR	P-Value	CI	N (Exposed : Unexposed)	Proportion Exposed (Cases: Control)
Cardio	.414	.14	[.1, 1.57]	15 : 53	15% : 29%
Ear, nose, and throat	1.17	.78	[.34, 4.1]	51 : 17	76% : 74%
Endocrine					
Eye	1	1	[.28, 3.58]	16 : 52	24% : 24%
Gastrointestinal	.62	.33	[.21, 1.8]	38 : 30	50% : 62%
Hematologic	2.06	.55	[.1, 125.1]	3 : 65	6% : 3%
Infectious	.38	.05	[.13, 1.13]	34 : 34	38% : 62%
Neoplasia	1.39	.57	[.39, 5.09]	16 : 52	26% : 21%
Nervous	1	1	[.21, 4.86]	10 : 58	15% : 15%
Other	2.06	.56	[.1, 125.1]	3 : 65	6% : 3%
Reproductive	1	1	[.26, 3.85]	14 : 54	21% : 21%
Skin	.68	.45	[.22, 2.06]	43 : 25	59% : 68%
Urinary	.78	.62	[.26, 2.32]	26 : 42	35% : 41%
Bone fractures	1	1	[.01, 80.84]	2 : 66	3% : 3%
Other musculoskeletal	4.67	.01	[1.34, 17.05]	23% : 45%	50% : 18%

Table 3.8: Ruptured and controlled cases odds ratios for diseases condition groups for all males (neutered and intact)

	Rupture OR	P-Value	CI	N (Exposed : Unexposed)	Proportion Exposed (Cases: Control)
Cardio	.3	.29	[.01, 4.24]	4 : 36	5% : 15%
Ear, nose, and throat	1.24	.74	[.29, 5.41]	25 : 15	65% : 60%
Endocrine					
Eye	1.91	.33	[.44, 8.63]	15 : 25	45% : 30%
Gastrointestinal	3.5	.06	[.8, 15.98]	18 : 22	60% : 30%
Hematologic					
Infectious	1	1	[.24, 4.25]	16 : 24	40% : 40%
Neoplasia	1.42	.68	[.2, 11.13]	7 : 33	20% : 15%
Nervous	.63	.63	[.05, 6.29]	5 : 35	10% : 15%
Other					
Reproductive	2.11	.55	[.1, 130.98]	3 : 37	10% : 5%
Skin	1.56	.51	[.35, 7.1]	26 : 14	70% : 60%
Urinary	.47	.55	[.01, 10.02]	3 : 37	5% : 10%
Bone fractures					
Other musculoskeletal	2.15	.29	[.42, 12.15]	11: 29	35% : 20%

Data was originally stratified by each sex status group, but due to low case numbers for both female and male intact groups, the Mantel-Haenszel test defaulted to the Tarone test of homogeneity in a few cases, but in most cases the odds ratio could not be computed. Data was then split into subgroups for each sex status group and analyzed separately, however the low case numbers for both intact groups again did not produce sufficient results and the separate female and male results. Data was then only stratified by female and male for calculations. Tables 3.7 and 3.8 report the odds ratio for female and male, respectively.

Females showed significant association between rupture and infectious diseases (OR = .38, p = .05) and rupture and other musculoskeletal conditions (OR = 4.67, p = .1). In the ruptured cases, 38% had at least one infectious disease, while infectious disease was protective in 62% of the control cases.

Males did not show a significant association in between rupture and any disease condition group; however, it is worth reporting that rupture and gastrointestinal conditions are trending (OR = .3.5, p = .06). In the ruptured cases, 60% had a gastrointestinal issue, while it was protective in 30% of the control cases. Cases numbers were too low to report results for endocrine, hematologic, other, and bone fracture conditions.

Combined odds ratios, test of modification, and stratified analysis between sexes (female and male) are reported in Table 3.9. Since the test of homogeneity rejected the null hypothesis for rupture and gastrointestinal (χ^2 = 4.36, p = .04), it cannot be concluded that there is a common odds ratio across strata in this condition group. Therefore, the Mantel-Haenszel combined estimated of the odds ratio is not interpretable and the specific strata are only reported. The results show there is significant evidence to conclude sex-specific odds ratios are different between the two groups. Other condition groups could not reject the null hypothesis that the stratum specific odds ratios are equivalent and there is not enough evidence to conclude sex-specific strata odds ratios are different

Table 3.9: Stratfied odds ratios between female and male for ruptured and controlled cases

	Female OR	Female CI	Male OR	Male CI	Effect Modification	Combined OR	Combined CI
Cardio	0.41	[.1, 1.57]	0.3	[.01, 4.24]	0.81	.39	[.13, 1.12]
Ear, nose, and throat	1.17	[.34, 4.1]	1.24	[.29, 5.41]	0.95	1.2	[.52, 2.76]
Endocrine	[redacted]						
Eye	1	[.28, 3.58]	1.91	[.44, 8.63]	0.46	1.3	[.57, 3.07]
Gastrointestinal	0.62	[.21, 1.81]	3.5	[.8, 15.98]	0.04	-	-
Hematologic	[redacted]						
Infectious	0.38	[.13, 1.13]	1	[.24, 4.26]	0.24	.55	[.26, 1.18]
Neoplasia	1.39	[.39, 5.09]	1.42	[.20, 11.13]	0.99	1.4	[.55, 3.54]
Nervous	1	[.21, 4.86]	0.63	[.05, 6.3]	0.7	.86	[.29, 2.55]
Other	[redacted]						
Reproductive	1	[.26, 3.85]	2.11	[.1, 130.99]	0.6	1.16	[.4, 3.31]
Skin	0.68	[.23, 2.06]	1.56	[.35, 7.1]	0.33	.92	[.42, 2.02]
Urinary	0.78	[.26, 2.32]	0.47	[.01, 10.02]	0.72	.76	[.29, 1.8]
Bone Fractures	[redacted]						
Other Musculoskeletal	4.67	[1.38, 17.05]	2.15	[.43, 12.15]	0.4	3.5	[1.47, 8.35]

3.5 Discussion

In the total population, the most common conditions were related to skin, ear, nose, and throat, infectious disease, and gastrointestinal issues. Infectious diseases were more common than gastrointestinal conditions in both the female and male intact groups. Overall, the intact groups had significantly less reported conditions than the gonadectomized groups. This contributes to the ongoing gonadectomy risks and benefits debate (Howe et al. 2001; McKenzie 2010; Reichler 2009; Yates & Leedham 2019). It should be noted however that these results may be confounded by age as many diseases do not appear until later in life, such as mammary cancer which is the most common cancer type in females with 50% malignancy and cited as being higher risk in intact or late spayed females (Gilbertson et al. 1983; Howe et al. 2001; Philibert et al. 2003; Sorenmo

2003; Sorenmo et al. 2000). These results may shift as the population ages, further supporting the GRLS functionality by observing new patterns associated with aging.

The group with the most reported conditions were spayed females but was not significantly different from neutered males. Intact females and males did not significantly differ. These limited numbers of cases in both the female and male intact groups resulted in the disease condition odds ratios being separated into the general categories of female and male.

In the total GRLS population, musculoskeletal ranked between sixth and eighth in all measured groups and males surprisingly had a higher total than females. However, spayed females had the greatest reported musculoskeletal conditions when examined by sex status. This is comparable with spayed females being more likely than any other group to rupture their cranial cruciate ligament (CCL) and aligns with human females rupturing their anterior cruciate ligament (ACL) more than men (Haida et al. 2016; Hewett et al. 2010; Taylor-Brown et al. 2015; van Diek et al. 2014; Witsberger et al. 2008).

Odds ratios were calculated to measure the relatedness of rupture to other diseases for discerning potential linkage. There was significant association between other musculoskeletal conditions and rupture. This relationship was also significant in females when the data was analyzed by sex, however it was not significant in males. This suggests previous or concurrent musculoskeletal injuries are a greater risk to females than males and may require more treatment and rehabilitation than males, in both dogs and humans.

Previous research has shown patellar luxation, another musculoskeletal condition, to influence rupture susceptibility (Griffon 2010; Johnson & Johnson 1993; Langenbach & Marcellin-Little 2010). Medial patellar luxation (MPL) is primarily congenital and found in small breed dogs with a range of 9-35% of cases in large breeds; MPL is cited as a predisposition factor to CCL rupture (Di Dona, Della Valle, & Fatone 2018; Gibbons et al. 2006; Harasen 2003; Langenbach & Marcellin-Little 2010). Females and gonadectomized dogs are more likely to have congenital patellar luxation, which aligns with the data presented here with females having a higher chance of musculoskeletal conditions in relation to CCL rupture (Di Dona, Della Valle, & Fatone 2018; O'Neill et al. 2016). However, golden retrievers are large breed dogs, which are less susceptible. This suggests there may be other musculoskeletal factors influencing rupture in large breed dogs.

Females also showed trending results for infectious diseases where it was protective against rupture in 62% of the cases. Females and males immune responses are different, and has been shown how human women experience significantly lower rates of infection than men (Straub 2007). Estrogen up-regulates immunoglobulin levels and partially explains why females react better to certain diseases than males, but also suffer more from hyper-immune responses (Jones et al. 2019). Estrogen acts as a protective factor in immune response and aids in proper growth and development. There is a close relationship between growth hormone and estrogen and is a factor in why early spayed dogs have longer limbs than their intact counterparts (Leung et al. 2004; Salmeri et al. 1991).

The age at spaying and the early removal of reproductive organs without estrogen replacement may impact infection rates and subsequently, CCL rupture. Estrogen may be protective of CCL rupture, to an extent, which is why intact and late spayed dogs have lower rupture rates than spayed females (Hart et al. 2014, 2016; Torres de la Riva et al. 2013). The estrogen aids in proper growth and development of the limb, while early spayed female dogs may have affected limb structures, such as their tibial plateau angle, which is cited to be a factor in CCL rupture (Duerr et al. 2007; Wilke et al. 2002).

Dogs that are late spayed or intact may produce enough estrogen to accurately fight infections when needed but suppress the quantity when not fighting an infection, indicating there is enough estrogen to support proper growth and development and proper hormone regulation. Early gonadectomized dogs may not have the same hormone regulation and may continuously hyper-produce estrogen without the suppression, which influences limb structure and rupture susceptibility.

Lastly, gastrointestinal conditions produced trending results in males. This suggests the gut and diet may play a critical role in ligament health. Diet is known to be essential for ligament health, directly and indirectly (e.g., obesity on joint health) (Close et al. 2019; Hansen et al. 2006; Logan 2006; Mooney et al. 2011). Vitamin D functions like a hormone and plays a role in estrogen synthesis. Dogs must obtain vitamin D from their diet as they cannot synthesize it from sunlight like humans (Guy et al. 2015). In humans, a lack of vitamin D has been reappearing in some populations for cultural reasons (e.g., clothing, reduced physical activity, working inside), causing rickets in children (Foo et al. 2009; Soliman et al. 2014). Vitamin D is essential for calcium homeostasis and skeletal mineralization throughout life (Soliman et al. 2014). The lack of vitamin D, in both humans and dogs, may influence limb structure, especially regarding estrogen synthesis in skeletal development and maintenance.

There were trending results between rupture and cardiac conditions in the total sample population, but none when separated by sex. The odds ratio was the same across sex stratum, suggesting a larger sample size in each sex is needed to better understand the relationship.

3.5.1 Limitations

There were two main limitations of this study. The first is the general usage of cruciate ligament rupture as a proxy for cranial cruciate ligament rupture. The database only recorded cruciate ligament rupture and not specifically cranial cruciate ligament rupture. However, caudal cruciate ligament injuries are rare and associated with traumatic injury (Harari 1993; Sumner et al. 2010; Zachos 2002). This is also similar in humans where posterior cruciate ligament without concurrent ACL injury is not common and is estimated of only 3% of knee ligament injuries in the general population (Harner & Höher 2016; Schulz et al. 2003).

The second limitation is the use of Boolean indicators in the GRLS database. Boolean indicators were used to indicate the presence or absence of a condition listed in the general conditions category. Therefore, if a dog had two or more conditions in a category, it was only marked as present and totaled as one. Notionally, the odds ratios should not have been impacted because if one condition was more prevalent in a certain category related to CCL rupture, there would have been a stronger presence in the sampled group and a higher odds ratio.

Despite these shortcomings, this study was conducted using a nationwide, longitudinal, non-veterinary school database, which is rare in similar studies. Previous studies have mainly used veterinary databases to obtain and analyze CCL data due to the easier accessibility, cost, and attainment of large sample sizes. However, there are inherent biases when using such databases, especially veterinary databases since there are only 32 accredited veterinary schools in the United State and results in regional clustering, referral biases (especially for severe cases), and inconsistent reporting standards across university hospitals (Bartlett et al. 2010; Egenvall et al. 2009; O'Neill et al. 2014).

3.6 Conclusion

The comparative anatomy approach of a human and dog knee disease follows the One Health Initiative with the goal to aid in the overall health of both humans and dogs. Evolution of the expansive breed varieties and formations, temperaments, sizes, and diseases identify them as excellent medical models as almost every human disease exists in dogs and the physiology, disease symptoms, and clinical phenotypes many times reflect human diseases (Kukekova et al. 2008). Beyond sharing similar diseases with humans, dogs are a useful model due to their accessibility, diverse living environments, shared environment with humans (exposure to same bacteria, pollutants, and toxins), exhibition of breed-specific diseases (some that are rare in humans, such as sarcomas), the appearance of spontaneous diseases (as opposed to induced, as in mice), and they age five to eight times faster than humans, aiding in a disease timeline, observation of disease onset, and condensed study time (Momozawa 2019; Oláh et al. 2021; Rowell et al. 2011). Cranial cruciate ligament (CCL) rupture in dogs is an excellent model for understanding anterior cruciate ligament (ACL) in humans.

Overall, this study showed females had more significant and trending results between CCL rupture and other disease conditions. Infectious diseases and other musculoskeletal conditions were related to CCL rupture in females and gastrointestinal conditions were related to rupture in males. These differences may be due to hormonal reasons, specifically estrogen. The low baseline estrogen levels in gonadectomy dogs may be further impacted by diet since dogs need to obtain the vitamin D hormone from their food. The lower estrogen levels in neutered males may not be compensated enough through diet, leading to gastrointestinal issues, and impacting skeletal structure. Spayed females may produce enough estrogen, along with dietary intake, to not have high levels of gastrointestinal conditions associated with rupture, but instead they may hyper-produce hormones that impact skeletal development.

The low levels of vitamin D in human populations may impact estrogen production, which then influences skeletal health. This leads to increased rupture susceptibility. Studies have seen that athletes do not normally meet the dietary intake needs of vitamin D and lack sufficient sun exposure due factors like indoor training, early or late training times, and sunscreen use (Larson-Meyer & Willis 2010). Musculoskeletal weakness is a symptom of vitamin D deficiency (Cannell et al. 2007). Athletes may push themselves mentally and physically to perform despite this weakness, which is supported by rupture occurring more during games than practice (Paterno et al. 2012). Females utilize their quadriceps over their hamstrings, which is vis versa in males, and this extra strain on the weakened muscular in females may impact rupture susceptibility (Griffin et al. 2000; Hewett et al. 2010a, 2010; Myer et al. 2009). More research is needed on sex differences in vitamin D levels in athletes and how it impacts the skeleton. Longitudinal studies would be beneficial for observing points in time when events occurred and how that impacts individuals over time. This will show if there is a genetic linkage between conditions or influenced by environmental factors.

4.0 Paper 3: Translational anterior cruciate ligament model: Longitudinal study of reproductive variables associated with cranial cruciate ligament rupture in dogs

Toth, SA & Grosso, AR. Translational anterior cruciate ligament model: Longitudinal study of reproductive variables associated with cranial cruciate ligament rupture in dogs. Manuscript prepared for submission to the *Development Dynamics.*

4.1 Overview

Non-traumatic cranial cruciate ligament rupture (CCL) in dogs and anterior cruciate ligament (ACL) rupture in humans occur in similar rupture discrepancy patterns. Gonadectomized dogs rupture their CCL at a greater rate than intact dogs and similarly, women rupture their ACL at a 2-8 times greater rate than men and it is not certain why these discrepancies occur in either species. This study used dogs as an anatomical model for humans to identify which reproductive variables are associated with rupture, while mutually benefitting dog health. Longitudinal data was obtained from the Golden Retriever Lifetime Study and analyzed using mixed-effects linear regression and general linear regression. Overall, height, weight, and age of gonadectomy were significantly associated with cruciate rupture in the total population (n = 3,044), males (n = 1,540), and females (n = 1,504). There were significantly different probabilities of rupture in those gonadectomized at $\leq$6 months and $\geq$13 months in the total population (OR = .12, $p < .01$), males (OR = .13, $p = .01$), and females (OR = .12, $p = .01$). Likewise, in those gonadectomized at 7-12 months and $\geq$13 months in the total population (OR = .15, $p < .01$) and females (OR = .12, p =

.01). All breeds are recommended to be gonadectomized at the same age despite different ossification rates and this may impact entire limb conformation. More research is needed to better understand ligament health and injury in women experiencing amenorrhea as it would provide additional insight into estrogen changes on ligaments.

Keywords: Cranial cruciate ligament, anterior cruciate ligament, gonadectomy, estrogen

4.2 Introduction

The National Institute of Health (NIH) mandated the inclusion of women in NIH-funded clinical trials through the NIH Revitalization Act to better understand sex health disparities less than 30 years ago in 1993 (Clayton & Collins 2014). One such disparity is women rupturing their anterior cruciate ligament (ACL) at a two to eight times greater rate than men, and this sex discrepancy is not substantial until the onset of puberty, suggesting biological factors related to the development of secondary sex characteristics may be a critical component to rupture susceptibility (Carter et al. 2018; Lin et al. 2018; Voskanian 2013).

Females between 15-19 years old are the most susceptible group to ACL rupture and the younger the individual, the higher chance of reinjury (Sanders et al. 2016; Vavken & Murray 2013; Webster & Feller 2016). An ACL tear increases lifelong issues, such as the likelihood of reinjury to the same ligament, contralateral injury, and osteoarthritis later in life (Grindem et al. 2016; Lohmander et al. 2007; Øiestad et al. 2009; Paterno et al. 2012, 2014; Swärd, Kostogiannis, & Roos 2010). The reason for these rupture discrepancies is not well understood and predisposition reproductive factors are examined here through comparative anatomy of the dog cranial cruciate ligament (CCL) to aid in understanding more about the multifactorial etiology of CCL rupture for mediation of future tears and associated secondary injuries.

The lack of socialized national healthcare databases creates difficulties in obtaining patient ACL data. National registries in other countries connect ACL injury with other patient data, such as age and sex, and are connected to identification numbers. Similar databases have been created in the US but are run by for-profit entities like healthcare companies, which inherently have conflicts of interest in preventative research studies (Vavken & Murray 2013). Vanderbilt University Medical Center has two ACL patient databases – (1) Multicenter Orthopedic Outcomes Network (MOON) and (2) Multicenter ACL Revision Study (MARS). The MOON study focuses on long-term outcomes (mainly osteoarthritis onset) after ACL reconstruction surgery and MARS examines longitudinal outcomes of revision ACL reconstruction (as opposed to primary reconstruction) (MARS Group 2019; MOON Knee Group 2018). These databases are beneficial for learning about the aftermath of injury but not for prophylactic studies. Only 3% of ACL publications pertain to prevention (Griffin et al. 2000; Uhorchak et al. 2003). Therefore, to better understand ACL rupture causation, longitudinal animal studies provide an excellent, steady data modeling source that provide more controlled biomechanics between the sexes and was used here to best understand the biological underpinning of ACL rupture.

The ACL is a homologous structure to the CCL in the canine knee (Meeson et al. 2019; Milachowski, Weismeier, & Wirth 1989). Spayed female dogs are at the highest risk for CCL rupture, and gonadectomized dogs have significantly higher rupture rates than sexually intact dogs (Hart et al. 2014, 2016; Hart et al. 2020b; Hart et al. 2020a; Reiter, Jagoda, & Capellini 2016; Slauterbeck et al. 2004; Taylor-Brown et al. 2015; Witsberger et al. 2008). Female dogs spayed before one year of age have the highest rupture risk (Torres de la Riva et al. 2013). The reason for these rupture disparities in dogs is unknown, like in humans. Given the ACL and CCL are homologous structures, they signify potential relationships between rupture factors and can be used to better understand rupture patterns in each other and is examined here.

4.3 Methods

Research that utilizes sentient animals is a controversial topic, but because CCL rupture in dogs is naturally occurring, there are less ethical concerns when studying its relation to human medicine (Ribitsch et al. 2020). This research used a database from the Golden Retriever Lifetime Study (GRLS), a breed-specific longitudinal study to better identify CCL rupture factors that may predispose certain dogs to injury and translates it to human medicine. The comparative anatomy approach of a human and dog knee disease follows the One Health Initiative with the goal to aid in the overall health of both humans and dogs. These following sections regarding GRLS data collection have been previously published in Toth 2021a.

4.3.1 Sample population

This study used data collected for the GRLS and articles outlining the structure, collection procedure, and goals of the study have been previously published and a brief synopsis is mentioned here (Guy et al. 2015; Simpson et al. 2017). The GRLS is a longitudinal study with the primary goal to understand developmental factors in four prominent cancer types in dogs – (1) high-grade mast cell tumors, (2) osteosarcoma, (3) hemangiosarcoma, and (4) lymphoma (Simpson et al. 2017). All 3,044 GRLS enrolled dogs are purebred with at least two prior generations of pedigree proof; 95% of the enrolled dogs were from a breeder and no more than two littermates per household were enrolled (Guy et al. 2015; Simpson et al. 2017).

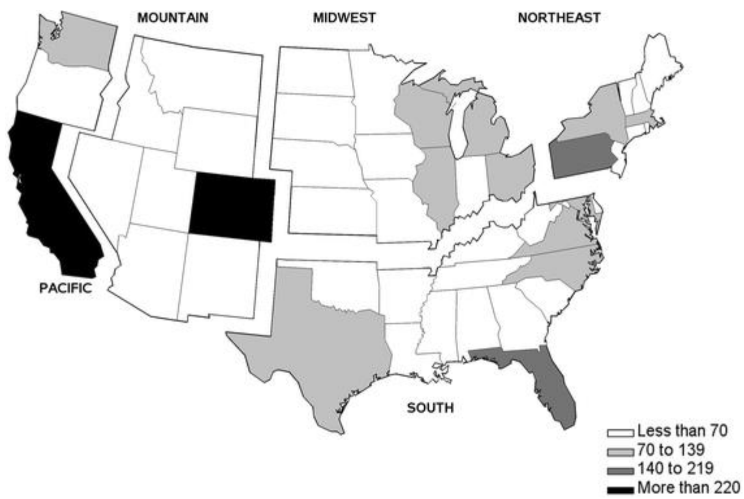

Figure 4-1: "Recruitment regions and number of dogs enrolled by state in the Golden Retriever Lifetime Study" by Simpson et al. 2017 CC BY 4.0

Golden retrievers were selected because of their overall popularity as being one of the most prevalent breeds in the United States based on American Kennel Club (AKC) registrations, allowing sufficient enrollment numbers and a high diversity of geographical environments and ownership, reducing selection biases in the study population [Figure 4.1] (Guy et al. 2015; Reisen 2021). While cancer is the leading cause of morbidity and mortality in all dogs, golden retrievers are noted of being at a greater risk resulting in them being used as a translational model for human cancer studies (Guy et al. 2015; Kent et al. 2018; Schiffman & Breen 2015). This breed is also noted to be susceptible to other diseases, including muscular dystrophy, sensory ataxic neuropathy, and hip dysplasia, while being low in cases of diabetes mellitus, all of which have been used as translational models for the comparable human disease (Barthélémy, Hitte, & Tiret 2019; Gaiad et al. 2011; Pascual-Garrido et al. 2018; Yoon et al. 2020). Presented in this study is the use of golden retriever cruciate ligament rupture as a model for human ACL rupture.

4.3.2 Study structure

Guy (2015) outlined the specific criteria fulfilled by the owner, dog, and participating veterinarian for partaking in the GRLS [Table 4.2]. A fundamental aspect of the study data collection is the annual physical examination, biological sample collection, and completed online study questionnaire by the veterinarian and owner. A study reported only a 6.3% non-compliance rate in the first year and identified the dog sleeping location variable (garage or in the bedroom) as being the prominent indicator of non-compliance (Ruple et al. 2020).

The GRLS will continue until nearly 500 cases of each of the four studied cancers (high-grade mast cell tumors, osteosarcoma, hemangiosarcoma, and lymphoma) are reached (Guy et al. 2015). The primary endpoint of the GRLS is to determine the incidence, genetic, and environmental and lifestyle factors of the four cancer types, in addition to establishing an extensive database with biological samples for future studies related to golden retriever health. The secondary endpoints are to explore other cancer types and to provide incidence rate and risk factors in other diseases common in golden retrievers (Guy et al. 2015). This database provides substantial information for analyzing cruciate ligament rupture and its correlation to other conditions.

Table 4.1: "Inclusion and exclusion criteria for the GRLS enrollment" by and adapted from Guy et al. 2015 CC BY 4.0

OWNER INCLUSION CRITERIA	
—	Must be 18 years of age or older, reside in the contiguous USA, be willing to provide registration and identification information for enrolled dog and dam and sire
—	Permit study team to access appropriate registry to retrieve full pedigree
—	Complete online questionnaires successfully prior to enrolment
—	Sign informed consent to participate with study requirements including annual veterinary visits, laboratory evaluations and sample procurement for biorepository storage
—	Consent to have samples of tumor tissue and normal tissue sampled and stored at the time of biopsy or surgery
—	Consent to relinquish rights to biological samples from their pet
—	Consider necropsy (post-mortem) evaluation at the time of death owner exclusion criteria
—	Inability to complete pre-enrolment online survey/questionnaire successfully Golden
GOLDEN RETRIEVER INCLUSION CRITERIA	
—	American Kennel Club, United Kennel Club, other kennel club registration or service dog organization registration as a Golden Retriever with three generations of pedigree documentation
—	Less than two years of age at the time of study application
—	No more than two littermates may be enrolled from the same household
—	Microchip in place or owner willing to allow microchip or alternate permanent identification such as a tattoo
GOLDEN RETRIEVER EXCLUSION CRITERIA	
—	Prior malignancy (benign lipomas or papillomas are allowed)
—	Prior diagnosis with a life-threatening condition that may substantially shorten expected lifespan
—	Inability to demonstrate a verified three-generation pedigree
VETERINARIAN INCLUSION CRITERIA	
—	Licensed in the contiguous United States to practice in their State and in good standing
—	Agree to care for Golden Retriever enrolled in the GRLS including commitment to complete study questionnaires online, biological sample collections and physical examinations
—	Provide medical records at the request of the owner to the owner or the GRLS study team
—	Perform and document diagnostic sample collection procedures, as appropriate, to confirm a diagnosis of cancer or refer dog to a specialty center for collection of tumor tissue
—	Participate with the GRLS Team regarding education of client and hospital staff about the importance of this study to future canine health by providing counselling, in-service training and dissemination of GRLS communications

4.3.3 Variables and statistical analyses

This study analyzed the reproductive longitudinal data from the GRLS. The available reproduction variables are listed in Tables 4.2 and 4.3 The asterisk denotes which variables were analyzed here. The variables not used in this study, but available in the GRLS study, were not investigated because they were either not relevant to this study or there were not enough cases for proper analyses.

There were three variables analyzed for females that were not used in males – (1) currently pregnant for study year (pregnant status), (2) experienced heat cycles last year for study year (heat last year) and (3) spayed before or during a heat cycle for study year (spayed while in heat).

Physical exam details were also examined in this study, which included weight (controlling for height), height (controlling for weight), and body condition score. These variables were available for all categories analyzed (total population, females only, males only), except body condition score was available for any individual in the baseline year.

Table 4.2: Variables available from the GRLS for female reproductive history (*variables used in this study)

Female Reproductive History (most in Boolean indicators)
Experienced heat cycles last year for study year *
Given hormone therapy to prevent heat in the last year for study year
Any breedings not result in pregnancy for study year
Number of times the dog required mis-mating management (pregnancy termination) for study year
Ever had any breedings not result in pregnancy for study year
Currently pregnant for study year *
Date dog was spayed for study year *
Reason dog was spayed for study year
Spayed before or during a heat cycle for study year *
Given hormone therapy to prevent heat ever for study year
Pregnant ever for the year for study year
Spayed while pregnant in the last year for study year

Table 4.3: Variables available from the GRLS for male reproductive history

(*variables used in this study)

Male Reproductive History (most in Boolean indicators)
Intact dogs, if they bred last year for study year
Intact dogs, count of breedings last year for study year
Intact dogs, count of females they have bred with last year for study year
Intact dogs, count of natural litters they have ever sired for study year
Intact dogs, any fertility concerns in the last year for study year
Intact dogs, fertility evaluation in the last year for study year
Intact dogs, outcome last year for study year
Date dog was neutered for study year *
Reason for neutering for study year
Ever bred for study year
Neutered dogs, count of breedings last year for study year
Neutered dogs, count of females they have bred with for study year
Neutered dogs, count of natural litters they have ever sired for study year
Neutered dogs, any fertility concerns in the last year for study year
Neutered dogs, fertility evaluation in the last year for study year
Neutered dogs, outcome last year for study year
Neutered dogs, whether semen was collected for study year
Neutered dogs, count of semen collected for study year
Neutered dogs, how many litters have been sired by insemination in the last year for study year
Intact dogs, whether semen collected for study year
Intact dogs, count of intact dogs semen collected in the last year for study year
Intact dogs, how many litters have been sired by insemination in the last year for study year

Body condition score refers to the Nestle Purina Body Condition System, which is a scale from 1-9 with 4-5 being the optimal physique of the dog. Scores from 1-3 are too thin and scores 6-9 are too heavy. The scale provides a description and front and side image of each weight category (underweight, normal weight, and overweight) and many times is posted within a veterinary exam room for patients to view. These numbers are also usually reported on a dog's SOAP chart and standardized across veterinary practices. This study categorized scores 4-6 as normal weight to control for interobserver error. Normal weight and underweight (1-3) were combined due to the low sample size of underweight dogs in the study.

Gonadectomized age was available for all categories and measured in months and analyzed by the individual gonadectomy date and by grouped age range. The grouped age ranges were split into three groups – (1) $\leq$6 months, (2) 7-12 months, and (3) $\geq$13.

Mixed effect general linear regression was used to analyze the associations overtime between ligament rupture and weight (controlling for height), height (controlling for weight), being in heat the previous year, pregnancy status, and body condition score. General linear modeling (logit) was used to measure the associations between rupture and gonadectomy age, gonadectomy age range group(s), and spayed while in heat. All significance was set at $p < .05$. Statistical results were reported for the total population, males only, and females only since rupture is known to vary by sex. In general, the total female population (both spayed and intact) have more relevant, readily reproductive (non-invasive) data available than males, therefore more analyses were performed for females.

The data shows there was a total of 64 cases of cruciate ligament rupture over the five reported years, with 10 dogs having multiple rupture cases. Contralateral rupture is a risk factor primarily one to two years after sustaining an initial rupture and the repeated cases here occurred predominately one reported year apart from each other (DeCamp 1997; Ragetly et al. 2011; Robinson et al. 2006). These 10 cases were composed of six spayed females, three neutered males, and one intact male. These repeated cases align with the literature that gonadectomized dogs are more likely to rupture their CCL than their intact counterparts (Hart et al. 2016, 2020; Torres de la Riva et al. 2013). The repeat cases were not removed from analyses since the mixed effects regression model accounted for the probability that the event could occur multiple times.

4.4 Results

4.4.1 Total population

All subject data available from the GRLS study was used in this analysis. The total population of the GRLS is 3,044 dogs. The study target goal was 3,000 dogs and the additional 44 were included because they met eligibility and applied for the study prior to the closing date (Simpson et al. 2017). There is nearly an equal number of females (n = 1,504) and males (n = 1,540) (sex ratio = .98).

Table 4.4 reports the longitudinal results for the entire population. There was a significant association between cruciate ligament rupture and height (controlling for weight) overtime (OR = 1.21, p = .02). Dogs with rupture were taller on average (24.09 $\pm$ 2.31 in) than dogs without rupture (22.88 $\pm$ 1.98 in) overtime. There was also a significant relationship between rupture and weight (controlling for height) overtime (OR = 1.04, p = .01) with dogs with ruptured cases being heavier (77.24 $\pm$ 15.03 lbs) on average than dogs that did not rupture their cruciate ligament (67.01 $\pm$ 12.86 lbs). However, there was no significance between rupture and body condition score (OR = 1.23, p = .66).

Additionally, there was significant association between rupture and the age each individual dog was gonadectomized (OR = .9, p < .01), and the grouped age ranges for when the dog was gonadectomized (p < .01).

Since the p-value for the grouped age ranges was p < .01, the null hypothesis is rejected, and it is concluded there is a significant difference that the probability of cruciate ligament rupture differs among the gonadectomized age groups. There were significantly different probabilities of rupture in those gonadectomized at $\leq$6 months and those at $\geq$13 months (OR = .12, p < .01), and those between 7-12 months and at ≥13 months (OR = .15, p < .01). There was no significant difference between ≤6 months and 7-12 months (OR = .84, p = .59).

Table 4.4: Associations between ligament rupture and physical exam and reproduction variables in the total GRLS population overtime.

	Odds Ratio	P-Value	CI
Height (controlling for weight) (in)	1.21	.02	[1.04, 1.42]
Weight (controlling for height) (lbs)	1.04	.01	[1.01, 1.07]
Body condition score	1.23	.66	[.49, 3.08]
Spayed/neutered age (individual) (months)	.9	.00	[85, .96]
Spayed/neutered age (group age ranges)		.00	
≤6 months vs 7-12 months	.84	.59	[.45, 1.58]
≤6 months vs 13+ months	.12	.00	[.04, .36]
7-12 months vs 13+ months	.15	.00	[.05, .43]

4.4.2 Males

Data for males is reported in Table 4.5. Similar results were found in males and in the total population. Significance was found between cruciate ligament rupture and height (controlling for weight) (OR = 1.33, p = .02) with ruptured dogs being taller (24.97 ± 1.79 in) than non-ruptured dogs (23.58 ± 1.87 in). There was significance between rupture and weight (controlling for height) (OR = 1.04, p = .07). Dogs that sustained a rupture were heavier (83.99 ± 17.29 lbs) on average overtime than dogs that did not sustain a rupture (71.98 ± 12.55 lbs). Comparable to the total population however, there was no significant difference in cruciate ligament rupture and body condition score in males (OR = .77, p = .76)

Rupture was found to be significantly associated with the age each individual dog was neutered (OR = .91, p = .03), and the grouped age ranges for when the dog was neutered (p = .01).

The *p*-value for the grouped age ranges was .01 < .05, rejecting the null hypothesis, and concluding there is a significant difference that the probability of cruciate ligament rupture differs among the neutered age groups. There was significantly different probabilities of rupture in those neutered at ≤6 months and those at ≥13 months (OR = .13, p = .01), and trending results in those neutered between 7-12 months and at ≥13 months (OR = .2, p = .05). There was no significant difference between gonadectomy at ≤6 months and 7-12 months (OR = .66, p = .45).

Table 4.5: Associations between ligament rupture and physical exam and reproduction variables in the male GRLS population overtime.

	Odds Ratio	P-Value	CI
Height (controlling for weight) (in)	1.33	.02	[1.04, 1.72]
Weight (controlling for height) (lbs)	1.04	.07	[.1, 1.09]
Body condition score	.77	.76	[.14, 4.19]
Neutered age (individual) (months)	.91	.03	[.84, .99]
Neutered age (group age ranges)		.01	
≤6 months vs 7-12 months	.66	.45	[.23, 1.93]
≤6 months vs 13+ months	.13	.01	[.03, .62]
7-12 months vs 13+ months	.2	.05	[.04, .99]

4.4.3 Females

Results for females in Table 4.5 include more variables than for the total population or males alone. Significance was reported between cruciate ligament rupture and height (controlling for weight) (OR = 1.22, p = .04). The results are similar to the total population and males with ruptured cases being taller (23.5 ± 2.46 in) than non-ruptured cases (22.16 ± 1.83 in). There was also a significant association between cruciate ligament rupture and weight (controlling for height) (OR = 1.06, p < .01). Females with rupture were heavier (77.74 ± 11.58 lbs) than their non-ruptured counterparts (61.85 ± 11 lbs).

There was a significant association between rupture and if the female was spayed while in heat (OR = 5.62, p < .01), the age each individual dog was spayed (OR = .9, p = .01), and the grouped age ranges for when the dog was spayed (p < .01).

Like the total population and males, the p-value for the grouped age ranges was p < .01, concluding there is a significant difference that the probability of cruciate ligament rupture differs among the spayed age groups. There was significantly different probabilities of rupture in those spayed at ≤6 months and those spayed at ≥13 months (OR = .12, p = .01), and in those spayed between 7-12 months and spayed at ≥13 months (OR = .12, p = .01). There was no significant difference between gonadectomy at ≤6 months and 7-12 months (OR = .98, p = .97).

There was no significance between cruciate ligament rupture and pregnancy status (OR = 1.07, p = .96), being in heat the previous recorded year (OR = .72, p = .81), or body condition score (OR = .64, p = .34).

Table 4.6: Associations between ligament rupture and physical exam and reproduction variables in the female GRLS population overtime.

	Odds Ratio	P-Value	CI
Pregnant status	1.07	.96	[.07, 15.49]
Heat last year	.72	.81	[.05, 10.58]
Height (controlling for weight) (in)	1.22	.04	[1.01, 1.48]
Weight (controlling for height) (lbs)	1.06	.00	[1.02, 1.11]
Body condition score	1.64	.34	[.55, 4.91]
Spayed while in heat	5.62	.00	[1.94, 16.31]
Spayed age (individual) (months)	.9	.01	[.83, .97]
Spayed age (group age ranges)		.00	
≤6 months vs 7-12 months	.98	.97	[.45, 2.13]
≤6 months vs 13+ months	.12	.01	[.03, .55]
7-12 months vs 13+ months	.12	.01	[.03, .56]

4.5 Discussion

Cruciate ligament rupture was associated with height (controlling for weight), weight (controlling for height), individual age each dog was gonadectomized, and the gonadectomized group age ranges in the total population, males only, and females only. No significance was found for body condition score or gonadectomized age between $\leq$6 months and 7-12 months in any group. In females, there were no associations between rupture and pregnancy status, being in heat the previous year, or body condition score.

Dogs that sustained a cruciate ligament rupture at some point in their life were taller and heavier on average than dogs that had not injured the ligament. Dogs with rupture were about two inches taller and 10 pounds heavier than dogs without rupture. It has been found that the earlier the dog is gonadectomized, the taller it will be in adulthood due to extended plate closure time (Salmeri et al. 1991). Dogs gonadectomized before 6 months of age are at the greatest risk for CCL rupture, followed by one year of age (Duerr et al. 2007; Hart et al. 2014). Overall, dogs that are gonadectomized have higher CCL rupture rates than their intact counterparts (Hart et al. 2016, 2020; Torres de la Riva et al. 2013).

Females spayed while in heat were shown to have a significant relationship with cruciate ligament rupture, furthering supporting hormone disruption as a possible susceptibility to CCL rupture. However, it is commonly known that females experience their first heat cycle around six months of age, which is the traditional age advised for gonadectomy. Therefore, spaying around six months of age and spaying while in heat are confounding variables in this study. More research is needed to better understand the orthopedic consequences of spaying a dog in heat.

Studies have shown prepubertal gonadectomy encounters short-term problems, such as increased surgical and anesthetic complications that can occur days or weeks after the procedure

(Yates & Leedham 2019). However, there are substantially more long-term negative effects, including increased chance of obesity, certain cancer types (prostatic carcinoma, lymphosarcoma, transitional cell carcinoma, mast cell tumors, hemangiosarcoma, and osteosarcoma), urinary incontinence, and musculoskeletal conditions (hip and elbow dysplasia and CCL rupture) (Root Kustritz 2014; Yates & Leedham 2019). Conversely, there are some long-term positive effects of early neutering besides population control, such as no chance of pregnancy associated complications, tumors in organs that were removed (ovarian, uterine, testicular neoplasia), cryptorchidism, pyometra (life-threatening infection in females), and positive behavioral traits (Root Kustritz 2014; Yates & Leedham 2019).

Conversely, many rescue groups and shelters mandate their animals are gonadectomized before adoption as a method for population control (Howe et al. 2001). Veterinarians have historically supported this view because it is estimated humane societies acquire 5-7 million dogs and cats per year and 3-4 million are euthanized (Root Kustritz 2014).

Another leading reason for early ovariectomy is the reduced chance of mammary cancer, the most common type of tumor in female dogs with 50% malignancy (relatedly, breast cancer is also the most common cancer type in women worldwide) (Gilbertson et al. 1983; Howe et al. 2001; Philibert et al. 2003; Sorenmo 2003; Sorenmo et al. 2000; World Health Organization 2021a). There are conflicting studies suggesting the earlier the age of ovariectomy (<2.5 years), the less chance of mammary cancer, but the results are not consistent (Sorenmo, Shofer, & Goldschmidt 2000; Yates & Leedham 2019). The risks and benefits of early gonadectomy are a debated issue, especially regarding CCL rupture. Early spaying may increase the most common cause of lameness in dogs but decrease the foremost cancer in female dogs.

Other orthopedic issues, such as patellar luxation, femoral angle, and tibial plateau angle have also been shown to impact rupture rates (Griffon 2010; Johnson & Johnson 1993; Langenbach & Marcellin-Little 2010). The hormonal effects of early gonadectomy may impact limb conformation. New research is showing the growth charts used for dogs are based on older studies that used small sample sizes that only used select breeds and did not document their methods well, and this new research is showing not all dog breeds mature at the same rate (Roccaro et al. 2021; Salt et al. 2017).

Patella ossification time has been found to verify among breeds with large breeds having longer ossification times than small breeds (Roccaro et al. 2021). Early gonadectomy may affect the entire limb conformation due to changes in patella ossification time. The patella acts as a leverage system in the knee and changes in its ossification time and morphology may produce subsequent changes in the tibia, femur, and associated ligaments. This may more greatly impact large breeds since they are recommended to undergo gonadectomy at the same age as small breeds despite having longer ossification times.

Hormones also affect tissue-level health and physiological activities (i.e., ligament stiffness and laxity). Knee stability is under both passive and active control. In the CCL and ACL, the hamstrings and gastrocnemius actively stabilize the posterior knee, while the four main ligaments (ACL, PCL, LCL, and MCL) passively provide stability. Estrogen influences muscle retention and may affect this stability. More research is needed to better understand ligament health and injury in women experiencing amenorrhea as it would provide additional insight into estrogen changes on the ligaments and better identify which specific knee structures are directly and indirectly impacted by these changes.

Rupture is a multifactorial process and early gonadectomy is only one factor associated with higher predisposition (Griffin et al. 2000; Hewett, Myer, & Ford 2006; Toth & Siegel 2021). No single variable can account for the rupture majority; a study examining genetic predisposition in Newfoundlands, a highly susceptible breed to CCL rupture, found only 27% of the phenotypic expression of CCL rupture could be attributed to genetics and the remaining 73% was caused by environmental factors, further supporting the idea that rupture is multifactorial (Griffon 2010; Wilke et al. 2006).

4.5.1 Limitations

There were two main limitations of this study and has been previously mentioned in Toth 2021a. The first is the general usage of cruciate ligament rupture as a proxy for cranial cruciate ligament rupture. The database only recorded cruciate ligament rupture and not specifically cranial cruciate ligament rupture. However, caudal cruciate ligament injuries are rare and associated with traumatic injury (Harari 1993; Sumner et al. 2010; Zachos 2002). This is also similar in humans where posterior cruciate ligament without concurrent ACL injury is not common and is estimated of only 3% of knee ligament injuries in the general population (Harner & Höher 2016; Schulz et al. 2003).

A second limitation was the absence or confounding of data in the database. Not all variables were reported for all dogs and this reduced sample size in some of the analyses or it could not be performed. For instance, there was only a few cases of low body weight and could not be calculated and, therefore, it was grouped in with average body weight and compared against overweight cases. There were also repeat occurrences of actions that could only take place once, such as being spayed while in heat. In those cases, general linear regression was used to assess if the event ever occurred during the dog's life instead of calculating it over time.

Despite these limitations, this study was conducted using a nationwide, non-veterinary school or industry-based database, which is uncommon in other CCL studies. Previous research has been conducted using these other database methods due to the accessibility of obtaining large sample sizes. While these types of databases are useful, they are inherently biased in some regards, such as regional clustering of samples, referral biases to the teaching hospitals, random breed samples (including mixed-breed dogs), and non-uniform reporting across hospitals (Bartlett et al. 2010; Egenvall et al. 2009; O'Neill et al. 2014). The GRLS reduced such biases in its study collection, providing useful analyses for dogs, especially golden retrievers, across the country.

4.6 Conclusion

Nearly 90% of veterinary drugs are identical or very similar to those used in humans because of the high relatedness of diseases (Barré-Sinoussi & Montagutelli 2015). The co-evolution of humans and dogs suggest the same evolutionary selection pressures acted on both species. It has been observed that dog domestication mirrors human evolution and the sequencing of both genomes reveal convergent evolutionary processes (Barthélémy et al. 2019; Mazzatenta et al. 2017). This makes them an excellent model for humans.

This study found height, weight, and gonadectomized age were significantly associated with CCL rupture. Spayed while in heat for females was also found to correlate significantly, but that may be confounded by age spayed. This suggests early gonadectomy impacts CCL rupture susceptibility and the age of performing gonadectomies should be reconsidered in dogs, especially those classified as large breed. This work translates to human ACL rupture and the growing need for this research in female-focused medicine.

Female sports participation has increased more than 1000% since the passing of the 1972 Title IX Education Assistance Act that mandates all institutions that receive federal money must offer equal access to curricular and extra-curricular activities for men and women (Mall et al. 2014; Traina & Broomberg 1997). Nearly 8 million students currently participate in high school sports with continuously rising numbers over the past 29 years with female participation at an all-time high with more than 3.4 million athletes (NFHS 2018). The increase in female participation suggests ACL studies prior to Title IX were focused mainly on male rupture factors and only in the past 50 years, at most, has the literature and research grown to include female athletes to address their rupture rate. This work is necessary for scientific value and for continued equality among the sexes.

5.0 Conclusion

The knee is the largest joint in the body and despite that prominence and its importance in locomotion, there are mechanisms that are still not well understood. The anterior cruciate ligament (ACL) is one of the four main ligaments in the knee, and it sustains high rupture rates consistent throughout the world with women rupturing it at a greater rate than men (Carter et al. 2018; Lin et al. 2018). The reason for this rupture discrepancy is not well identified and was investigated here throughout these three papers.

Dogs were used as an anatomical model for identifying variables associated with rupture since they rupture their homologous structure, the cranial cruciate ligament (CCL), similar to patterns observed in humans. Gonadectomized dogs rupture their CCL more than their intact counterparts with spayed females having the highest rupture rate (Hart et al. 2014, 2016; Hart et al. 2020b; Hart et al. 2020a; Reiter, Jagoda, & Capellini 2016; Slauterbeck et al. 2004; Taylor-Brown et al. 2015; Witsberger et al. 2008). The reason for this is not well understood and was recognized as an excellent model for humans because of the comparable rupture discrepancies and anatomical similarities. Additionally, using dogs as an animal model supports the Centers for Disease Control and Prevention's (CDC) One Health, One Medicine Initiative.

Dog CCL rupture was used as an anatomical model for human ACL rupture throughout the three papers to identify which variables cause certain individuals to become more susceptible to rupture. Biological variables were assessed here because rupture varies by sex in both humans and dogs, which indicates biological reasons for rupture discrepancies. The three papers examined multiple biological variables and their relationship to rupture for an extensive analysis of identifying variables that predispose rupture. The first paper verified the relationship between variables previously identified in the literature for rupture by using data from the Oklahoma State Veterinary Hospital. The second and third papers both used data from the Golden Retriever Lifetime Study (GRLS). The second paper measured the association between broad medical categories (e.g., cardiac, endocrine, infectious disease) and rupture to identify potential new variables associated with rupture. The third paper analyzed longitudinal reproductive data and rupture to identify how variables impact susceptibility overtime. The major findings throughout the three papers are that size (height and weight), sex status (intact or gonadectomized), gonadectomized age, and having additional musculoskeletal conditions are significantly related to CCL rupture. These results substantiate there are biological underpinnings to rupture susceptibility.

The association between these variables and rupture is a multifactorial process. The overall, cohesive conclusion is that early gonadectomy produces elongated limb bones and impacts ossification rates due to the removal of essential growth hormones [paper 3]. This changes the overall limb structure, including the tibial plateau angle [paper 1]. This impacts ligament anatomy and health, which is affected by low vitamin D levels [paper 2]. Vitamin D and estrogen aid in regulating immune response, and proper immune response is protective of CCL rupture [paper 2]. The compromised ligament is more susceptible to secondary microtraumas that are influenced by the increase in the cranial tibial thrust, which weakens the CCL and makes it more prone to rupturing [paper 1]. This is more prevalent in large breed dogs because they are gonadectomized at the same age as small breeds despite requiring longer development time [paper 3].

These results indicate hormone levels combined with behavioral factors, such as vitamin D uptake, are essential components for ACL rupture in humans. The hormonal changes observed in females during puberty and their relationship with other biological systems impact overall health and wellbeing, especially for proper mechanical loading and movement maintenance. The biological susceptibility to weakened ACLs due to hormonal changes is further impacted by being quadriceps dominate instead of hamstring dominate, increasing strain on an already weakened ligament and leading to higher rupture rates. Additional factors still need continued research in this area, such as genetics, diet, and behavior, including hormonal changes with behavior (e.g., cortisol). More research is needed in the interaction of estrogen, ligament health, and vitamin D, as vitamin D research is a recently new focus area. This is necessary from both dietary studies and genetics to better identify how much hormones and hormonal regulations are influenced by personal choices and by genetics. Overall, this dissertation concludes ACL rupture is a multifactorial process predisposed through biological means with hormone levels being an essential component.

5.1 Research questions reflection

These three papers examined and answered the three research questions initially asked at the beginning of this study. The answers are integrated within the papers with the final conclusions coalesced into one conclusive analysis in the previous section. Below is a brief review of the individual questions and the associated answers.

5.1.1 First question: Overarching question

Is there a singular cause (e.g., landing stance) for why some individuals, especially adult women, rupture their ACL at a greater rate than others?

Synopsis: The data reported throughout these papers continuously suggested rupture is a multifactorial process. No one variable was solely associated with rupture in the dog CCL cases, suggesting it is the same for humans. Specific variables, such as age of gonadectomy and sex status, were prevalent throughout the papers and indicate they are important factors in the observed rupture patterns. This translates to human hormonal fluctuations, specifically estrogen, and its impact on overall knee conformation and health. Hormones effect all aspects of the body and their changes to the joint structure are most likely multifactorial.

5.1.2 Second question: Animal models

Using dogs as an anatomical model for human ACL rupture, which variables are associated with CCL rupture in dogs?

Synopsis: The variables most associated with rupture in dogs, as assessed through these three papers, are size (height and weight), sex status (intact or gonadectomized), age at gonadectomy, and having additional musculoskeletal conditions. Early gonadectomy influences height and weight, as seen in them being taller and heavier than their intact counterparts. It also modifies ossification rates in the limb bones, causing the dogs to become taller and impacting patella formation. These changes may influence the CCL and make it weaker by overstretching the ligament on the elongated limbs. Additionally, vitamin D aids in physiological health and poor absorption effects estrogen levels, which is why rupture may be more prevalent in females. The rupture discrepancy in dogs is generally noted between gonadectomized and intact dogs, while in humans it is between females and males. Human females may have higher rupture rates because of their quadriceps dominance stance, as opposed to hamstring dominance as seen in males, in addition to the hormonal changes.

5.1.3 Third question: Genetics

Is there a genetic predisposition to rupture as observed through greater breed prevalence in rupture data, or are other biological factors more prevalent?

Synopsis: Based on the three papers written here, there is not enough data to conclusively argue that any one specific breed or genetic cause is associated with rupture, as originally hypothesized. The first paper was a multi-breed sample where most dogs with rupture were listed as "mixed-breed." However, when categorized by size, large dogs were more associated with rupture than small dogs. The second and third paper both used a single breed database based on golden retrievers, which are categorized as a large breed. Overall, there were only 54 independent cases of CCL rupture out of the total population of 3,044, making it only 1.8% of the population. Golden retrievers are exemplified as being highly susceptible to rupture, but the data did not display a large proportion of rupture cases. This suggests that certain breeds may not be particularly susceptible to rupture, instead it is the ossification rates of large breed dogs. Certain breeds, like golden retrievers, are well utilized in research studies because they are ubiquitous and have a non-reactive demeanor suitable for researchers. This alone may bias the data towards certain breeds. Additional research on ossification rates in different breeds may also indicate that early gonadectomy impacting ossification times is impacted by size and breed.

5.2 Future directions

This research further supports anterior cruciate ligament (ACL) and cranial cruciate ligament (CCL) ruptures are caused partially by biology means and multifactorial by nature.

However, additional research is needed to further identify more specific variables and any external factors. Mentioned briefly here are three future directions for this research – (1) targeted human studies on estrogen, (2) continued updates on the GRLS study, and (3) analyses of diet and behavior in both humans and dogs.

There are numerous human ACL studies that focus on a plethora of rupture variables, nevertheless more research is needed specifically on estrogen and rupture. Studies assessing birth control type have been studied, but they are not consistent in their results and many times have small sample sizes. As previously mentioned in paper 3, studies examining rupture rates in females experiencing amenorrhea would be beneficial to see how those hormonal changes impact rupture susceptibility to identify how that transforms the ligament. Additionally, longitudinal studies measuring hormonal levels on a consistent basis in both men and women would also be of great value. This would allow for more accurate assessments of how hormones fluctuate overtime in relation to rupture, in addition to overall knowledge of hormonal fluctuations and general health.

A second research direction is to follow up with the Morris Animal Foundation Golden Retriever Lifetime Study (GRLS) data and compare the ending results of that data with this dissertation. The GRLS is set to continue until it reaches 500 cases of each of its targeted cancers. The CCL data can be reassessed to see if the same conclusions here hold true when the GRLS data is completed. This may allow for examination of additional variables that did not have large enough sample sizes in this study and it will provide an opportunity for measuring osteoarthritis rates, which is a common occurrence after ACL or CCL rupture, as previously mentioned. The data is still too young currently to accurately see those rates since osteoarthritis does not usually set in until later in life. Lastly, when this data is finished, a more definitive argument can be made about the role of genetics and rupture in terms of breed and hereditary genetics.

A third future direction is additional studies on GRLS variables and their relation to CCL rupture, especially those related to diet and behavior. The GRLS provides an extensive amount of information and there are many opportunities to expand research within CCL studies. Diet and behavior are of particular interest because it was seen that vitamin D levels may be a rupture factor and dogs must receive the vitamin through food, therefore studying dietary intake would be an excellent way to measure those level, in addition to being a proxy for the human diet. Behavior can also be measured to better understand how activity level impacts physiology, diet, and rupture rates. The bodily and hormonal changes associated with behavior may be an important factor in rupture and should be further studied.

These are just a few future directions, but there are ample opportunities to expand on this research, especially with usage of the GRLS. More research is needed to identify specific variables related to rupture. Dogs are an excellent and reliable model for human ACL studies and should continue to be utilized for human translational medicine.

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