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Determining the Effects of Exogenous Human Chorionic Gonadotropin and Pregnant Mare Serum Gonadotropin on Plasma Testosterone, Testicular Volume, and Semen Production in Leopard Geckos (Eublepharis macularius)

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DETERMINING THE EFFECTS OF EXOGENOUS HUMAN CHORIONIC GONADOTROPIN AND PREGNANT MARE SERUM GONADOTROPIN ON PLASMA TESTOSTERONE, TESTICULAR VOLUME, AND SEMEN PRODUCTION IN LEOPARD GECKOS (*EUBLEPHARIS MACULARIUS*)

A Thesis

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ABSTRACT

Planet earth has now entered into its sixth mass extinction as a result of expanding human populations, posing an unprecedented threat to biologic diversity. Climate change, habitat fragmentation, wildlife trade, pollution, and biological invasions are just a few consequences of human activities that have directly contributed to unprecedented species extinctions. While widespread amphibian declines have gained more recent attention, it has been proposed that reptiles may be in even greater danger of extinction worldwide. Unfortunately, many reptiles are destined to go extinct before many of their basic biologic traits, including reproductive methods, have even been determined.

In order to mitigate the substantial loss to biodiversity that the impending decline of reptiles represents, development and employing additional methods of in-situ and ex-situ conservation strategies will be paramount. Although assisted reproductive technologies (ART) have been developed and successfully utilized in a handful of wildlife and domestic species, these techniques have been historically underutilized in reptiles. ART will not only enhance our understanding of reptile reproductive biology and physiology, but will facilitate conservation efforts in these species to overcome reproductive barriers and by preserving genetic material to protect biodiversity in the future.

The goal of this research was to use exogenous hormones in the male leopard gecko (Eublepharis macularius) as a model for the development of ART in geckos and other endangered lizard species. The study was conducted from September-December in the Northern Hemisphere. Single injections of human chorionic gonadotropin (hCG) at 50 IU or 100 IU were ineffective at increasing plasma testosterone concentrations within 24 hours of injection. Electrostimulation was successfully used to collect serial semen samples. Pregnant mare serum
gonadotropin (PMSG) (20 IU, 50 IU) effectively increased testicular volume, spermatozoa concentration, motility, and the prevalence of morphologically normal sperm. Unilateral orchidectomies were successfully completed and allowed for histologic assessment of the testicles and epididymides without necropsy. PMSG was not successful at increasing circulating plasma testosterone concentrations. Ultimately, there is still time to prepare and slow the rate of global reptile declines. The development and refinement of ART in reptiles can aid in this mission to preserve biodiversity for future generations.
CHAPTER 1. INTRODUCTION

While only an estimated 2% of all of the species that ever lived are alive today, the absolute number of species is greater now than ever before (Raup and Sepkoski, 1982). However, the diversity of life on earth is being dramatically affected by human alterations to ecosystems (Baillie et al., 2004). Biodiversity loss represents the most serious consequence of the current environmental crisis, threatening both ecosystem health and human well-being (Ceballos et al., 2015; Barnosky et al., 2011). Reptiles are at the forefront of species extinctions, with recent estimates suggesting that one in five species of reptiles is currently threatened with extinction and 20% of all reptiles are currently classified as threatened (Böhm et al., 2013). However, current estimates are likely an under representation of the true number of reptiles faced with extinction due to a paucity of data for most species, large geographical gaps that have not been assessed, and ongoing discovery of new species as molecular technologies continue to be developed (Böhm et al., 2013). Thus, conservation of this vulnerable group of animals is of paramount importance. At this time, averting a dramatic decay of biodiversity and the subsequent loss of ecosystem services is still possible through intensified conservation efforts, but that window of opportunity is rapidly closing (Ceballos et al., 2015).

While a “One Plan Approach” of developing multi-disciplinary conservation strategies including in-situ and ex-situ management practices is ideal for the preservation of biodiversity in the long run, challenges, including effects of habitat degradation and climate change, make in-situ work for reptiles difficult. Thus, the short-term survival of many threatened species depends on ex-situ conservation strategies (Mestanza-Ramón et al., 2020), with assisted reproductive technologies (ART) playing a key role in many ex-situ plans. However, variations in anatomy, physiology, and endocrinology across reptile species is pronounced, posing major challenges for
the development of ART in these animals (Clulow and Clulow, 2016). Ultimately, more research is required across a variety of reptile species to aid in our understanding of their reproductive biology so that ART may be developed and we can provide a more certain future.

Geckos are one of three mega-diverse lineages of squamate reptiles known today, and, as a group, represent one fourth of all described lizards (Uetz, 2010; Uetz et al., 2020). As of January 2021, 185 (24.3%) of 762 extant species of geckos are included on the International Union for Conservation of Nature (IUCN) Red List and categorized as threatened (i.e., critically endangered, endangered, vulnerable) or near-threatened. Geckos began diversifying around 200 million years ago as a result of the major squamate radiation (Uetz et al., 2020). If current trends of human propagated biodiversity loss continue, 200 million years of gecko evolution and diversity will be obliterated in roughly 200 years of human industrialization. Currently, no assisted reproductive programs exist for this vulnerable group of animals; thus, there is a need to develop methods to understand the reproductive physiology of geckos.

Exogenous human chorionic gonadotropin (hCG) and pregnant mare serum gonadotropin (PMSG) have been used to manipulate the reproductive hormones of vertebrates, and have been integral to the development of assisted reproductive programs for mammals, birds and amphibians. However, the administration of exogenous mammalian gonadotropins to male lizards to stimulate reproductive functions, such as spermatogenesis and steroidogenesis, have only been assessed in less than 0.15% (9/6687) of all lizard species (http://www.reptile-database.org/db-info/SpeciesStat.html). PMSG has been determined to have FSH-like activity, whereas hCG has been noted to demonstrate more LH-like activity in reptiles, although conflicting findings exist on gonadal and endocrine responses to hCG. In reptiles, a one gonadotroph, two-cell theory is generally accepted, with the primary gonadotroph being a
“follicle stimulating hormone (FSH)-like protein”. In squamates, FSH has been found to stimulate both spermatogenesis and steroidogenesis, while LH has been found to have a similar qualitative effect, although to a lesser degree (Licht and Pearson, 1969; Reddy and Prasad, 1970a,b; Eyeson, 1971; Licht and Papkoff, 1971; Licht 1772a, b, 1974; Lance and Vilet, 1987; Vijaykumar et al., 2002; Jadhav and Padgaonkar, 2010). It is clear based on these previous results that additional research is needed if we hope to develop assisted reproductive programs to preserve these evolutionary sentinel species.

The overall goal of this thesis was to contribute to our current understanding of leopard gecko reproductive physiology by exploring the impacts of exogenous hormones (hCG and PMSG) on the male gecko reproductive system. The aim was to evaluate the effectiveness of these hormones for potential future implementation of ART in other geckos and endangered lizards. Leopard geckos were selected for use in both studies due to their abundance in captivity and minimal husbandry requirements. The objectives of the experiments were: 1) to determine if a single dosage of 50 IU or 100 IU hCG could increase circulating testosterone concentrations within 24 hours of administration, 2) determine if serial semen samples could be collected from leopard geckos using electrostimulation, 3) determine if ultrasound could be used to successfully measure testicle size in leopard geckos, and 4) determine if nine weekly doses of 20 IU or 50 IU PMSG could be used to increase plasma testosterone concentrations, testicular volume and weight, and spermatozoa concentration and motility over time.
CHAPTER 2. LITERATURE REVIEW

2.1. Gecko Taxonomy and Diversity

Lizards are the most widely distributed and diverse group of reptiles. The order Squamata comprises 95% of reptiles (Helmer and Whiteside, 2005) and is divided into three suborders: Lacertilia (lizards), Serpentes (snakes), and Amphisbaenia (worm lizards). Just over half of all known reptiles fall into three families: the colubrid snakes (25%), skinks (16%), and geckos (14%) (Uetz, 2000). Gecko diversity is further reflected by their classification into seven families, including Gekkoninae, Pygopodidae, Diplodactylidae, Eublepharidae, Phyllodactylidae, Sphaerodactylidae, and Carphodactylidae. They are among the most species-rich and geographically widespread terrestrial vertebrates, with nearly 2,000 described species, and represent one fourth of all described lizards (Uetz, 2010; Uetz et al., 2020).

Geckos inhabit five continents, with Australasia, Southeast Asia, Africa, Madagascar, and the West Indies representing the most species-rich locations (Uetz et al., 2020). The majority of gecko species live in subtropical to tropical forests, followed by rocky mountainous regions and shrub land (IUCN, 2020). Other species have been found to live in urban areas, caves, and dry savannas (IUCN, 2020). As a result of their diverse habitat ranges, geckos have developed a unique set of evolutionary adaptations that have allowed them to thrive in a wide variety of ecosystems. However, despite their wide habitat diversity, geckos continue to remain at increased risk of extinction. Because of the threats facing these lizards, it is important that we develop long term in situ and ex situ conservation plans to protect them. However, in order to be successful creating realistic plans, it is important to understand the unique traits of these animals, their environmental needs, and their reproductive cycle to develop appropriate assisted reproduction techniques. The purpose of this review is to provide an overview of gecko diversity
and some of the adaptions that have made this group of animals successful, a review of the challenges that they face, and an overview of their reproductive anatomy and physiology.

2.2. Adapt and Succeed

2.2.1. Pedal Diversity

Structural innovations in anatomical systems are associated with the diversification of many groups of vertebrates (Frazzetta, 1975). The gecko’s adhesive toe pads originated as early as the mid Cretaceous period (Bauer, 2019) and have been instrumental in their ability to occupy otherwise inaccessible regions through enhanced climbing and the ability to cling to surfaces (Higham et al., 2015). As geckos have become ecologically diverse, they have also developed a compendium of pedal specializations, including webbed feet, paraphalanges, and adhesive toepads (Bauer and Russell, 1991). These traits have enabled them to successfully inhabit a wide range of habitats through the enhancement of organism-substrate interactions (Higham et al., 2019). Setae, microscopic hair-like outgrowths of the superficial layer of the subdigital epidermis, are the key component of the adhesive apparatus in lizards (Autumn et al., 2002; Gamble et al., 2012; Manderson, 1964; Russell, 2002). Setae evolved from the microscopic spinules found on the outer epidermis on all limbed geckkotans and are hypothesized to aid in skin shedding (Manderson, 1970; Alibardi and Manderson, 2003). These setae have been determined to promote gecko adhesion via van der Waals forces and complex reactional interactions (Autumn et al., 2002; Gamble et al., 2012; Russell, 2002), allowing them to climb up vertical surfaces and occupy otherwise inaccessible regions of the locomotor habitat (Higham et al., 2015). In addition to setae, the gekkotan adhesive system also includes scansors (expanded digital scales) and modified skeletal elements of the foot and proximal parts of the limb; associated locomotor kinematics permit the system to be deployed (Higham et al., 2015).
Furthermore, numerous morphological traits and behaviors have evolved to facilitate deployment of the adhesive mechanism, maximize adhesive force, and enable release from substrate (Gamble et al., 2012). Approximately 60% of gecko species possess adhesive toe pads (Pianka and Vitt, 2003). It is currently thought that adhesive toepads originated independently eleven times and have been lost at least nine times (Gamble et al., 2012). During the periods of loss, adaptations to a terrestrial lifestyle that required the animals to run faster and burrow may have occurred, explaining the 40% of gecko species without adhesive pads (Higham et al., 2015). While the adhesive system opened a previously unobtainable environment to geckos, the loss of adhesion in terrestrial species has further contributed to the overall diversity of this group of specialized lizards.

2.2.2. Ocular Adaptations

The most recent common ancestor to squamates, prior to the divergence of geckos, was considered diurnal (Anderson and Wiens, 2017), although geckos are the only major lizard group to consist mostly of nocturnal species (Gamble et al., 2015). Nocturnality is thought to have evolved early in gecko evolution and these lizards possess many adaptations that have allowed them to function in low light and at low temperatures (Gamble et al., 2015). One of the most notable adaptations to nocturnality is the modifications that have occurred in the gecko eye. Geckos’ ability to see in low light conditions are enhanced by large eyes that allow for extreme degrees in constriction and dilation, retinas that lack fovea, multifocal color vision, short visual focal length, and rod-like photoreceptor cells in the retina that lack oil droplets (Gamble et al., 2015; Underwood, 1951, 1970; Kröger et al., 1999; Röll, 2000, 2001; Roth and Kelber, 2004). Most nocturnal vertebrates have retinas comprised of high numbers of rods that enhance visual sensitivity in dim lighting but cause them to be color blind at night. Geckos are unusual among
nocturnal vertebrates because they have no true rods in their retinas and instead have three cone types that provide them with color vision in dim light (Roth and Kelber, 2004). These cone cells are larger and more rod-like than diurnal lizards (Crescitelli, 1972; Röll, 2000). In addition to their ocular adaptations, nocturnal geckos also lack a parietal eye (Ralph, 1975), have enhanced acoustic communication (Marcellini, 1977) and a heightened sense of olfaction (Schwenk, 1993), and can sustain locomotion at low temperatures (Autumn et al., 1999; Gamble et al., 2015).

Conversely, there are over 430 species of diurnal geckos that have their own adaptations that enable them to thrive in warmer and brighter lit environments (Gamble et al., 2015). It is thought that these diurnal species have independently transitioned from nocturnality over their evolutionary history (Walls, 1942; Autumn, 1999; Röll, 2001) and that the shift in activity pattern may be a result of climate, predators, or competition (Gamble et al., 2015). The visual cells of diurnal geckos have undergone a second transmutation from the larger rod-like cells seen in nocturnal species to smaller cones (Röll, 2000). Other ocular adaptations in diurnal geckos include small, circular pupils and small eyes relative to body size (Werner, 1969), a new fovea in the temporal aspect of the retina (Röll, 2001), and lenses containing an ultraviolet filter consisting of vitamin A₂ bound to ι-crystallin (Röll et al., 1996; Röll and Schwemer, 1999). Additionally, diurnal geckos have returned to a higher energetic cost of locomotion compared with their nocturnal relatives (Autumn et al., 1999). Historically, gecko digit morphology, including toepads, and the shape of the pupil have been used to distinguish gecko genera (Uetz et al., 2020). These evolutionary changes further highlight the diversity of the Gekkonidae family and aid them in fulfilling multiple evolutionary and environmental niches.
2.2.3. Tail Autonomy

Lizards fall into two categories regarding tail adaptations: those that use tail autotomy (self-induced loss) and regeneration and those that do not (Vitt et al., 1977). Tails serve a variety of functions, including predator distraction, sexual displays, defense, balance, fat storage, stabilization, and climbing (Vitt et al., 1977). Species that undergo tail autotomy possess a vertical fracture plane through the body and part of the neural arch of each caudal vertebrae. This fracture plane is comprised of a plate of cartilage or connective tissue and develops after ossification (Barten and Simpson, 2019). Tail autotomy often occurs during predatory attacks, and it may increase an individual’s chance of escaping from predators (Congdon et al., 1974; Dial and Fitzpatrick, 1984; Daniels et al., 1986; Kelehear and Webb, 2006). Many gecko species wave or raise their tails during encounters with predators (Bustard, 1965) to direct attacks away from their torso and increase their likelihood of survival (Congdon et al., 1974, Daniels et al., 1986, Kelehear and Webb, 2006). However, recent evidence has shown that, in addition to exhibiting autotomy to avoid predation, geckos also drop their tails in response to intraspecific aggression between geckos (Itescu et al., 2016). Lost tails are replaced with a cartilaginous rod that lacks fracture planes, so subsequent tail autotomy generally occurs at any remaining vertebral fracture plane cranial to the previous break (Bellairs and Bryant, 1985) and regenerated tails are typically as large or larger than the original tails (Vitt et al., 1977). Despite the immediate benefit of tail autotomy, this behavior is not benign, and costs associated with it include: increased susceptibility to subsequent predation attempts (Daniels et al., 1986; Dial and Fitzpatrick, 1984; Downes and Shine, 2001), increased energy expense with regenerating lost tissue, potential social impacts, and costs to locomotion and behavior (Bateman and Fleming,
Tail autotomy has further contributed to the uniqueness of geckos and aided them in their broad environmental success.

2.3. Reproduction

2.3.1. Reproductive Methods

Gecko reproductive methods are as diverse as the geckos themselves. Squamates are the most recently evolved reptiles and exhibit the most diversity in morphology, physiology, and life-history traits (Lance, 2003). Most gecko species are oviparous (egg-laying), although there are also viviparous (live-bearing) and parthenogenic (asexual reproduction) species. Oviparity is thought to be the ancestral reproductive modality for vertebrates and reptiles, although this point has been argued by some who believe that viviparity in reptiles is the ancestral condition (Webb and Cooper-Preston, 1989). The typical squamate egg consists of a flexible eggshell, extraembryonic membranes (amnion, allantois, and chorion), and yolk, but may either lack, or have a varying degree of albumin component that is more largely present in the eggs of turtles and crocodilians (Girling, 2002; Packard et al., 1988). Additionally, geckos are the only known squamate today capable of producing fully calcified and rigid-shell eggs (Packard and Hirsch, 1988; Fernandez et al., 2015). Viviparity appears to have evolved a minimum of 108 times in squamate reptiles and occurs in roughly 20% of all squamate species (Sites et al., 2011). While there are several hypotheses for the origin of viviparity, the “cold climate hypothesis” (Tinkle and Gibbons, 1977) remains the preferred working hypothesis today (Sites et al., 2011). The cold climate hypothesis posits that cold regions, often times at high latitudes or elevations, favor viviparity (and therefore the incidence of viviparous squamates is increased in these regions) because viviparous females can use thermoregulatory behavior to shorten embryonic developmental time and reduce exposure of embryos to stressful temperatures (Tinkle and
Gibbons, 1977; Ma et al., 2017). In addition to avoiding the low viability of eggs laid in the nest and exposed to the cold (Tinkle and Gibbons, 1977), viviparity reduces embryo exposure to highly variable temperatures (Shine, 2002), or drought (Packard et al., 1977). Among vertebrates, true parthenogenesis, or the production of viable offspring in the absence of spermatozoa, has evolved only in squamates (Kearney et al., 2009). The origin of parthenogenesis is usually associated with a historical hybridization event between two closely related, sexual species (Sites et al., 2011); however, there are exceptions to this rule and the common tropical asexual gecko, Lepidodactylus lugubris, is thought to have originated spontaneously from a single species (Cuellar and Kluge, 1972; Cuellar, 1984; Pasteur et al., 1987). In addition to the diverse modes of reproductive strategies used by geckos, some species of gecko possess genetic sex determination (GSD), with both male and female heterogamety, while other species have temperature-dependent sex determination (TSD) (Gamble, 2010). In species with GSD, the sex of the developing embryo is determined by genetic factors at fertilization and depends on whether males (XY) or females (ZW) are heterozygous for the master sex determining gene (Gamble, 2010). Additional sex chromosomes, such as XXY, have also been found in Gekkonidae (Warner, 2011). Homozygosity leads to development of one sex with heterozygosity determining the other. In species with environmental sex determination (ESD), environmental factors during embryonic development determine the sex, with TSD being the most common environmental cue (Gamble, 2010). Geckos with TSD may either completely lack sex chromosomes or have homomorphic, poorly differentiated sex chromosomes (Pokorná et al., 2010).
2.3.2. Male Reproductive Anatomy

The male reproductive system in lizards consists of the gonads (testes), gonadoducts (testicular ducts), the sexual segment of the kidney (SKK), and the cloaca (Rheubert et al., 2010). The testes are intra-coelomic elongated, cylindrical in shape, and covered by a thin fibrous tunica albuginea (Gribbins and Rheubert, 2011). The testes lie caudodorsally to the liver, are suspended by the mesorchium, and are comprised of seminiferous tubules, which are composed of germinal epithelium and associated lumina (Rheubert et al., 2010). From the seminiferous tubules of squamates, spermatozoa sequentially pass through the rete testis, ductuli efferentes, ductus epididymis, ductus deferens, and, in some species, the ampulla ductus deferentis (Akbarsha et al., 2006, 2007; Server, 2010). The epididymides are tubular structures that lie lateral to each testicle and comprise the cranial portion of the ductus deferens (Stahl and DeNardo, 2019). In lizards, the primary role of the epididymis is secretion of cellular products into the lumen that is modulated by testosterone and other androgens (Haider and Rai, 1986; Depeiges and Dufaure, 1977), with most secretory activity occurring during the active breeding season (Rheubert et al., 2010). While the role of the secretory material is unknown, epididymal secretions in other amniotes function in the final maturation of spermatozoa. In lizards, the epididymis serves as the region for sperm storage, while in snakes the ductus deferens stores sperm (Rheubert et al., 2015). Posteriorly, the epididymis transitions into the ductus deferens, which joins the ureter posterior to the kidney and anterior to the cloaca, forming a ductus deferens-ureter complex that enters the cloaca at the urogenital papilla (Rheubert et al., 2015). Sperm usually mix with secretions from the SSK at this ductus deferens-ureter complex prior to entering the cloaca (Rheubert et al., 2015). The SSK has been found in the kidney ducts of all examined male squamates, although the location of the SSK differs between species (Rheubert et
In Gekkonidae, the collecting duct serves as the site of the SSK, and these structures hypertrophy during times of reproductive activity (Rheubert et al., 2011). While the function of the SSK has yet to be fully determined, seasonal variation in the SSK has shown strong correlation with spermatogenic activity, mating, and increased androgen concentrations (Bishop, 1959; Pandha and Thapliyal, 1964; Misra et al., 1965; Prasad and Sanyal, 1969; Krohmer, 1966; Rheubert et al., 2011). The secretions of the reproductive and urinary tracts drain into the urodeum of the cloaca through common pores associated with the urogenital papillae (Rheubert et al., 2015). The lizard copulatory organs (hemipenes) are paired, and each structure can be used interchangeably (Fox, 1977). The hemipenes are sac-like structures devoid of erectile tissue and are located laterally within the ventral tail, held in place by a retractor muscle (Denardo, 1996; Barten and Simpson, 2019; Rivera, 2008). The hemipenes serve to direct the ejaculate from the urodeum by capillary action into the sulcus spermaticus to facilitate spermatozoa transfer during copulation (Rivera, 2008; Johnson, 2004).

### 2.3.3. Testicles, Testosterone, and Spermatozoa

Reptiles have transitioned from anamniotes to amniotes, and this transition has been characterized by the move from external to internal fertilization, freeing them completely from water for breeding (Pudney, 1990; Gist et al., 2000). Testes have two important roles within reproductive morphology and physiology, the production of spermatozoa and the secretion of sexual steroids (Kumar et al., 2011). Lizard testes are comprised of tubular compartments (seminiferous tubules) and interstitial compartments (including steroid producing Leydig cells, blood and lymphatic vessels, nerves, and immune cells) (Kumar et al., 2011). Tight junctions between Sertoli cells occur within the germinal epithelium providing an effective blood-testis barrier in squamates (Baccetti et al., 1983). Seminiferous tubule epithelium in all amniotes...
studied to date has a permanent population of Sertoli cells; therefore, irrespective of the seasonal activity of the testes, Sertoli cell components are easily identified within the seminiferous epithelium (Gribbins and Rheubert, 2011). Sertoli cells are considered “nurturing cells” for developing germ cells and synthesize and store lipids to provide nutritional provisions for developing germ cells (Perry et al., 2020). Within Leydig cells, cholesterol constitutes the primary but probably not the only building block needed for the synthesis of steroid hormones (Limerman and Prasad, 1990; Saez, 1994). During spermatogenically inactive times, Leydig cells contain large lipid inclusions within their cytoplasm. The disappearance of these inclusions during spermatogenesis suggests that these cells are using lipids for steroid synthesis and spermatogenesis (Gribbins and Rheubert, 2011). Testosterone is required for the expression of secondary sexual characteristics, reproductive behavior in seasonal animals, and for the promotion and maintenance of spermatogenesis, acting at the androgen receptors on the peritubular and Sertoli cells (Sharpe, 1994; McLachlan et al., 1996). Lizards generally demonstrate elevated androgen concentrations in their plasma during spermiogenesis (Villagrán-SantaCruz et al., 2014).

2.3.4. Spermatogenesis and Breeding Strategies

Spermatogenesis is a complex process in which progenitor cells (spermatogonia) mitotically divide, undergo meiosis, and then differentiate into one of the few motile cells, spermatozoa, within the male vertebrate (Gribbins, 2011). Different germ cell development strategies exist between fishes and amphibians, birds and mammals, and reptiles. Fish and amphibians exhibit a temporal germ cell development strategy, whereby the testes are comprised of tubules/lobules lined with cysts where germ cells develop as a single population through the phases of spermatogenesis, and cyst eruption at the end of the maturation phase leads to a single
spermiation event releasing spermatozoa into a centralized lumen (Lofts, 1964; Rheubert et al., 2009). In contrast, in the spatial germ cell development strategy displayed by birds and mammals, germ cells proceed through phases of spermatogenesis in association with Sertoli cells, with new generations of spermatogonia displacing more advanced germ cells centrally towards the lumen of the seminiferous tubules; at any given time three to five generations of germ cells can be consistently found together (Gribbins and Gist, 2003). The consistent spatial relationships among germ cells are referred to as stages and a complete series of stages within the seminiferous epithelium is called a spermatogenic cycle (Russel et al., 1990). This cycle allows for multiple waves of spermiation during spermatogenesis (Gribbins and Gist, 2003). Reptiles possess amniotic testes comprised of seminiferous tubules lined with seminiferous epithelia; however, their germ cell development strategy has been previously described as intermediate. While the testicular structure resembles that of mammals and birds, their germ cell development more closely resembles that of an amphibian, with the majority of germ cell generations progressing through the stages of spermatogenesis as a single population (Gribbins and Gist, 2003; Gribbins, 2011). This temporal germ cell strategy has been observed in the Mediterranean gecko (Hemidactylus turcicus) and the European wall lizard (Podarcis muralis) (Rheubert et al., 2009; Gribbins and Gist, 2003). In seasonal breeders, including many temperate reptile species, once a single major spermiation event occurs in the summer or late fall, the testes enter a quiescent period where spermatogenesis slows or ceases altogether and pieces of Sertoli cells slough into the lumina of the seminiferous tubules (Gribbins, 2011). Conversely, in continuously breeding reptiles, once a cycle of spermatogenesis is complete, the basal compartment shows an increased rate of mitosis and meiosis and new generations of germ cells enter spermiogenesis and spermiation again as a single wave or cohort (Gribbins, 2011). Most
gecko species exhibit a seasonal reproductive cycle, including the Mediterranean gecko and rock gecko (*Phyllopus pollicaris*) (Rheubert et al., 2009; Righi et al., 2012), whereas others, including the Javanese house geckos and other neotropical species (Kluge’s dwarf gecko [*Lygodactylus klugei*], naked-toed gecko [*Gymnodactylus geckoides*]) (Church, 1962; Vitt, 1986) breed continuously regardless of season. Based on seasonal timing of spermatogenesis and mating, two major patterns for testicular cycles are recognized in reptiles: prenuptual and postnuptual (Saint Girons, 1963). In prenuptual species (majority of lizards), spermatogenesis takes place before or during the mating season, and a single annual peak of testosterone is generally seen corresponding to the culmination of spermatogenesis (Kumar et al., 2011). In postnuptual species (majority of chelonians), spermatogenesis takes place after the mating season and sperm is stored in the male or female ducts until the next mating season (Kumar et al., 2011).

There are three phases of sperm development: proliferative, meiotic, and maturational (spermiogenic). The proliferative phase is characterized by rapid successive mitotic divisions of the spermatagonia that lead to a large clonal population of germ cells, followed by the meiotic phase where spermatocytes undergo reduction divisions, segregation, and recombination of genetic material. The final aspect of spermatogenesis is the maturational or spermiogenic phase, where haploid undifferentiated spermatids transform into specialized sperm cells that are equipped and capable of reaching and fertilizing eggs (Gribbins, 2011). Spermiogenesis is the longest phase of germ cell development and includes the development of the acrosome complex, elongation of the nucleus, and condensation of chromatin material (Perry, 2020). Once spermiation is complete, mature spermatozoa are shed from the seminiferous epithelium and enter the lumen of the seminiferous tubules (Gribbins and Rheubert, 2011).
2.4. The Challenge Ahead

2.4.1. Decline of Reptiles

Most gecko species have only been discovered over the last 40 years; however, as new technologies are developed to aid in our understanding of gecko diversity on a molecular basis, many species may already have gone extinct (Uetz et al., 2020). Geckos are a highly diverse group of animals, and many recently discovered species have only been found in limited habitats such as small islands or areas of exposed karst (Uetz et al., 2020). Unfortunately, the combination of a small native range and narrow niche requirements makes reptiles susceptible to anthropogenic threats such as habitat loss and degradation, unsustainable trade, introduced invasive species, environmental pollution, and climate change (Böhm et al., 2013; Gibbons et al., 2000; Todd et al., 2010). In fact, many reptiles are destined to disappear before anything is discovered of their basic biology, including reproduction (Lance, 2003).

The IUCN Red List of Threatened Species has been a global leader in assessing the status of many floral and fauna species (Baillie et al., 2004), serving as a standard to assess global species’ risk of extinction based on quantitative criteria. While the IUCN has comprehensively assessed birds, mammals, and amphibians to date, a global reptile assessment was more recently initiated with reptiles remaining one of the least studied vertebrate taxa (Todd et al., 2010). Unfortunately, through these methods, only 61% (6,680/10,793) of known reptiles have been evaluated (Perry, 2020). Due to the rigorous and time intensive process of determining a species’ conservation status, counts of “officially” recognized endangered and threatened species are likely to grossly underestimate the actual number of imperiled species (Gibbons et al., 2000). Thus, other studies relying on Red List data as a means to evaluate at risk reptile populations are also gross underestimates. Without taking this underestimation into account, by the year 2050, as
many as 76% of reptiles could be committed to extinction based on projections of species’ distributions for future climate scenarios (Thomas et al., 2004). Other studies evaluating extinction rates in reptiles have estimated that one in five reptile species are currently threatened with extinction and that about 20% of reptiles are threatened (Böhm et al., 2013).

2.4.2. Loss of Biodiversity

Loss of biodiversity is the most serious consequence of the current environmental crisis (Ceballos et al., 2015). Biodiversity is the number, abundance, composition, spatial distribution, and interactions of genotypes, populations, species, functional type and traits, and landscape units in a given system (Díaz et al., 2006). Biodiversity provides priceless ecosystem services, such as pollination, regulation of climatic conditions, control of agricultural pests and diseases, and support of the production of food, potable water, shelter, and medicines (Díaz et al., 2006). Understanding the causes of population declines and extinctions is of vital importance in the quest to stop currently rampant biodiversity loss (Kotiaho et al., 2005). Modern extinction rates have increased sharply over the past 200 years and correspond to the rise of industrial society (Ceballos et al., 2015). The loss of global biodiversity is not occurring at random, but instead is a consequence of climate change, biological invasions, and unsustainable land use (Díaz et al., 2006; Baillie et al., 2004; Mace et al., 2005; Kotiaho et al., 2005; McKinney and Lockwood, 1999). Anthropogenic impacts, including habitat fragmentation and environmental pollution, directly impact species survival by interfering with their ability to reproduce successfully through restriction of natural ranges, limiting their ability to find mates, and increased inbreeding that results in impairment of physiologic functions and pregnancy sustainability (Holt and Pickard, 1999). Many environmental contaminants and by-products of modern manufacturing processes (e.g., plastics) have also been found to induce weak estrogenic or anti-androgenic
activity, which may have deleterious effects on endocrine functions in many species (Holt and Pickard, 1999; Turner and Sharpe, 1997). For example, the reproductive abnormalities (e.g., smaller phallus size, lower plasma androgen concentrations, and lack of responsiveness of the phallus to plasma androgens) observed in alligators living on Lake Apopka have been linked to environmental exposure to the antiandrogenic DDT breakdown product \( p,p' \)-DDE (Guillette et al., 1996). The negative synergistic effects of inbreeding and environmental contamination on fertility, in addition to the other above-mentioned anthropogenic influences, contribute to artificially accelerated population decreases, and along with further climatic changes through increased greenhouse gas emissions, will be difficult to reverse (Holt and Pickard, 1999). When the number of individuals in a population or species drops too low, its contributions to ecosystem functions and services become unimportant, its genetic variability and resilience is reduced, and its contribution to human welfare may be lost (Ceballos et al., 2020). The rapid loss in biodiversity over the last few centuries indicates that a sixth mass extinction is already under way, and only through intensified conservation efforts (Ceballos et al., 2015) and implementation of technologies to decrease greenhouse gas emissions and strategies for carbon sequestration, may we avert the dramatic decay of biodiversity and loss of ecosystem services.

\subsection*{2.4.3. Need for Conservation}

The overall goal of conservation is to mitigate the loss of biodiversity and preserve ecosystem services, species, and genetic diversity for the future (Schwartz et al., 2017). Currently, there is a great need for a “One Plan Approach” to develop multi-disciplinary conservation strategies, including integration of \textit{in-situ} and \textit{ex-situ} management practices (Schwartz et al., 2017; Byers et al., 2013). \textit{In-situ} (on-site) strategies focus on protecting and managing natural habitats (wildlife reserves, on-farm and community-based), whereas \textit{ex-situ}
(off-site) conservation focuses on captive breeding and sampling, transfer, and storage of species (e.g., botanic or zoological gardens, seed/semen/ovule storage, or gene banks maintained under special artificial conditions) (Mestanza-Ramón et al., 2020). While in-situ conservation strategies provide the best long-term options for biodiversity conservation, the short-term survival of many threatened species depend on ex-situ conservation strategies (Mestanza-Ramón et al., 2020). Development of assisted reproductive technologies (ART) is a key component of ex-situ conservation strategies. However, successful application of assisted reproductive techniques, such as artificial insemination, in vitro fertilization, embryo transfer, and germplasm cryobiology, for enhancing propagation is directly related to the amount of basic reproductive information available from each species (Comizzoli and Holt, 2014), making the successful implementation of ART in wild and endangered species challenging.

Reproduction is core to species survival, so understanding how an animal breeds is fundamental to conserving species, populations, and, indirectly, the vitality of entire ecosystems (Wildt et al., 2003). However, the variation in gross anatomy, physiology, and endocrinology across reptile species is pronounced, posing major implications for the development of reproductive technologies in these animals (Clulow and Clulow, 2016). Despite the bewildering array of reproductive modes exhibited by reptiles, including TSD, parthenogenesis, and viable sperm retention in the female tract for up to five years, there is little information on the physiology and hormonal control of reproduction for most species (Lance 2003). Ultimately, reptiles are a neglected taxon in wildlife and non-domestic animal assisted reproductive technology research (Clulow and Clulow, 2016). However, spermatology research combined with simultaneous efforts in endocrinology, embryology, and cryopreservation (among others) can lead to the successful application of assisted reproduction (Wildt et al., 1995).
2.5. Developing Assisted Reproductive Technologies in Lizards

2.5.1. Spermatozoa Collection and Storage

Currently, our working knowledge of spermatozoa collection in male reptiles is limited to our ability to collect semen from less than 0.2% of all reptile species (Perry, 2020). In lizards, successful semen collection has only been achieved in 9 species.Electrostimulation has been used to collect semen from green iguanas (Iguana iguana), Grand Cayman blue iguana hybrids (Cyclura lewisi x nubila), leopard geckos (Eublepharis macularis), veiled chameleons (Chamaeleo calyptratus), panther chameleons (Furcifer pardalis), Chaco spiny lizards (Tropodurus spinulosus), and Texas rock lizards (Sceloporus torquatus) (Zimmerman et al., 2013; Funcke et al., 2015; Perry et al., 2019; Perry, 2020; López et al., 2018; Martínez-Torres et al., 2019), while coelomic massage has been used to collect semen from McCann’s skinks (Oligosoma maccanni) and common house geckos (Hemidactylus frenatus) (Molinia et al., 2010; Todd, 2003). The ability to collect spermatozoa from reptiles is an important step in being able to develop ART in these animals. Furthermore, it has been projected that spermatozoa cryopreservation will be the ART that yields the most progress in the near future, almost certainly resulting in the production of live young when combined with improvement and optimization of artificial insemination protocols (Culow and Culow, 2016). However, while it is relatively easy to define spermatozoa structure, there are few data on membrane biophysical properties, even in common domestic and laboratory species (Comizzoli and Holt, 2014). The need for comparative/systematic approaches extends to cryopreservation studies of gametes, embryos, gonadal tissues, and stem cells (Comizzoli et al., 2012). Methods for freezing, storing, and thawing these biomaterials are well established for improving breeding efficacy in livestock, to sustain specific laboratory animal genotypes, and for addressing certain subpar fertility
conditions in humans (Mazur et al., 2008). Systematic studies can lead to successful post-thaw recovery, especially after determining: 1) membrane permeability to water and cryoprotectant, 2) cryoprotectant toxicity, 3) tolerance to osmotic changes, and 4) resistance to cooling and freezing temperatures (Comizzoli et al., 2012). There are currently few studies in reptiles evaluating short-term semen extension or long-term biobanking. Semen extenders have only been studied in three lizards to date. In the McCann’s skink, spermatozoa stored in Ham’s F-10 maintained 70% motility after five days incubation at 4°C (Molina et al., 2010). In green iguanas, spermatozoa motility was found to decrease during refrigeration from an initial motility of 78% to 33% after 48 hours; the samples were stored in Ham’s F-10 with albumin (Zimmerman et al., 2013). Spermatozoa motility was also poor in green anole (Anolis carolinensis) samples extended in both commercial extenders and crystalloids (Perry et al., 2020). Potential reasons for the poor response in the green anoles were attributed to the possibility of intrinsic spermatozoa mechanisms being responsible for motility in this species, that an important substrate was absent in the extenders, or that the spermatozoa concentrations in the samples were too low to be successful (Perry, 2020). Ultimately, additional research is needed to assess the specific mechanisms required to successfully extend lizard spermatozoa to develop future ART for these animals. Attempts at cryopreservation in lizards are also limited, with only two examples in Argentine black and white tegus (Tubinambis merianae) and Eastern water skinks (Eulamprus quoyii) (Hobbs et al., 2018; Young et al., 2017). In the Argentine black and white tegu, substantial levels of motility (~40%) were recovered using the lowest cooling rate (0.3°C min⁻¹), suggesting that lizards may need a slower cooling rate for cryopreservation. The minimum spermatozoa quality parameters necessary for successful fertilization are not currently known for any reptile (Young et al., 2017), and successful fertility using cryopreserved spermatozoa has not
been confirmed in any reptile (Culow and Culow, 2016). Artificial insemination (AI) is another potential ART that may be used in reptiles; however, there has only been one unsuccessful attempt in a lizard to date (Molina et al., 2010). AI in reptiles is currently an inefficient process due to challenging logistics associated with simultaneously organizing both semen collection and the availability of females for insemination (Culow and Culow, 2016). Additionally, validation of AI trials requires genetic verification of the parentage of the offspring due to other potential complicating factors, including parthenogenesis (Groot et al., 2003; Neaves and Baumann, 2011; Neaves, 2014) or from stored spermatozoa in the female oviducts from previous copulations. Ultimately, additional studies are needed to further evaluate effective semen extenders and cryopreservation techniques, and to reduce obstacles to AI for lizards.

2.5.2. Hormonal Manipulation in Developing ART

In addition to the above-mentioned tools for assisted reproduction, hormonal manipulation has also been evaluated for ART in reptiles. The administration of exogenous mammalian gonadotropins to male lizards has been previously used by researchers in order to stimulate spermatogenesis and steroidogenesis in less than 0.13% (9/6687) of all lizard species (http://www.reptile-database.org/db-info/SpeciesStat.html). Current studies on exogenous hormonal administration in male lizards are limited to the following species: little brown skinks (Leiолopisma laterale), common agamas (Agama agama), green anoles, common wall lizards (Lacerta muralis), broadhead skinks (Eumeces laticeps), Indian spiny-tailed lizards (Uromastix harkwickii), Indian wall lizards (Hemidactylus flaviviridis), oriental garden lizards (Calotes versicolor), and veiled chameleons (Arslan et al., 1977; Edwards et al., 2004; Eyeson, 1971; Haider et al., 1987; Jalali et al., 1976; Jones, 1973; Licht and Pearson, 1969; Licht and Papkoff, 1971; Perry, 2020; Prasad and Sanyakl, 1969; Reddy and Prasad, 1970a, 1970b; Sonar and Patil
Exogenous hormones have varying results when administered to reptiles. It has been proposed that the squamate pituitary gland may secrete only one gonadotropin (or a gonadotropin complex), with biological properties of both follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Licht, 1984). If true, it would make snakes and lizards the only amniotes with such an unusual reproductive system (Lance, 2003). This idea is referred to as the one gonadotroph, two cell theory. In squamates, FSH has been found to stimulate both spermatogenesis and steroidogenesis, while LH has been found to have a similar qualitative effect, although to a lesser degree (Licht and Pearson, 1969; Reddy and Prasad, 1970a,b; Eyeson, 1971; Licht and Papkoff, 1971; Licht 1772a, b, 1974; Lance and Vilet, 1987; Vijaykumar et al., 2002; Jadhav and Padgaonkar, 2010).

Exogenous pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) are used to alter reproductive hormones in vertebrates, and have been integral to the development of ART for mammals, birds, and amphibians; however, our understanding of the potential roles of these hormones in reptiles is limited. Like FSH, PMSG is capable of stimulating testicle growth, spermatogenesis, and steroidogenesis in some squamates, including Indian spiny-tailed lizards (Arslan et al., 1975, 1977; Jalali et al., 1976), oriental garden lizards (Sonar and Patil, 1994), and common agamas (Eyeson, 1971). hCG has been previously thought to possess similar properties to LH, although conflicting findings exist in reptiles on the gonadal and endocrine responses to hCG. In oriental garden lizards, hCG alone was effective at stimulating spermatogenesis and steroidogenesis, but to a lesser effect than PMSG alone or when combined with PMSG (Sonar and Patil, 1994). These results were similar to what was observed in the spiny tailed lizard, where hCG (50 IU/animal) administered on two consecutive days was effective at increasing plasma testosterone concentrations; however, PMSG administered in the
same manner yielded plasma testosterone concentrations that were twice as high. These findings suggest that hormones with FSH-like activity have a more generalized effect on reptile testicles (Arslan et al., 1977). In the New Guinea bockadam (Cerebrus rhinchops), daily administration of hCG at 50IU/day for 14 and 28 days did not induce any major changes in the spermatogenic compartment of the testicles, but the interstitial cells, epididymal epithelium, and ductus deferens epithelium were stimulated, indicating increased androgen production from the Leydig cells (Jadhav and Padgaonkar, 2010). hCG was also found to have a greater stimulatory effect on the interstitial cells of little brown skinks compared to administration of LH (Jones, 1973). Recent findings in veiled chameleons confirm that a single dose of hCG (100 IU/animal) can increase circulating plasma testosterone concentrations; however, no impact on spermatozoa production was observed (Perry, 2020).
CHAPTER 3. MEASURING THE EFFECTS OF A SINGLE DOSE OF HUMAN CHORIONIC GONADOTROPIN (HCG) ON PLASMA TESTOSTERONE CONCENTRATIONS IN LEOPARD GECKOS (*EUBLEPHARIS MACULARIUS*)

3.1. Introduction

Anthropogenic activity is currently accelerating earth’s 6th mass extinction. The combination of small home ranges and narrow niche requirements has increased the susceptibility of reptiles to anthropogenic threats, including: habitat loss, trade, invasive species, pollution, and climate change (Böhm *et al*., 2013; Gibbons *et al*., 2000; Todd *et al*., 2010). Current estimates suggest one in five lizard species are in danger of extinction, and that about 20% of reptiles are threatened (Böhm *et al*., 2013). As of January 2021, 185 (24.3%) of 762 extant species of geckos are included on the International Union for Conservation of Nature (IUCN) Red List and categorized as threatened (i.e., critically endangered, endangered, vulnerable, or near threatened) or near-threatened. Geckos are among the most species-rich and geographically widespread terrestrial vertebrates, with nearly 2,000 described species that represent one fourth of all described lizard species (Uetz, 2010; Uetz *et al*., 2020). Most gecko species were only discovered over the last 40 years; however, as new molecular technologies are developed to aid in our understanding of gecko diversity, many species may have already gone extinct (Uetz, *et al*., 2020). Despite geckos representing one of the most diverse groups of reptiles on earth, clinical assisted reproductive programs are non-existent for these animals but will be needed for future conservation efforts.

Exogenous human chorionic gonadotropin (hCG) and pregnant mare serum gonadotropin (PMSG) are used to alter the reproductive hormones of vertebrates and have been integral to the development of assisted reproductive programs for mammals, birds, and amphibians. However, studies on exogenous hCG or PMSG administration in male lizards are limited to the following
nine species: little brown skinks (*Leiolopisma laterale*), common agamas (*Agama agama*), green anoles (*Anolis carolinensis*), common wall lizards (*Lacerta muralis*), broadhead skinks (*Eumeces laticeps*), Indian spiny-tailed lizards (*Uromastix harkwickii*), Indian wall lizards (*Hemidactylus flaviviridis*), oriental garden lizards (*Calotes versicolor*), and veiled chameleons (*Chamaeleo calyptratus*) (Arslan et al., 1977; Edwards et al., 2004; Eyeson, 1971; Haider et al., 1987; Jalali et al., 1976; Jones 1973; Licht 1969,1971; Perry, 2020; Prasad and Sanyakl,1969; Reddy and Prasad, 1970a, 1970b; Sonar and Patil 1994; Vijaykumar et al., 2002). These examples represent <0.13% (9/6687) of all lizards (http://www.reptile-database.org/db-info/SpeciesStat.html), reinforcing the need for additional research.

Exogenous mammalian gonadotrophins administered to reptiles do not appear to have the same actions reported in mammals. In contrast to mammals, FSH has been found to stimulate both spermatogenesis and steroidogenesis in squamate reptiles; mammalian LH has a similar qualitative effect, although it is much less potent (Licht and Pearson, 1969; Reddy and Prasad, 1970a,b; Eyeson, 1971; Licht and Papkoff, 1971; Licht 1772a, b, 1974; Lance and Vilet, 1987; Vijaykumar et al., 2002; Jadhav and Padgaonkar, 2010). A one gonadotroph, two cell theory has been suggested for squamates, whereby a single gonadotropin or gonadotropin complex performs functions that are carried out by FSH and LH independently in mammals. However, not all reptiles adhere to this theory (Licht and Hartree, 1971; Jones, 1973; Jalali et al., 1975; Arslan, 1977). In some reptiles, two gonadotrophs have been found to work synergistically, with PMSG serving FSH-like functions and hCG demonstrating more LH-like activity. Conflicting reports on the activity of hCG on gonadal and endocrine responses in squamates suggests further study is warranted.
Recent findings in veiled chameleons confirm hCG (100 IU/animal) can increase circulating plasma testosterone concentrations; however, no impact on spermatozoa production was observed, suggesting this species may not follow a one gonadotroph, 2-cell theory (Perry, 2020). In the spiny tailed lizard, hCG (50 IU/animal) administered on two consecutive days was effective at increasing plasma testosterone concentrations; however, PMSG administered in the same manner yielded plasma testosterone concentrations that were twice as high, indicating that hormones with FSH-like activity have a more generalized effect on reptile testes in this species (Arslan et al., 1977). Furthermore, PMSG was found to stimulate a four-fold increase in testicular weights and luminal diameter of the testis tubules in the common agama compared to controls, while hCG had a much smaller qualitative effect on testicle size and tubule diameter (Eyeson, 1971). Unfortunately, endogenous hormone concentrations were not measured in the study. hCG and PMSG were both found to increase seminiferous tube diameters, leading to increased testicular weights, during the quiescent phase in the oriental garden lizard. The results also confirmed that these hormones stimulated germ cell development (Sonar and Patil, 1994). Similar findings were reported for little brown skinks treated with 1 IU of ovine luteinizing hormone (oLH), hCG, ovine follicle stimulating hormone (oFSH), oFSH and LH, and PMSG intracoelomically during the quiescent stage of their reproductive cycle. Necropsies 15 days after treatment revealed increased interstitial cell numbers, interstitial cell hypertrophy, cytoplasmic granulation, and increased epididymal and sexual segment epithelial height (Jones, 1973). The authors did not measure testosterone concentrations in these animals, although the gonadal data suggests there was a physiological stimulation of testosterone. A histological and histochemical study of the epididymides of Indian wall lizards, a species of gecko, treated with LH, oFSH, hCG, PMSG, and testosterone propionate concluded that FSH by itself was capable of
stimulating the growth and secretory activity of the epididymides based on the variation in epididymal cell height and luminal diameter during the different phases of the reproductive cycle (Prasad and Sanyal, 1969; Reddy and Prasad, 1970a, b; Haider and Rai, 1987). Additional studies performed in green anoles, broadhead skinks, and common wall lizards (Herlant, 1933; Evans, 1935; Turner, 1935) have yielded similar results. Based on these findings, PMSG may have FSH-like activity in reptiles and suggests that FSH stimulation is secondary to the preparation of the male reproductive tract with hCG.

The purpose of this study was to determine if an exogenous hormone, hCG, could be used to increase circulating testosterone concentrations in a model species of gecko, the leopard gecko (Eublepharis macularius). Our objective was to determine an effective dose of hCG for increasing plasma testosterone concentrations within 24 hours of administration. The hypotheses tested in this study were that: 1) administering exogenous hCG would increase circulating testosterone concentrations in leopard geckos within 24 hours of administration, 2) testosterone concentrations would be highest in the animals administered 100 IU of hCG, and 3) testosterone concentrations would be highest at 24 hours post administration of hCG.

3.2. Materials and Methods

3.2.1. Ethics Statement

This longitudinal experimental study was performed in accordance with the rules and regulations established by Louisiana State University’s institutional animal care and use committee (protocol # 20-043).

3.2.2. Study Species

Twelve captive-bred, adult, male leopard geckos were used for this study. The sample size was determined using the following a priori information: an alpha=0.05, a power=0.80, a 2:1
ratio of hCG: control animals, an expected >40 pg/mL difference in testosterone concentrations between hCG and control animals, and a 20 pg/mL standard deviation (SD) for each group.

3.2.3. Husbandry

Animals were individually housed at Louisiana State University in 43 x 21 x 25 cm clear, plastic containers. The environmental temperature range and humidity in the climate-controlled room were 28-29°C (83-85°F) and 30-40%, respectively. The geckos were housed on a paper substrate and provided a hiding area and water bowl. A 12-hour photoperiod was provided with standard fluorescent lighting. The geckos were fed a diet consisting of gut-loaded house crickets (*Acheta domestica*) (Fluker Farms, Port Allen, LA) three times weekly; the amount offered was based on 3% of the geckos’ body weight. Physical examinations were performed on each leopard gecko prior to initiating the study to confirm that they were healthy.

3.2.4. hCG Dose Determination Study

A prospective experimental study was conducted in September 2020. Timing for the study was based on the expected non-breeding season (quiescent period) for leopard gecko reproduction in the Northern Hemisphere (September-December) (de Vosjoli *et al.*, 2005). Twelve adult male leopard geckos weighing at least 75g were randomly divided into three treatment groups (group 1: control [saline], n=4; group 2: 50 IU hCG/animal, n=4; group 3: 100 IU hCG/animal, n=4) using a random number generator (random.org). The chosen doses of hCG (human chorionic gonadotropin, lyophilized powder, 2,500 IU, Sigma Aldrich, C1063, St Louis, MO, USA) were based on previous work done with amphibians and reptiles (Eyeson, 1971, Kouba, 2012, Perry, 2020). The hCG was reconstituted with 5 ml of sterile water (Hospira Inc., RL-4428 Lake Forest, IL, USA) to 500 IU/mL and each treatment animal administered the appropriate dose intramuscularly in the right triceps (50 IU, 0.1 mL) or divided between both
triceps (100 IU, 0.2 mL). Control animals received an injection of sterile 0.9% saline (Abbott Laboratories, North Chicago, IL, USA) at either 0.1 mL in the right triceps or 0.2 mL divided between both triceps. Whole blood was collected from each individual from the ventral tail vein (Figure 3.1) or cranial vena cava prior to the hCG injection (time 0, baseline) and then 12 hours and 24 hours post-injection using a heparinized 25-gauge needle fastened to a 1 mL syringe. The geckos were anesthetized for venipuncture using an induction chamber with 5% isoflurane and 3 L oxygen/minute. Once the geckos lost their righting reflex, they were removed from the chamber and maintained on 4% isoflurane and 2 L oxygen/minute administered via face mask. A total of 0.2 ml whole blood was collected at each time point, ensuring total blood volume collected was <0.8% body weight (all geckos >75 grams). Blood samples were placed into lithium heparin microtainers (B-D Vacutainer Systems, Franklin Lakes, NJ, USA) and separated into components using centrifugation at 4,000 x g for 8 minutes. Plasma was aliquoted into cryovials and frozen at -80°C (-112°F) until it was analyzed for plasma testosterone concentrations.

![Figure 3.1. Blood collection via ventral tail vein.](image)
3.2.5. Testosterone Assay

An enzyme immunoassay kit (EIA) (Arbor Assay DetectX Testosterone K032-H5, Ann Arbor, MI, USA) was used to measure plasma testosterone concentrations. First, the EIA kit was optimized for this species. Plasma testosterone was extracted using a liquid extraction method based on the manufacturer’s protocol. Plasma samples (50µL) were aliquoted into microcentrifuge tubes, 400µL of ethyl-acetate (Sigma Aldrich, St. Louis, MO, USA) added to each tube, and the samples vortexed for two minutes. Following vortexing, samples were allowed to rest for 5 minutes before being submerged into liquid nitrogen. Following freezing, the ethyl acetate layer was poured off into a new microcentrifuge tube; this process was repeated three times. The pooled solvent layer was dried in a Speedvac (Thermo Fisher Scientific, Waltham, MA, USA) before being reconstituted with 300 µL of assay buffer prior to analysis. For analysis, samples were diluted at a ratio based on 50% binding for the assay with the assay buffer determined from a parallelism procedure as described below. In brief, the microtiter plates were loaded with 50 µL of the plasma extracts, controls, and standards in duplicate; this was followed by the addition of the conjugate and antibody to each well. Plates were then incubated at room temperature for two hours on a microplate shaker (VWR International, Radnor, PA, USA) at 300 rpm. Following incubation, the plates were washed with the wash buffer, aspirated four times, and allowed to dry. Next, substrate was added to the wells and allowed to incubate for 30 minutes at room temperature without shaking; this was followed by the addition of a stop solution. Absorbance was measured at 450 nm using an Epoch Microplate Spectrophotometer (Bioteck Instruments Inc., Winooski, VT, USA). Final plasma concentrations were calculated based on the plate output, dilution factors (see parallelism), and extraction efficiency. The
published sensitivity for this assay is reported to be 9.92 pg/mL, with a limit of detection at 30.6 pg/mL.

3.2.6. Parallelism

Parallel displacement between the standard curve (Figure 3.2) and serial dilutions of plasma extracts were used to determine immunological similarities between the standard and sample hormones. A pooled sample of plasma extracts previously collected from the LSU leopard gecko colony and stored at -80°C was serially diluted from 1:2 to 1:1024 in assay buffer and run with the standard curve. Parallel displacement of the curve was observed with the serial dilution. The ideal dilution with approximately 50% binding was 1:16; based on this finding, dilutions were performed at 1:20.

![Figure 3.2. Parallelism graph for leopard gecko testosterone EIA.](image)

3.2.7. Precision and Accuracy

To determine the repeatability of the assay, intra- and inter-assay coefficients of variation (CV) were measured. Intra-assay CV were measured by examining the CV of each sample run in duplicate. Inter-assay CV were measured by examining CV of select samples run on different plates. Values with CV >15% were re-analyzed; samples with CV <15% were considered data.
3.2.8. Statistical Analysis

Testosterone concentrations were evaluated for normality using the Shapiro-Wilk test, skewness, kurtosis, and Q-Q plots. Normally distributed data are reported by the mean, SD, and minimum-maximum values, while non-normal data are reported by the median, 25-75%, and min-max values. Non-normal data were log transformed for parametric analysis. A repeated measures ANOVA was used to determine whether treatment group or time had an impact on testosterone concentrations. Mauchly’s test for sphericity was used to test covariance. Gecko was the random variable in the model, time was the within subjects variable, and treatment group the between subjects variable. An observed range for testosterone concentrations was generated following outlier detection testing with the Tukey test. SPSS 24.0 (IBM Statistics, Armonk, NY, USA) (data distribution, data transformation, repeated measures ANOVA) and MedCalc Statistical Software version 19.1.3 (MedCalc Software bv, Ostend, Belgium;) (outlier detection) were used to analyze the data. A p ≤0.05 was used to determine statistical significance.

3.3. Results

There were no significant differences in testosterone concentrations based on time (F= 0.592, p= 0.564) or treatment group (F= 1.97, p= 0.195) (Table 1). Because there were no significant differences, the three testosterone concentrations from each gecko were averaged to create baseline descriptive statistics. A single outlier was detected (gecko 5, testosterone: 210.7 ng/mL) and was removed. The following represents observed testosterone concentrations for captive male leopard geckos in September in the Northern hemisphere: mean±SD, 61.0±42.5; min-max, 5.5-149.0.
Table 3.1. Plasma testosterone concentrations (ng/mL) at times 0 (prior to injection), and 12 and 24 hours post injection of saline, hCG 50 IU, and hCG 100IU. There were no significant differences by time or treatment group.

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment Group</th>
<th>Median 25-75%</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>Saline</td>
<td>52.3</td>
<td>22.3-117.8</td>
</tr>
<tr>
<td></td>
<td>50 IU</td>
<td>18.5</td>
<td>0.44-24.8</td>
</tr>
<tr>
<td></td>
<td>100 IU</td>
<td>124.6</td>
<td>21.1-178.8</td>
</tr>
<tr>
<td>12 hours</td>
<td>Saline</td>
<td>81.5</td>
<td>5.9-146.5</td>
</tr>
<tr>
<td></td>
<td>50 IU</td>
<td>37.1</td>
<td>9.6-140.9</td>
</tr>
<tr>
<td></td>
<td>100 IU</td>
<td>43.7</td>
<td>.92-179.5</td>
</tr>
<tr>
<td>24 hours</td>
<td>Saline</td>
<td>109.7</td>
<td>63.3-148.5</td>
</tr>
<tr>
<td></td>
<td>50 IU</td>
<td>14.7</td>
<td>5.3-25.8</td>
</tr>
<tr>
<td></td>
<td>100 IU</td>
<td>91.4</td>
<td>12.4-393.0</td>
</tr>
</tbody>
</table>

3.4. Discussion

The results of this study did not support the authors’ original hypotheses. The administration of exogenous hCG did not increase circulating testosterone concentrations 12- or 24-hours post-injection, and there was no significant difference in the testosterone concentrations between the animals receiving saline, 50 IU hCG, or 100 IU hCG. This study did validate the use of a commercial EIA for measuring testosterone in leopard geckos, which will be useful for future studies using this species as a model, and is the first to objectively assess the proposed non-breeding season of leopard geckos, finding that these animals are not in quiescence in September in the Northern hemisphere.

The findings of this study are in contrast to others in reptiles that directly measured testosterone concentrations and testicular histology following administration of hCG (Jadhav and Padaonkar, 2010; Sonar and Patil, 1994; Jones, 1973; Eyeson, 1971; Perry, 2020). In these previous studies, significant increases in circulating testosterone were measured and histology of the testicles noted an increase in the interstitial cells at the level of the epididymis, suggesting probable androgen production. However, in these aforementioned studies, sampling was
conducted during the quiescent phases of reproduction for each species. In the present study, we selected a time (September) that has been proposed as the non-breeding season, and assumed it would represent the beginning of the quiescent phase of the reproductive cycle for captive leopard geckos (de Vosjoli et al., 2005); however, based on our results, the authors suggest that these animals were more likely to be in the recrudescent phase than in true quiescence. In the house gecko (*Hemidactylus flaviviridis*), three phases of the reproductive cycle were described, including the quiescent, recrudescent and active phases. The recrudescent phase was from September-October and was characterized by increasing testicular mass, corresponding to early stages of spermatogenesis, and a rise in plasma steroid concentrations (Al-Amri et al., 2013). The seasonally breeding Caspian bent-toed gecko (*Cyrtopodion caspium*) was also described as having three phases of spermatogenesis: the active, transitional, and inactive phases (Hojati et al., 2013). The findings of the present study suggest that leopard geckos follow a similar pattern of spermatogenesis, and were likely in the recrudescent, or transitional, phase of spermatogenesis at the time of this study. This theory would also support the lack of gecko response to hCG, as they were already at a stage in their reproductive cycle that they were producing elevated testosterone concentrations and would be unlikely to respond further to added stimulus.

The mean testosterone concentrations in the leopard geckos (61 ng/mL) were 3.4 times higher than baseline values in veiled chameleons during their quiescent phase, and approximately ½ the chameleons’ mean value post-hCG (Perry, 2020). The min-max values for the leopard geckos in the present study were also similar to the min-max values for the chameleons post-hCG, further reinforcing that the leopard gecko testosterone concentrations were already elevated. Leopard gecko testosterone concentrations were also higher than green iguanas during their reproductive season (29.7 +/- 14.4ng mL^-1^) and house geckos at 15 ng/mL.
(Moyano et al., 2020; Al-Amri et al., 2013). However, the range of the leopard gecko testosterone concentrations observed in this study were similar to previously reported testosterone concentrations for this species (87.6-139.69 ng/mL) (Crews et al., 1997) and the Madagascar ground gecko (*Paroedura picta*) (3.30 to 144.22 ng/mL) (Golinski et al., 2014). Based on these results, some species of gecko may normally exhibit a high variability in testosterone concentrations between individuals, or it is possible that the breeding season of these animals is less well defined than previously thought in captive situations. Unfortunately, we do not have comparative data across the reproductive and quiescent phases of the reproductive cycle for the leopard gecko. Measuring testosterone concentrations over the course of the year will be necessary to determine if leopard geckos have a three-phase reproductive cycle, characterized by active, recrudescent and quiescent phases, and to better understand comparisons with other species.

A single dose of hCG was administered in this study based on the previously demonstrated success at increasing plasma testosterone concentrations in veiled chameleons using a single dose (Perry, 2020). However, in other studies evaluating the effect of exogenous hCG administration in the spiny-tailed lizard (Arslan et al., 1977), the little brown skink (Jones, 1973), common agama (Eyeson, 1971), oriental garden lizard (Sonar and Patil, 1994), and the New Guinea bokadam snake (Jadhav and Padgaonkar, 2012), animals were given serial injections of hCG. Testosterone concentrations were only measured in one of these studies, and while hCG was confirmed to increase plasma and testicular testosterone concentrations in the spiny tailed lizard, PMSG was found to elevate them to a higher degree (Arslan et al., 1977). In all previous studies listed, the animals were considered to be in their quiescent phase of reproduction (other than the spiny tailed lizard, who was in the recrudescent phase).
Unfortunately, it is not possible to determine whether the number of hCG injections impacted the results, as the plasma concentrations in the present study were much higher than reported in other species and suggest that the geckos’ testicles were already primed and producing testosterone. It is possible that multiple dosages of hCG may be required to stimulate the interstitial cells of the leopard gecko to produce androgens; however, this will need to be tested in the quiescent phase or their cycle.

In addition to serial dosing, leopard geckos may require higher doses of hCG to stimulate testosterone production than the two doses tested in this study. In previous reptile studies, effective dosages of hCG have ranged from 1 IU in little brown skink (Jones, 1973) to 100 IU in the common agama (Eyeson, 1971). In these studies, histological changes were observed and reported; however, no attempt was made to measure circulating testosterone concentrations. The dosages of hCG utilized in the present study were selected based on the results of a single dosage of hCG delivered to the veiled chameleon, with the goal being to use the lowest effective dose of hCG to stimulate testosterone production in the leopard gecko. While there was no significant difference in the testosterone concentrations of veiled chameleons given doses of 100IU, 200 IU, and 300 IU hCG (Perry, 2020), a dose dependent response was reported in the American toad (Anaxyrus americanus) (Kouba et al., 2012). Toads administered the highest concentration of hCG, 300 IU, produced spermatozoa more consistently and had higher spermatozoa concentrations compared to animals receiving lower dosages (Kouba et al., 2012). It is possible that leopard geckos may require higher dosages of hCG in order to stimulate the interstitial cells, and future studies should consider higher dosages (e.g., 300 IU/animal) to determine dosing efficacy.
While hCG has been shown to be effective at stimulating androgen production in other reptiles, the leopard geckos’ lack of response to this hormone may also be due to the one gonadotropin, two cell theory. This species may require FSH stimulation for both steroidogenesis and spermatogenesis. In mammals, the biological effects and immunological cross-reactivity of hCG and LH are similar (Belle et al., 1969). However, in the majority of lizard species studied, interstitial cells appear to be more sensitive to hCG than LH (Jones, 1972), making it unlikely, based on the results of this study, that LH or LHRH would be effective at stimulating androgen production in the leopard gecko. Alternative hormones that have been found to demonstrate FSH-like activity, like PMSG, should be considered in future studies to test the one gonadotroph, two cell theory in leopard geckos.

There were several limitations to this pilot study. The leopard geckos were housed individually at a set temperature, humidity, and twelve-hour photoperiod. Normal breeding season for leopard geckos in the wild is dictated by fluctuations in the season, although how this impacts testosterone in these animals has not been studied. However, in the house gecko, peak plasma testosterone concentrations strongly correlated with the active spermatogenesis phase and breeding period (Al-Amri et al., 2013), so it is possible that this would be similar in the leopard gecko as well. Leopard geckos are one of the most popular lizard species in the pet trade and are commonly bred in captivity between January and September under a variety of artificial conditions, but the effect of these conditions on gecko physiology has not been established. One method that could be pursued is to measure testosterone concentrations bi-monthly over the course of a year, similar to a previous study in veiled and panther chameleons (Perry, 2020). The geckos used in this study were also not exposed to females. Previous work has established that male leopard geckos that have had previous sociosexual experience had higher circulating
androgen concentrations than naïve males (Crews et al., 1997). The ages and previous sexual histories of the geckos used in this study were not known; therefore, how prior sociosexual experiences could have impacted this study could not be determined. However, all of the geckos used in this study were housed individually in a single room consisting of only males without exposure to females for at least three years. Dividers were also utilized to decrease visualization of other males. It is possible that other cues existed that kept the animals’ testosterone concentrations high. Lastly, other studies evaluating response to exogenous hormonal administration commonly evaluated histology to determine the effect of these hormones at the cellular level. However, the geckos used in this study could not be sacrificed or altered due to plans for future studies, so this was not performed. Based on the lack of change in circulating testosterone concentration following hCG administration, it is unlikely that changes to the interstitial cells would have been observed or that they would have existed in all three treatment groups, including saline controls, because of the high circulating testosterone concentrations.
CHAPTER 4. DETERMINING THE EFFECT OF SERIAL INJECTIONS OF PREGNANT MARE SERUM GONADOTROPIN (PMSG) ON PLASMA TESTOSTERONE CONCENTRATIONS, TESTICULAR DYNAMICS, AND SEMEN PRODUCTION IN LEOPARD GECKOS (*EUBLEPHARIS MACULARIUS*).

4.1. Introduction

Geckos are one of three extant mega-diverse lineages of squamate reptiles originating from the major radiations that began diversifying around 200 million years ago (Uetz et al., 2020). While most gecko species have only been discovered over the last 40 years, as new molecular technologies are developed to aid in our understanding of gecko diversity, many species may already have gone extinct (Uetz et al., 2020). Modern extinction rates have increased sharply over the past 200 years and correspond to the rise of the industrial society (Ceballos *et al.*, 2015). Reptiles are especially susceptible to anthropogenic threats, including habitat loss and degradation, unsustainable trade, introduced invasive species, environmental pollution, and climate change (Böhm *et al.*, 2013; Gibbons *et al.*, 2000; Todd *et al.*, 2010). Extinction model estimates that include expected climate changes and habitat losses suggest that as many as 76% of reptiles will be committed to future extinctions by 2050 (Thomas, 2004), while current estimates have determined that one in five reptile species are already threatened with extinction (Böhm *et al.*, 2013). However, due to the rigorous and time intensive process of determining a species’ conservation status, counts of “officially” recognized endangered and threatened species are likely to grossly underestimate the actual number of imperiled species (Gibbons *et al.*, 2000).

Reproduction is core to species survival, so understanding how an animal breeds is fundamental to conserving species, populations, and, indirectly, the vitality of entire ecosystems (Wildt *et al.*, 2003). Successful application of assisted reproductive techniques for enhancing
propagation, such as artificial insemination, *in vitro* fertilization, embryo transfer, and germplasm cryobiology, are directly related to the amount of basic reproductive information available for each species (Comizzoli and Holt, 2014). However, despite the bewildering array of reproductive modes exhibited by reptiles, there is little information on the physiology and hormonal control of reproduction for most species (Lance, 2003). For example, the ability to collect spermatozoa in reptiles is a key initial step in developing assisted reproductive technologies (ART) for these animals; however, in lizards, successful semen collection has only been achieved in 9 species through either electrostimulation or manual massage, including: green iguanas (*Iguana iguana*), Grand Cayman blue iguana hybrids (*Cyclura lewisi x nubila*), leopard geckos (*Eublepharis macularis*), veiled chameleons (*Chamaeleo calyptratus*), panther chameleons (*Furcifer pardalis*), Chaco spiny lizards (*Tropodurus spinulosis*), Texas rock lizards (*Sceloporus torquatus*), McCann’s skinks (*Oligosoma maccanni*) and common house geckos (*Hemidactylus frenatus*) (Zimmerman et al., 2013; Perry et al., 2019; Funcke et al., 2015; Perry, 2020; López Jury et al., 2018; Martínez-Torres et al., 2019; Molinia et al., 2010; Todd, 2013). Furthermore, the administration of exogenous mammalian gonadotropins to male lizards in order to stimulate steroidogenesis and spermatogenesis has only been attempted in < 0.13% (9/6905) of all lizard species (http://www.reptile-database.org/db-info/SpeciesStat.html) (Arslan et al., 1977; Edwards et al., 2004; Eyeson, 1971; Haider et al., 1987; Jalali et al., 1976; Jones, 1973; Licht and Pearson, 1969; Licht and Papkoff, 1971; Perry et al., 2020; Prasad and Sanyakl, 1969; Reddy and Prasad, 1970a, 1970b; Sonar and Patil, 1994; Vijaykumar et al., 2002). Prior studies attempting to evaluate the effects of exogenous hormone administration on squamate testes have been terminal, and most of these studies have not examined the effects of the exogenous
hormones on circulating testosterone concentrations or semen parameters, which will be paramount in establishing ART in threatened and endangered species in the future.

In mammals, follicle stimulating hormone (FSH) acts at the Sertoli cell to stimulate spermatogenesis, while luteinizing hormone (LH) stimulates steroidogenesis at the level of the Leydig cell. In contrast, a one gonadotropin two cell theory is generally accepted in reptiles, whereby one gonadotropin, or a gonadotropin complex, is responsible for stimulating both spermatogenesis and steroidogenesis, functions carried out by FSH and LH independently in mammals (Licht and Hartree, 1971; Jones, 1973; Jalali et al., 1976; Arslan, 1977). In squamates, FSH has been found to stimulate both spermatogenesis and steroidogenesis, while LH has been found to have a similar qualitative effect, although to a lesser degree (Licht and Pearson, 1969; Reddy and Prasad, 1970a,b; Eyeson, 1971; Licht and Papkoff, 1971; Licht 1772a, b, 1974; Lance and Vilet, 1987; Vijaykumar et al., 2002; Jadhav and Padgaonkar, 2010). Exogenous hormones, including pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG), have been previously used with success to develop assisted reproductive programs for mammals, birds, and amphibians. In reptiles, PMSG has been found to have FSH-like activity, whereas hCG has been noted to demonstrate more LH-like activity, although conflicting findings exist on gonadal and endocrine responses to hCG.

PMSG has been found to be capable of stimulating testicle growth, spermatogenesis, and steroidogenesis in some squamates, including Indian spiny-tailed lizards (Uromastix hardwicki) (Arslan et al., 1975, 1977; Jalali et al., 1976), oriental garden lizards (Calotes versicolor) (Sonar and Patil, 1994), the common agama (Agama agama) (Eyeson, 1971), and the little brown skink (Leiolopisma laterale) (Jones, 1973). In Indian spiny-tailed lizards, PMSG induced marked elevations of both testicular and plasma androgen concentrations (Jalai et al., 1976), and was
several times more potent than hCG at stimulating testosterone synthesis (Arslan et al., 1977). PMSG was also found to stimulate a four-fold increase in testicular weights and luminal diameter of the testis tubules in common agamas compared with controls, while hCG had a much smaller qualitative effect on testicle size and tubule diameter (Eyeson, 1971). Unfortunately, testosterone concentrations were not measured in the common agamas. Male oriental garden lizards administered either hCG, PMSG, or a combination of the two hormones during the quiescent phase were found to have increased testicular weights and diameters, in addition to increased seminiferous tubule diameters and spermatids as the abundant germ cell element (Sonar and Patil, 1994). Additionally, cholesterol levels in the testicles were found to be lower in animals receiving PMSG and hCG, suggesting that both exogenous hormones possessed the ability to stimulate spermatogenesis and steroid hormone production in this species of lizard. Exogenous LH, hCG, FSH, FSH+ LH, or PMSG administered to the male little brown skinks during the quiescent phase found that all of the mammalian gonadotropins, with the exception of LH, increased the interstitial cell number, stimulated interstitial cell hypertrophy and cytoplasmic granulation, and increased epididymal and sexual segment epithelial heights (Jones, 1973). Based on these findings, PMSG may have FSH-like activity in reptiles and suggests that FSH stimulation is secondary to preparation of the male reproductive tract with hCG in some species.

The purpose of this study was to determine the effects of serial injections of an exogenous hormone, PMSG, at two different concentrations, 20 IU and 50 IU, on circulating testosterone concentrations, testicular dynamics, and semen production in a model species of gecko, the leopard gecko. Our primary objective was to determine an effective dose of PMSG to increase plasma testosterone concentrations, testicular volumes and weights, the likelihood for semen collection, and spermatozoa motility and concentration with weekly dosing over nine
weeks. The hypotheses tested in this study were: 1) PMSG administration would increase testicular volumes and weights; 2) testicular volumes measured on ultrasound would positively correlate with actual testicular volumes; 3) testicular volumes and testosterone production could be effectively determined through non-lethal methods; 4) semen samples could be consistently collected from the leopard geckos by means of electrostimulation; 5) administering exogenous PMSG would increase semen collection success, spermatozoa concentration, and motility; 6) PMSG administration would be associated with a higher prevalence of morphologically normal spermatozoa; 7) PMSG treated geckos would have significantly more histologic changes in the testis and epididymis consistent with spermatogenesis; and 8) PMSG would significantly increase circulating plasma testosterone concentrations.

4.2. Materials and Methods

4.2.1. Ethics Statement

This longitudinal experimental study was performed in accordance with the rules and regulations established by Louisiana State University’s institutional animal care and use committee (protocol # 20-043).

4.2.2. Study Species

Twenty-four captive-bred, adult, male leopard geckos were used for this study. The sample size was determined using the following a priori information: 1) an alpha=0.05, a power=0.80, a 2:1 ratio of PMSG to control animals, an expected difference of >40 pg/mL testosterone concentrations between PMSG and control animals, and a standard deviation (SD) of 20 pg/mL for each group, and 2) an alpha=0.05, a power =0.80, a 2:1 ratio of PMSG to control animals, an expected difference of at least 1 x 10^6 spermatozoa/mL between PMSG and control animals, and a SD of 7.5 x10^5 spermatozoa/mL for each group.
4.2.3. Husbandry

Animals were individually housed at Louisiana State University in 43 x 21 x 25 cm clear, plastic containers that were separated with dividers to prevent visualization of other geckos. The environmental temperature range and humidity in the climate-controlled room were 28-29°C (83-85°F) and 30-40%, respectively. The geckos were housed on a paper substrate and provided a hiding area and water bowl. A 12-hour photoperiod was provided with standard fluorescent lighting. The geckos were fed a diet consisting of gut-loaded house crickets (Acheta domesticus) (Fluker Farms, Port Allen, LA) three times weekly; the amount offered was based on 3% of the geckos’ body weight. Physical examinations were performed on each leopard gecko prior to initiating the study to confirm that they were healthy.

4.2.4. Experimental Design

A prospective experimental study was conducted from October – December (2020); timing was based on the expected non-breeding period for leopard gecko reproduction in the Northern Hemisphere (September- December) (de Vosjoli et al., 2005). Twenty-four adult male leopard geckos were randomly divided into three treatment groups (group 1: control [saline], n=8; group 2: 20 IU/animal [PMSG], n=8; group 3: 50 IU/animal [PMSG], n=8) using a random number generator (random.org). The chosen dosages of PMSG (Pregnant mare serum gonadotropin, sterile filtered white lyophilized powder, 1,000IU, ProSpec-Tany TechnolGene LTD, Rehovot, Israel) were based on previous work performed in reptiles (Arslan, 1975, 1977; Jalali, 1976). A new bottle of PMSG was reconstituted with 2mL sterile water (Hospira Inc., RL-4428 Lake Forest, IL, USA) to 500 IU/mL for use in the geckos each week, and each animal was administered the appropriate dose subcutaneously over the left epaxial region (shoulder) once weekly for 9 weeks. Control animals received a subcutaneous injection of sterile 0.9% saline at
either 0.04mL or 0.1mL at the same injection site to mimic the volumes of the 20IU and 50IU PMSG doses, respectively. All injections were administered under manual restraint.

All other procedures, including non-invasive testicular measurements, electrostimulation, and venipuncture, were performed under general anesthesia with isoflurane prior to the first PMSG and saline injections (time 0, baseline) and then again once every three weeks (days 21, 42, and 63) of the experiment. Right unilateral orchidectomies with epididymectomy were performed in all animals on day 63. The geckos were anesthetized using an induction chamber with 5% isoflurane (Fluriso, VetOne, Boise, ID, USA) and 3 L oxygen/minute. Once the geckos lost their righting reflex, they were removed from the chamber and maintained on 3% isoflurane and 2 L oxygen/minute administered via face mask. Heart rates and respiratory rates were monitored throughout sample collection, with geckos spontaneously ventilating throughout the procedure.

4.2.5. Non-Invasive Testicular Measurement

Testicular measurements of the right testicle were recorded using the Sonoscape S8 (Sonoscape, Centennial, CO, USA) with the 10-15 mHz linear array hockey stick probe as previously described (Figure 4.1) (Perry et al., 2019, Perry, 2020). The right testicle was selected for measurement because it was more easily visualized on ultrasound; visualization of the left testicle was challenging due to superimposition of the gastrointestinal tract. Testicular length and width were recorded on days 0, 21, 42, and 63 of the study (Figure 4.2). The distance from the cranial to caudal poles of the testicle represented the length. Width was measured in the same view and included the distance from the dorsal to ventral borders of the testicle at its midpoint. Testicular volume was estimated using the following equation: \( V(\text{mm}^3) = 0.52 \times LW^2 \) (Watson-Whitmyre and Stetson, 1985).
4.2.6. Blood Collection

Whole blood was collected from the ventral tail vein or cranial vena cava of each gecko using a heparinized 25-gauge needle fastened to a 1mL syringe on days 0, 21, 42, and 63 of the study. A total of 0.2 ml whole blood was collected at each time point, ensuring total blood volume collected was <0.8% body weight. Blood samples were placed into lithium heparin microtainers (B-D Vacutainer Systems, Franklin Lakes, NJ, USA) and separated into
components using centrifugation at 4,000 x g for 8 minutes. Plasma was aliquoted into 2mL cryovials (VWR International, Radnor, PA, USA) and frozen at -80°C (-112 °F) until it was analyzed for plasma testosterone concentrations.

4.2.7. Electrostimulation/Semen Collection/Semen Evaluation

Semen was collected using electrostimulation on days 0, 21, 42, and 63 of the study. The vent and cloaca were cleaned with a kimwipe (Kimberly-Clark Professional, Corinth, MO, USA) to remove debris. While anesthetized, each animal was electrostimulated using a 360° circumferential metallic probe (20 mm length, 3 mm diameter) connected to a variable amperage power source (Perry et al., 2019). An intromission was defined as the process of fully inserting the metallic portion of the probe into the vent and directing it cranially. Based on ultrasound measurements, the probe length was sufficient to reach the caudal pole of the testicles. Animals were electrostimulated by performing three series of intromissions: 15 intromissions at 0.1 mAmps, 15 cloacal intermissions at 0.15 mAmps, and 15 intromissions at 0.2mAmps. A three-minute break was provided in between each series of intromissions. Electrostimulation was discontinued following the collection of a semen sample. Any fluid observed in the cloaca following a series of intromissions was collected with a 2-20µl single channel pipettor. Each sample was evaluated for the presence or absence of spermatozoa by placing the fluid directly on a glass slide with a cover slip and reviewing it under light microscopy (100x and 400x) at ambient temperature. If spermatozoa were visualized, the intromission number required for successful collection of an ejaculate, in addition to semen color and volume collected, were recorded. Motility of spermatozoa was determined by estimating the percentage of progressively motile spermatozoa to the nearest 5% in 5 high powered fields (magnification, 400x). The sample on the microscope slide and coverslip were washed into a 2mL microcentrifuge tube.
(VWR International, Radnor, PA, USA) with a 1:40 dilution of formal saline. Spermatozoa concentration was determined with a Neubauer hemocytometer with phase contrast microscopy (magnification, 400x). Spermatozoa were counted in all 25 cells on each side of the hemocytometer and the total number of spermatozoa calculated by multiplying by the dilution factor. Spermatozoa morphology were recorded for each sample when at least 50 spermatozoa could be assessed. The number of morphologically normal spermatozoa, in addition to sperm with folded tails, kinked midbodies, detached heads, retained proximal droplets, coiled tails, head defects, and distal droplets were evaluated.

### 4.2.8. Unilateral Orchidectomy and Epididymectomy Procedures

On day 63 of the study, all twenty-four geckos underwent a surgical procedure to remove their right testicle and epididymis for morphometric measurements and gross and histopathological assessment. Each gecko was already anesthetized with isoflurane inhalant gas for venipuncture, ultrasound, and electrostimulation, and was administered subcutaneous injections of dexmedetomidine (Zoetis Services LLC, Parsippany, NJ, USA) 0.025mg/kg, hydromorphone (Hospira, Inc., Lake Forest, IL, USA) 0.5 mg/kg, and meloxicam (OstiLox, VetOne, Boise, ID, USA) 0.3mg/kg for additional sedation and analgesia. Anesthesia was monitored throughout the procedure by measuring the respiratory rate, doppler heart rate, and presence/absence of muscle tone and reflexes. The geckos were placed in dorsal recumbency, and their surgical site (ventral right abdominal region) aseptically prepared with chlorohexidine scrub (VetOne, Boise, ID, USA) and 0.9% sterile saline. A #11 scalpel blade (Bard-Parker, Aspen Surgical Products, Inc, Caledonia, MI, USA) was used to make an initial paramedian incision on the right side of the abdomen, and Metzenbaum scissors were used to extend the body wall incision (3-4 cm). A Lone Star self-retaining retractor (Cooper Surgical Inc.,
Trumbull, CT, USA) was used to enhance visualization within the coelomic cavity. The ventral aspect of the intraabdominal fat pad was immediately visualized upon entering the coelomic cavity. Gentle retraction of the fat pad revealed that the thin-walled urinary bladder was adhered to the dorsal wall of the fat pad. Medial displacement of the intestines using a cotton tipped applicator (Puritan Medical Products, Guilford, ME, USA) revealed the right testicle (Figure 4.3) and epididymis along the dorsal body wall (Figure 4.4). Once in the visual field, the thin mesorchium at the cranial pole of the testicle was gently grasped with atraumatic forceps to aid in the exteriorization and visualization of the testicle. A small hemoclip (Titanium ligating clips, Weck, Morrisville, NC, USA) was placed on the testicular artery and vein to control hemostasis, and the testicle was dissected from the remainder of the mesorchium for removal. The remaining epididymis was then grasped at its cranial end and traced caudally so an additional hemoclip could be placed and the epididymis removed. Unfortunately, it was challenging to remove the testicle and epididymis en bloc. Sterile cotton tip applicators were used to apply pressure for additional hemostasis as necessary, and the abdomen was flushed with sterile saline prior to closure. The body wall was closed with 4-0 Maxon (Coviden, Mansfield, MA, USA) in a continuous pattern, and the skin was also closed with 4-0 Maxon using a horizontal mattress pattern. Sterile skin glue (GLUture, Zoetis, Kalamazoo, MI, USA) was applied to the incision to reduce seepage. A subcutaneous injection of atipamezole (Zoetis Services LLC, Parsippany, NJ, USA) 0.5 mg/kg was administered to reverse the dexmedetomidine. The geckos were monitored post-operatively until all reflexes had returned and they were able to ambulate normally. Each animal received an additional injection of hydromorphone 0.5mg/kg subcutaneously the following day, in addition to 0.3 mg/kg meloxicam subcutaneously once daily for three consecutive days to minimize discomfort. Animals were observed daily for 6 weeks post-
operatively for any negative side effects associated with the surgical procedure, including anorexia, depression, discharge or swelling at the incision site, dehiscence, and lack of energy or ambulation.

Figure 4.3. Visualization of right testicle in body cavity prior to removal.

Figure 4.4. Visualization of right epididymis in body cavity prior to removal.
4.2.9. Gross and Microscopic Assessment of the Reproductive Tract

Testicular and epididymal tissues were immediately rinsed with sterile 0.9% saline upon removal from the body cavity and pat dried using a kimwipe. The testicle and epididymis were weighed separately to the nearest milligram using an analytical balance, and testicle length and width were measured using digital calipers. A gonadosomatic index (GSI) was calculated using the following formula: (testicle weight [g]/body weight[g]) x 100. Snout-vent length (SVL) and snout-tail length (STL) were also obtained while the geckos were anesthetized.

The testicles and epididymides were fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. 5 μm sections were stained with hematoxylin and eosin for histological analysis. Germ cell identification was performed under light microscopy by reviewing five sections of seminiferous tubules per animal. For micrometric measurements, the slides were scanned using a digital slide scanner (Nanozoomer C9600-02, Hamamatsu Photonics, Hamamatsu City, Japan). The measurements were taken using Aperio ImageScope software (Leica Biosystems, Buffalo Grove, IL, USA). Five measurements were used to define the diameter of the epididymis; ten measurements to define epididymal epithelial height; twenty measurements to define the diameter of the seminiferous tubules; five measurements to define the numbers of interstitial cells; and ten measurements to define the interstitial cell nuclear diameter from each animal. The numbers of interstitial cells were counted from triangular interstitial areas formed by three sections of seminiferous tubules. For the diameter of seminiferous tubules, diameters of round sections or short axes of elongated sections were measured. Intraepithelial secretory granules of the epididymal epithelial cells, intraluminal spermatozoa in the epididymis, interstitial cell cytoplasm, and vacuolation in the testes were graded from 1 to 3.
4.2.10. Testosterone Assay

An enzyme immunoassay kit (EIA) (Arbor Assay DetectX Testosterone K032-H5, Ann Arbor, MI, USA) was used to measure plasma testosterone concentrations. This assay has been previously validated by the authors in leopard geckos, and the same methods were followed in the present study (Mason et al., in review). All samples were processed at the conclusion of the study. The published sensitivity for this assay is 9.92 pg/mL, with a limit of detection at 30.6 pg/ml. Based on previous validation, samples were diluted at 1:20 (Mason et al., in review). To determine the repeatability of the assay, intra- and inter-assay coefficients of variation (CV) were measured. Intra-assay CV was measured by examining the CV of each sample run in duplicate, while inter-assay CV was measured by analyzing the same samples on different plates. Values with CV <15% were considered data, while samples with CV >15% were re-analyzed.

4.2.11. Statistical Analysis

The distributions of the data were evaluated using the Shapiro-Wilk test, skewness, kurtosis, and q-q plots. Data that did not meet the assumption of normality were log transformed for parametric testing. Data that were normally distributed are reported by the mean, SD, and minimum-maximum values (min-max), while non-normally distributed data are reported by the median, 25-75%, and min-max. Mixed linear models were used to determine if there were differences in the body weight, spermatozoa concentrations, testicular volume, ejaculate volume, testosterone concentrations, and spermatozoa motility by time and treatment. Leopard gecko was included in the model as the random variable, while time and treatment were fixed factors. Separate models were created for spermatozoa concentration to evaluate the treatment variable with three levels (saline, 20IU, 50IU) and two levels (saline, PMSG). One-way analysis of variance (ANOVA) testing was used to determine if post-surgical testicular volume, weight, or GSI differed between the treatment groups and the controls. Least significant difference tests
were used for any post-hoc comparisons if the ANOVA was significant. Levene’s test was used to assess for homogeneity of variance. If no difference was noted when the three levels of treatment were compared, an independent samples t-test was used to determine if differences existed between the controls and PMSG treated animals (20 IU and 50 IU animals combined). One-tail testing was used for these comparisons. The same analyses were used to determine if seminiferous tubule diameter, epithelial height, interstitial cell nuclear diameter, interstitial cell number, and epididymal diameter differed by treatment groups. Kruskal Wallis tests were used to determine if secretory granule content, intraluminal spermatozoa, interstitial cell cytoplasm, and cytoplasmic vacuoles on post-surgical testicles differed by treatment groups. If not significant when comparing all three groups, a Mann-Whitney test was used to make the same comparisons for saline versus PMSG combined treated geckos. A generalized linear model for an ordinal logistic response was used to determine if the number of intromissions required to collect a semen sample differed by treatment group or time; the same model was used to determine if the number of mature spermatozoa differed by treatment group and sections samples. Generalized linear models for linear responses were used to determine if spermatozoa morphologic characteristics differed by treatment or time. Pearson’s correlation coefficients were calculated to determine if body weight, testicular weight and volume, and SVL were correlated. SPSS 24.0 (IBM Statistics, Armonk, NY, USA) was used to analyze the data. A P\textless{}0.05 was used to determine significance.

4.3. Results

4.3.1. Body Weights, SVL and STL

There was a significant difference in body weight over time (F=7.94, p<0.0001), but not by treatment (F=1.32, p=0.284) or interaction of treatment and time (F=0.33, p=0.888). Body
weights on day 63 were significantly lower than baseline (p<0.0001), 21 days (p<0.0001), and 42 days (p=0.012); there was no difference in body weights between the other sampling periods (all p>0.081) (Table 4.1). Body weight was positively correlated with snout vent length (R: 0.534, p=0.007), but not snout tail length (R:-0.09, p=0.673).

Table 4.1. Leopard gecko body weights over time.

<table>
<thead>
<tr>
<th>Time</th>
<th>Median</th>
<th>25-75%</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>79.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75.6-83.0</td>
<td>46.0-91.0</td>
</tr>
<tr>
<td>21 days</td>
<td>78.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>74.9-82.4</td>
<td>46.8-88.3</td>
</tr>
<tr>
<td>42 days</td>
<td>77.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>74.2-81.7</td>
<td>45.0-87.0</td>
</tr>
<tr>
<td>63 days</td>
<td>75.7&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>75.7-81.2</td>
<td>44.5-84.6</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>p=0.0001, <sup>c</sup>p=0.012

4.3.2. Testicular Volumes by Ultrasound

There was a significant difference in testicular volume measured by ultrasound by treatment (F= 4.62, p=0.006) and time (F=3.98, p=0.034), but not the interaction of treatment and time (F=0.73, p=0.625). Testicular volumes by ultrasound were significantly larger (p=0.011) in the 20 IU (median: 63.62, 25-75%: 49.14-76.14, min-max: 20.10-141.52) treatment group compared to baseline (median: 38.47, 25-75%:25.50-53.68, min-max: 13.05-81.47). There was no significant difference in testicular volume by ultrasound between the 50 IU (median: 52.77, 25-75%: 35.15-70.07, min-max: 14.72-122.91) and control groups (p=0.107) or the 50 IU and 20 IU groups (p=0.272). Testicular volumes by ultrasound were significantly larger on day 63 compared to baseline (p<0.0001) and day 21 (p=0.04), and day 42 testicular volumes were significantly higher than baseline (p=0.04) (Table 4.2).

Table 4.2. Leopard gecko testicular volume measured by ultrasound over time.

<table>
<thead>
<tr>
<th>Time</th>
<th>Median</th>
<th>25-75%</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>38.0&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>29.98-64.34</td>
<td>13.05-79.61</td>
</tr>
<tr>
<td>21 days</td>
<td>52.65&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32.91-67.52</td>
<td>14.65-122.91</td>
</tr>
<tr>
<td>42 days</td>
<td>54.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.89-61.9</td>
<td>15.78-112.05</td>
</tr>
<tr>
<td>63 days</td>
<td>66.01&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>35.36-79.62</td>
<td>20.33-141.52</td>
</tr>
</tbody>
</table>

<sup>a,c</sup>p=0.04, <sup>b</sup>p<0.0001
4.3.3. Post-Surgical Testicular Volume, Weight and GSI

There was a significant positive correlation between post-surgical testicular volume and testicular weight (R: 0.936, p<0.001); however, there was no correlation between post-surgical testicular weight and body weight (R: 0.014, p=0.949) or SVL (R: 0.095, p=0.659). There were significant differences in post-surgical testicular volume (F=3.53, p=0.024) and testicular weight (F= 2.76, p=0.043) by treatment group. The saline group had significantly smaller post-surgical testicular volumes (20 IU, p=0.009, 50 IU, p=0.036) and weights (20 IU, p=0.018, 50 IU, p=0.043) compared with the PMSG treated animals (Table 4.3). There were no significant differences in post-surgical testicular volumes (p=0.243) or weights (p= 0.314) between the PMSG treated geckos. The GSI was significantly higher (F= 4.1, p=0.028) in the PMSG (mean±SD: 0.13±0.05, min-max: 0.06-0.24) treated geckos compared with the control geckos (mean±SD: 0.08±0.06, min-max: 0.01-0.18).

Table 4.3. Leopard gecko post-surgical testicular volume and weight by treatment group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Median 25-75%</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testicular volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>66.6a, b</td>
<td>32.7-117.3</td>
<td>10.40-143.0</td>
</tr>
<tr>
<td>20 IU</td>
<td>124.3a</td>
<td>86.1-168.9</td>
<td>83.2-196.6</td>
</tr>
<tr>
<td>50 IU</td>
<td>97.6b</td>
<td>58.9-161.0</td>
<td>51.5-282.8</td>
</tr>
<tr>
<td>Testicular weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>58.0c,d</td>
<td>28.2-96.0</td>
<td>9.0-120.0</td>
</tr>
<tr>
<td>20 IU</td>
<td>95.0c</td>
<td>74.5-118.7</td>
<td>47.0-148.0</td>
</tr>
<tr>
<td>50 IU</td>
<td>68.0d</td>
<td>59.7-112.7</td>
<td>47.0-195.0</td>
</tr>
</tbody>
</table>

a p=0.009, b p=0.036, c p= 0.018, d p=0.043

4.3.4. Testicular Volume on Ultrasound Correlation to Post-Surgical Volume

Testicular volume by ultrasound was positively correlated (R:0.672, p<0.001) to post-surgical testicular volume. Post-surgical testicular volume was always higher than ultrasound measured testicular volume, except for one case. There was no significant difference in testicular volume difference between treatments (F=0.923, p=0.413). On average, post-surgical testicular
volumes were 1.7±0.62 (min-max: 0.13-2.54) times larger than ultrasound measured testicular volumes.

4.3.5. Electrostimulation Success and Ejaculate Volumes

Electrostimulation was successful in 85.4% (82/96) of the cases over 63 days. Semen was not collected in 6/32 (18.7%) events for the control and 20 IU groups, while only 2/32 (6.2%) attempts in the 50IU group were unsuccessful. The majority (10/14, 71.4%) of the unsuccessful electrostimulation events were during the baseline sampling; 3/14 (21.4%) and 1/14 (7.1%) negative events were from the 21 and 42 day sampling periods, respectively. There was no significant difference in the number of intromissions required to collect semen by treatment ($X^2=1.3$, $p=0.529$) or time ($X^2=2.4$, $p=0.502$). There was no difference in ejaculate volume by treatment ($F=0.34$, $p=0.718$), time ($F=1.25$, $p=0.296$) or the interaction of time and treatment ($F=0.24$, $p=0.961$). Because there were no differences in ejaculate volume, the data were combined for a single reference (median: 2.0 µl, 25-75%: 2.0-2.0, min-max: 0-5.0).

4.3.6. Spermatozoa Motility

There was a significant difference in motility by treatment ($F= 4.89$, $p=0.018$) and time ($F=4.7$, $p=0.014$). The interaction of treatment by time was not significant ($F=0.958$, $p=0.477$). Motility was significantly higher in the 20IU ($p=0.006$) and 50 IU ($p=0.049$) groups compared with the controls (Table 4.4). There was no significant difference in motility between the two PMSG groups ($p=0.345$). Spermatozoa motility was found to be significantly higher in the 42 and 63 day sampling periods compared to baseline and 21 day samples (Table 4.5).
Table 4.4. Leopard gecko spermatozoa motility over time.

<table>
<thead>
<tr>
<th>Time</th>
<th>Median</th>
<th>25-75%</th>
<th>Minimum-Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 0 days</td>
<td>0(^a,b)</td>
<td>0-10</td>
<td>0-80</td>
</tr>
<tr>
<td>21 days</td>
<td>3(^c,d)</td>
<td>0-10</td>
<td>0-80</td>
</tr>
<tr>
<td>42 days</td>
<td>25(^a,c)</td>
<td>5-60</td>
<td>0-90</td>
</tr>
<tr>
<td>63 days</td>
<td>45(^b,d)</td>
<td>5-67.5</td>
<td>0-90</td>
</tr>
</tbody>
</table>

\(^{a}=0.021, \(^{b}=0.01, \(^{c}=0.053, \(^{d}=0.005

Table 4.5. Leopard gecko spermatozoa motility by treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median</th>
<th>25-75%</th>
<th>Minimum-Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>5(^a,b)</td>
<td>0-21</td>
<td>0-90</td>
</tr>
<tr>
<td>20 IU</td>
<td>25(^a)</td>
<td>1-70</td>
<td>0-90</td>
</tr>
<tr>
<td>50 IU</td>
<td>10(^b)</td>
<td>0-60</td>
<td>0-90</td>
</tr>
</tbody>
</table>

\(^{a}=0.006, \(^{b}=0.049

4.3.7. Spermatozoa Concentrations

There was a significant difference in spermatozoa concentrations over time (F=6.7, p=0.002) and for the interaction of time by treatment (F=2.5, p=0.054). There was no significant difference in spermatozoa counts by treatment (F=3.25, p=0.064) when evaluating all three treatments (saline, 20 IU, 50 IU), but it approached significance. Spermatozoa counts were significantly higher at 42 (baseline, p=0.009; 20 days, p=0.002) and 63 days (baseline, p=0.006; 20 days, p=0.004) compared to baseline and 21 days (Table 4.6). There was no significant differences in spermatozoa counts between baseline and 21 days (p=0.703) or 42 and 63 days (p=0.749) (Table 6). When evaluating the model with the treatment variable at two levels (saline, PMSG [combined 20 IU, 50 IU]), treatment (F=4.34, p=0.042) and treatment by time (F=4.35, p=0.038) were found to be significantly different, while time was not (F=2.65, p=0.114). Spermatozoa concentrations were significantly higher in the PMSG treated animals (p=0.042) compared to the saline treated animals (Table 4.6).
Table 4.6. Leopard gecko spermatozoa concentrations over time and by treatment group.

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Median</th>
<th>25-75%</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>2.4 x 10^7</td>
<td>6.3 x 10^6-1.4 x 10^8</td>
<td>1.8 x 10^6-1.77 x 10^8</td>
</tr>
<tr>
<td></td>
<td>20 IU</td>
<td>1.7 x 10^7</td>
<td>1.1 x 10^7-6.8 x 10^7</td>
<td>1.0 x 10^7-8.5 x 10^7</td>
</tr>
<tr>
<td></td>
<td>50 IU</td>
<td>3.4 x 10^7</td>
<td>1.1 x 10^7-3.4 x 10^8</td>
<td>9.0 x 10^6-2.3 x 10^8</td>
</tr>
<tr>
<td>Day 21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>2.9 x 10^7</td>
<td>1.7 x 10^7-1.3 x 10^8</td>
<td>1.3 x 10^7-2.1 x 10^8</td>
</tr>
<tr>
<td></td>
<td>20 IU</td>
<td>1.8 x 10^7</td>
<td>6.2 x 10^6-7.0 x 10^7</td>
<td>5.3 x 10^6-1.4 x 10^8</td>
</tr>
<tr>
<td></td>
<td>50 IU</td>
<td>1.4 x 10^7</td>
<td>4.7 x 10^6-7.8 x 10^7</td>
<td>2.8 x 10^6-4.9 x 10^8</td>
</tr>
<tr>
<td>Day 42</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>9.2 x 10^6</td>
<td>4.8 x 10^6-5.6 x 10^7</td>
<td>2.0 x 10^6-1.1 x 10^9</td>
</tr>
<tr>
<td></td>
<td>20 IU</td>
<td>3.5 x 10^8</td>
<td>5.5 x 10^7-1.0 x 10^9</td>
<td>5.3 x 10^7-1.1 x 10^9</td>
</tr>
<tr>
<td></td>
<td>50 IU</td>
<td>2.8 x 10^8</td>
<td>1.1 x 10^8-7.1 x 10^8</td>
<td>2.0 x 10^6-4.3 x 10^9</td>
</tr>
<tr>
<td>Day 63</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>4.4 x 10^7</td>
<td>1.0 x 10^7-1.9 x 10^8</td>
<td>2.8 x 10^6-1.8 x 10^8</td>
</tr>
<tr>
<td></td>
<td>20 IU</td>
<td>1.2 x 10^8</td>
<td>2.4 x 10^7-1.0 x 10^9</td>
<td>2.1 x 10^7-2.7 x 10^9</td>
</tr>
<tr>
<td></td>
<td>50 IU</td>
<td>1.1 x 10^8</td>
<td>6.0 x 10^7-2.2 x 10^8</td>
<td>2.0 x 10^7-8.3 x 10^8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>Median</th>
<th>25-75%</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>2.4 x 10^7</td>
<td>6.3 x 10^6 - 1.4 x 10^8</td>
<td>1.8 x 10^6 - 1.8 x 10^8</td>
</tr>
<tr>
<td></td>
<td>PMSG</td>
<td>2.0 x 10^7</td>
<td>1.1 x 10^6 - 7.2 x 10^8</td>
<td>9.0 x 10^6 - 2.3 x 10^8</td>
</tr>
<tr>
<td>Day 21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>2.9 x 10^7</td>
<td>1.7 x 10^7-1.4 x 10^8</td>
<td>1.3 x 10^7-2.1 x 10^8</td>
</tr>
<tr>
<td></td>
<td>PMSG</td>
<td>1.5 x 10^7</td>
<td>6.2 x 10^6 - 7.0 x 10^7</td>
<td>2.8 x 10^6-4.9 x 10^8</td>
</tr>
<tr>
<td>Day 42</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>9.2 x 10^6</td>
<td>4.8 x 10^6 - 5.6 x 10^7</td>
<td>2.0 x 10^6 - 1.1 x 10^9</td>
</tr>
<tr>
<td></td>
<td>PMSG</td>
<td>3.0 x 10^8</td>
<td>9.0 x 10^7 - 7.5 x 10^8</td>
<td>2.0 x 10^6 - 4.3 x 10^9</td>
</tr>
<tr>
<td>Day 63</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>4.4 x 10^7</td>
<td>1.0 x 10^7 - 1.9 x 10^8</td>
<td>2.8 x 10^6 - 2.4 x 10^8</td>
</tr>
<tr>
<td></td>
<td>PMSG</td>
<td>1.1 x 10^8</td>
<td>3.6 x 10^7 - 4.9 x 10^8</td>
<td>2.0 x 10^7 - 2.7 x 10^9</td>
</tr>
</tbody>
</table>

4.3.8. Spermatozoa Morphology

The presence of normal spermatozoa was not impacted by time ($X^2=0.8$, $p=0.845$), but was significantly different by treatment ($X^2=10.0$, $p=0.007$) (Table 4.7). There were significant differences in the presence of folded tails ($X^2=22.8$, $p<0.001$) and kinked midbodies ($X^2=22.9$, $p<0.001$) by time, but not by treatment (folded tail: $X^2=0.7$, $p=0.698$; kinked midbody: $X^2=0.8$, $p=0.661$) (Table 4.8). There was no significant difference in the likelihood of distal droplets.
(time: $X^2=1.4$, $p=0.569$; treatment: $X^2=0.4$, $p=0.829$), head defects (time: $X^2=1.5$, $p=0.523$; treatment: $X^2=2.3$, $p=0.313$), detached heads (time: $X^2=2.8$, $p=0.422$; treatment: $X^2=0.9$, $p=0.623$), retained proximal droplets (time: $X^2=5.2$, $p=0.157$; treatment: $X^2=2.1$, $p=0.345$), or coiled tails (time: $X^2=2.9$, $p=0.393$; treatment: $X^2=1.1$, $p=0.561$) by time or treatment group (Table 4.9).

Table 4.7. Differences in normal spermatozoa morphology (% total morphology) by treatment group.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Mean</th>
<th>SD</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>17.4</td>
<td>8.0</td>
<td>4-36</td>
</tr>
<tr>
<td>20 IU PMSG</td>
<td>28.5</td>
<td>15.0</td>
<td>4-60</td>
</tr>
<tr>
<td>50 IU PMSG</td>
<td>22.0</td>
<td>10.5</td>
<td>4-44</td>
</tr>
</tbody>
</table>

Table 4.8. Abnormal spermatozoa morphology (% total morphology) that differed over time.

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>Time</th>
<th>Median</th>
<th>25-75%</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folded tail</td>
<td>baseline</td>
<td>26.0</td>
<td>21.5-40</td>
<td>14-60</td>
</tr>
<tr>
<td></td>
<td>21 days</td>
<td>30.0</td>
<td>20-36</td>
<td>14-60</td>
</tr>
<tr>
<td></td>
<td>42 days</td>
<td>37.0</td>
<td>27-41</td>
<td>25-46</td>
</tr>
<tr>
<td></td>
<td>63 days</td>
<td>45.5</td>
<td>40-49.5</td>
<td>17-72</td>
</tr>
<tr>
<td>Kinked midbody</td>
<td>baseline</td>
<td>19.0</td>
<td>10-26.7</td>
<td>2-34</td>
</tr>
<tr>
<td></td>
<td>21 days</td>
<td>18.0</td>
<td>14-25</td>
<td>2-36</td>
</tr>
<tr>
<td></td>
<td>42 days</td>
<td>12.0</td>
<td>3-15</td>
<td>0-24</td>
</tr>
<tr>
<td></td>
<td>63 days</td>
<td>7.5</td>
<td>5.7-13.7</td>
<td>0-24</td>
</tr>
</tbody>
</table>

Table 4.9. Abnormal spermatozoa morphology (% total morphology) that was not different by time or treatment group.

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>Median</th>
<th>25-75%</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distal droplet</td>
<td>0</td>
<td>0-0</td>
<td>0-12</td>
</tr>
<tr>
<td>Head defect</td>
<td>0</td>
<td>0-1.7</td>
<td>0-9</td>
</tr>
<tr>
<td>Detached head</td>
<td>0</td>
<td>0-2</td>
<td>0-12</td>
</tr>
<tr>
<td>Proximal droplet</td>
<td>12</td>
<td>7-16</td>
<td>0-33</td>
</tr>
<tr>
<td>Coiled tail</td>
<td>6</td>
<td>2-13.7</td>
<td>0-56</td>
</tr>
</tbody>
</table>

4.3.9. Histopathology of Testes and Epididymis

There was a significant difference in the seminiferous tubule diameters ($F=4.4$, $p=0.025$) by treatment group, with PMSG treated gecko seminiferous epithelial heights being higher
(mean: 229.9 μm, SD: 16.8, min-max: 200.5-264.9) than saline controls (mean: 205.6 μm, SD: 40.9, 135.1-255.6). There were no significant differences in epididymal diameter (F=0.9, p=0.356), interstitial cell nuclear diameter (F= 0.07, p=0.785), interstitial cell number (F= 0.629, p=0.436), or epididymal epithelial height (F=2.5, p=0.67) between saline and PMSG treated geckos, although epithelial height approached significance. There was a significant difference in intraluminal spermatozoa (z= -1.6, p=0.045) between treatment groups, with abundant spermatozoa found in 78.6% (11/14) of PMSG treated geckos and only 40% (2/5) of saline treated geckos. There were no significant differences in secretory granule content (Z=-0.394, p=0.347) between treatment groups. There were no significant differences in cytoplasmic vacuolization (Z=-2.43, p=0.417) or interstitial cell cytoplasm (Z=-2.57, p=0.417) between saline and PMSG treated geckos. There was no significant difference in the presence of mature spermatozoa in the post-surgical testicles based on sample section reviewed (X²=3.7, p=0.491) or treatment group (X²=3.9, p=0.142). Mature spermatozoa were found to be abundant (80.8%) in the post-surgical testicles; fewer samples were found to have moderate (10.8%), few (6.7%), or absent (1.7%) mature spermatozoa. Spermatogonia, round spermatids, elongate spermatids, and primary spermatocytes were present in all three groups of geckos.

4.3.10. Testosterone Concentrations

There was no significant difference in testosterone concentrations over time (F=2.1, p=0.139), treatment group (F=0.703, p=0.507), or the interaction of time and treatment group (F=1.6, p=0.220). Because there was no difference in time or treatment groups, leopard gecko testosterone reference intervals (Table 4.10) were established for the months of October, November, and December according to the American Society of Veterinary Clinical Pathologists (Friedrichs et al., 2012). The Tukey’s test was used to screen for outliers (Friedrichs et al.,
2012). For October, there were two outliers: gecko 19 (358.2 ng/mL) and gecko 4 (524.2 ng/mL); these data were removed for reference interval determination. There were no outliers for November or December. MedCalc 17 (MedCalc Software, Ostend, Belgium) was used to determine the central 95th percentile of the data. Because the data were not normally distributed, the central 95th percentiles of non-normally distributed data were determined using non-parametric methods established by the Clinical and Laboratory Standards Institute (Horowitz et al., 2008). 90% confidence intervals for the lower and upper limits could not be determined for this data set due to the number of samples being less than 120.
Table 4.10. Leopard gecko testosterone concentration reference intervals for October, November, and December in the Northern hemisphere. Testosterone concentrations were not found to meet the assumption of normality and are reported by the median, 10-90%, minimum-maximum values, 95% reference intervals using both the robust and nonparametric methods, and the 90% confidence intervals for the upper and lower limits of the reference interval nonparametric and minimum-maximum values. The nonparametric data are preferred for these reference intervals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Median</th>
<th>Min-Max</th>
<th>10-90%</th>
<th>95th Percentile Reference Interval (robust method)</th>
<th>90% CI for Lower Limit</th>
<th>90% CI for Upper Limit</th>
<th>95th Percentile Reference Interval (nonparametric)</th>
<th>90% CI for Lower Limit</th>
<th>90% CI for Upper Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone October (ng/mL)</td>
<td>82.1</td>
<td>29.5-216.12</td>
<td>32.0-287.2</td>
<td>-47.2 - 206.3</td>
<td>-76.4 - 10.2</td>
<td>163.3-259.1</td>
<td>29.5-216.1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>(n=22)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone November (ng/mL)</td>
<td>77.2</td>
<td>17.5-465.7</td>
<td>22.6-327.7</td>
<td>-195.2 – 388.3</td>
<td>-276.3 - 82.7</td>
<td>264.4-474.5</td>
<td>17.5-465.7</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>(n=24)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone December (ng/mL)</td>
<td>79.0</td>
<td>11.1-399.8</td>
<td>25.5-350.2</td>
<td>-183.4 – 340.6</td>
<td>-247.1 - 82.2</td>
<td>244.2-441.8</td>
<td>11.1-399.8</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>(n=24)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
4.4. Discussion

The results of this study confirmed the majority of the authors’ original hypotheses, except two: that animals administered PMSG would have histological changes associated with increased testosterone production, and that there would be higher circulating plasma testosterone concentrations in PMSG treated animals compared to controls. Administration of PMSG was found to increase testicular volume and weight, and final testicular volumes measured on ultrasound were positively correlated with actual testicular volumes. Testicular volume and circulating testosterone concentrations were determined non-lethally in this model species of gecko, giving hope for future conservation programs with threatened and endangered species. Electrostimulation was determined to be an effective method to collect semen repeatedly in leopard geckos, and semen collection, spermatozoa concentrations, and motility all increased over time in PMSG treated geckos. While seminiferous tubule diameters and epithelial heights were significantly increased in PMSG animals, indicating that there was a gonadal effect on spermatogenesis, there were no differences in interstitial cell number, nuclear diameter, cytoplasmic vacuolation, cytoplasm or secretory granule content in the Leydig cells. The results of this study confirm that PMSG can have a direct impact on the male leopard gecko reproductive tract from October-December in the Northern Hemisphere. The ability to obtain pharmacological control over the reproductive system of geckos will enable scientists to manipulate the reproductive cycle to reduce dependency on natural breeding seasons in these animals.

4.4.1. Body Weights, SVL and STL

Gecko body weights decreased over time. The diet offered to the geckos remained constant over the course of the study; thus, the weight loss noted could not be attributed to access to energy but instead some other factor(s). Physiologic stress, characterized by an increase in glucocorticoid
synthesis and catabolism of stored energy, can be associated with routine restraint and handling (Lance and Lauren, 1984; Grassman and Hess, 1992; Denardo, 2006; Silvestre, 2014;) The authors’ attempted to reduce gecko handling over the course of the study by limiting injections to once weekly and sampling frequency to once every three weeks. It is possible that a shorter study with more frequent dosing could reduce overall handling and should be considered in the future. Additionally, while behavior was not monitored over the course of this study, wild male reptiles often migrate short distances for breeding purposes (Southwood and Avens, 2010), which can be energetically costly. In the authors’ experience (SMP, MAM), it is not uncommon for captive male squamates to reduce food consumption during the breeding season. Based on the high testosterone concentrations measured in all geckos, regardless of treatment, it is possible that the weight loss was attributed to the prenuptial reproductive cycle of the geckos. Results of this study also indicate that gecko body weight and SVL are correlated, but that body weight and STL were not correlated. This discrepancy is likely due to the differences in tail length in these animals, and possible variation between normal and re-grown tails. Thus, SVL is a better indication of weight than STL in leopard geckos.

4.4.2. Testicular Sizes

PMSG increased testicular sizes in treatment animals compared to those administered saline, as measured by elevated GSI, testicular weights, ultrasound and post-operative testicular volumes, and correlating with a higher degree of sperm production in PMSG animals. Spermatozoa are produced in the testicles and testis size in reptiles is maximal at the time of spermiogenesis, suggesting that large testes are indicative of a high spermatozoa production at the individual level (Licht, 1984). In the common agama, a four-fold increase in GSI (mean GSI 0.88) was observed in animals receiving PMSG compared to control animals after 21 days (Eyeson, 1971). While the
difference in GSI was not as dramatic in the leopard geckos, a nearly two-fold increase was observed in animals administered PMSG compared to controls, suggesting that PMSG administration had a significant impact on spermatogenesis.

PMSG administered to leopard geckos was also successful at increasing testicular volumes as measured by ultrasound over time, but these results were not dependent on the dosage of PMSG administered. Other studies evaluating the use of PMSG in lizards did not vary their doses of PMSG in order to determine their effects (Eyeson, 1971; Jones, 1973; Arslan, 1975, 1977; Jalali et al., 1976; Sonar and Patil, 1994); however, PMSG was found to increase testicle size and promote spermatogenesis in these studies. Effective dosages of PMSG in lizards have ranged from 1 IU in *Leiolopisma laterale* (Jones, 1973) to 100 IU in the *Agama agama* (Eyeson, 1971) and demonstrated histologic changes at the level of the testis and epididymis. Thus, the effects of PMSG in lizards may not be dose dependent. While no current attempt has been made to standardize dosing, standardization will be necessary in order to develop functional reproductive programs in the future.

Testicular volume as measured on ultrasound was positively correlated to the post-surgical testicular volume, suggesting that ultrasound is a viable option for non-invasive monitoring of testicular volume in a small lizard species. Ultrasound was initially selected by the authors as a non-invasive method for measuring testicle size to develop a clinical, ante-mortem method to assess the reproductive cycles of male reptiles. Previous studies evaluating the effects of exogenous hormone administration on testicle size and function have relied on post-mortem measurements to determine their effectiveness (Eyeson, 1970; Reddy and Prasad, 1970a,b; Jones, 1973; Arslan et al., 1975, 1977; Jalali et al., 1976; Rai and Haider, 1986, 1991; Haider and Rai, 1987; Sonar and Patil, 1994; Vijaykumar et al., 2002; Jadhav and Padaonkar, 2010). By using
ultrasound, it is possible to conduct these types of studies on threatened and endangered species. The equation used in this study to estimate testicular volume was based on the volume of an ellipsoid (Watson-Whitmyre and Stetson, 1985) and only requires two measurements (length and width) to estimate volume. This was advantageous because obtaining a second image to evaluate testicle width was challenging in these small lizards. This method for obtaining non-invasive testicular measurements was also found to be effective in veiled and panther chameleons (Perry, 2019). However, in contrast to the chameleon study, the right testicles of all leopard geckos were removed, weighed, and measured to calculate actual testicular volumes, allowing for the validation of this equation and comparison between gross and ultrasound measured testicles. Actual testicular volumes were found to be higher than ultrasound measured testicular volumes (except in one animal); thus, ultrasound measurements may underestimate actual volume. Reptile testes are intracoelomic, elongated, and cylindrical in shape (Gribbins and Rheubert, 2011); lie dorsocaudal to the liver; and are suspended by the mesorchium (Rheubert et al., 2010). Due to their position in the body cavity, accurate identification of the testicle borders may be reduced by the superimposition of the gastrointestinal tract, urinary bladder, liver, and intra-abdominal fat pads. Initially, the authors of this study had planned to measure both testicles via ultrasound in each animal, and then randomly remove either the left or right testicle for histopathologic assessment. However, the left testicle was often difficult to visualize due to interference from the gastrointestinal tract, thus, the more consistently visualized right testicle was selected for routine measurement and histologic assessment in all geckos.

4.4.3. Electrostimulation

While a previous study successfully used electrostimulation in the leopard gecko to collect semen samples at a single time point (Funcke et al., 2015), the results of the current study
confirm that electrostimulation is a safe and effective means of reliably collecting repeated semen samples from leopard geckos over time. Electrostimulation was successful in producing semen samples in 85.4% of the attempts made in both the control and treatment gecko groups. Electrostimulation of Texas rock lizards (*Sceloporus torquatus*), Chaco spiny lizards (*Tropidurus spinosus*), and green iguanas produced similar successes, with semen collected in 77%, 94%, and 88% of samples collected, respectively (Martínez-Torres *et al*., 2018; López Juri *et al*., 2018; Zimmerman *et al*., 2013). Lower results were reported for panther (55%) and veiled (50%) chameleons (Perry, 2020). Sampling was performed during the non-breeding season for the geckos, but during the breeding seasons of the other lizards. These findings affirm that electrostimulation can be used to collect semen from leopard geckos with a high degree of success during the non-breeding season regardless of additional exogenous treatments.

Unlike mammals, the neuronal pathway that controls ejaculation in reptiles is unknown, but anatomic similarities between the urogenital systems of mammals and reptiles suggest they have similar innervations (Zimmerman *et al*., 2013). Additionally, a recent study in a porcine model suggested that electrostimulation directly activates pelvic musculature rather than neural mechanisms (Groh *et al*., 2018). While the results obtained in the leopard geckos and other lizards support this idea, more studies are ultimately needed to confirm the underlying mechanisms.

Isoflurane anesthesia was used in this study due to the perceived discomfort associated with electrostimulation (Zimmerman *et al*., 2013; Perry *et al*., 2019). While cattle routinely undergo electrostimulation without anesthesia, and electrostimulation was successfully utilized in the spiny lava lizard without sedation (López Juri *et al*., 2018), the authors find that the significant amount of muscle contraction associated with the procedure must cause some
discomfort. Follow-up studies attempting to objectively measure the degree of discomfort are warranted. When considering anesthetics for this type of procedure, it is important to consider potential sequellae to treatment. For example, opioids have been associated with adverse effects on spermatozoa and should be avoided (Xu et al., 2013; Drobnis and Nangia, 2017). The authors only used an opioid, hydromorphone, after the final semen collection (day 63) and immediately prior to the orchidectomy for pre-emptive analgesia to limit any impact on spermatozoa. More studies are needed to determine the potential impacts of anesthetics and analgesics on reptile spermatozoa.

4.4.4. Semen Analysis

Spermatozoa concentration and motility were found to increase over time and by treatment during the course of this study. However, no difference in ejaculate volume was observed based on treatment or time. PMSG has been found to possess FSH-like activity, promoting spermatogenesis in some lizard species (Eyeson, 1971; Jones, 1973; Arslan, 1975; Jalali, 1976; Sonar and Patil, 1994). Additionally, reptile spermatogenesis may take 5-8 weeks to complete (Gribbins, 2011). The results of the present studies coincide with these previous findings. Spermatozoa concentrations and motility increased over time in the PMSG treated geckos, with both being significantly higher 6 (42 days) and 9 weeks (63 days) after initiating treatment. The initial median motility of spermatozoa collected at baseline was 0%, but this improved to 45% at day 63 (9 weeks) of the experiment. In other lizard species, spermatozoa motilities were found to be 78% in both green iguanas and spiny lava lizards, 70% in McCann’s skinks, 51-93% in the tegus (Tupinambis merianae), and 0-100% in veiled and panther chameleons (Zimmerman et al., 2013; López Juri et al., 2018; Molina et al., 2010; Perry, 2020). While the potential mechanisms behind PMSG activity on spermatozoa motility are not currently
understood, motility is a trait of mature sperm and may be impacted by differences in anatomy and physiology. In lizards, spermatozoa pass from the epididymis into the ductus deferens and gain maximum motility in the distal segment where they accumulate; testicular spermatozoa have poor motility (1%) (Depeiges and Dacheux, 1985). PMSG may have a secondary impact on increasing leopard gecko spermatozoa motility as they undergo the maturation process and are expelled. The effects of PMSG on leopard gecko spermatozoa concentration and motility were not dose dependent, and future studies may aim to use the lowest effective dose of exogenous gonadotrophin to elicit an effect. Some authors believe that reptile spermatozoa motility is tied to some induction agent (Sirinarumitr et al., 2010), and thus may be why higher average motilities have been observed in other studies that have taken place during normal breeding seasons.

Median ejaculate volume in this study was 2.0 µl. Ejaculate volumes of the leopard gecko were similar to those obtained in veiled (2.0 µl) and panther chameleons (2.9 µl), but were lower than those observed in other studies (4.6 µl in the Texas rock lizard) (Perry, 2020; Martinez-Torres et al., 2018). However, even larger lizards, such as the green iguana, produce small ejaculate volumes (median 50 µl) (Zimmerman et al., 2013). Low semen volumes in reptiles have been associated with a lack of accessory sex glands (Zimmerman et al., 2013). However, despite the low ejaculate volumes, the concentration and motility of spermatozoa did not appear to be adversely affected. The small volumes can limit the number of tests that can be done to evaluate the sample; however, extending the sample to increase sample volume can help overcome this deficiency (Zimmerman et al., 2013).

There were more morphologically normal spermatozoa in PMSG treated leopard geckos compared with the control animals. Folded tails were the most common morphological defect, and
became more prevalent over time, while kinked midbodies were the second most common anomaly but improved over time. The electrostimulation technique used in this study was similar to that employed in green iguanas (Zimmerman et al., 2013) and chameleons (Perry et al., 2019); however, how electrostimulation affects the male lizard reproductive tract, and where the semen is dispelled from, is unknown. Manual manipulation techniques, like those employed in the New Zealand gecko (Todd, 2003), or a combination of manual manipulation and electrostimulation may be more successful at obtaining a physiologic ejaculate with less morphologic defects and contamination in the leopard gecko. Other squamate species have been found to have higher proportions of morphologically normal spermatozoa than were observed in the leopard geckos. In the Chaco spiny lizard, no morphological abnormalities were observed in semen samples (López Juri et al., 2018), whereas green iguanas, corn snakes (Pantherophis guttatus), veiled chameleons, and panther chameleons had 94%, 75%, 56.5%, and 55% morphologically normal sperm, respectively (Zimmerman et al., 2013; Fahrig et al., 2007; Perry, 2020). However, the majority of these studies were performed during the breeding season of the species examined, when the normal spermatogenic cycle occurs, and morphologically normal spermatozoa would be expected. Despite expected elevations in testosterone concentrations amongst leopard geckos, the authors’ suspect they had not officially entered into the breeding season yet (January-September, Northern Hemisphere) and their reproductive tracts were still being primed prior to spermatogenesis. Thus, electrostimulation may have expelled spermatozoa from the epididymydes prior to complete maturation, resulting in a larger proportion of secondary morphological defects. The higher proportion of morphologically normal spermatozoa in PMSG treated animals further supports the authors’ theory that this hormone has FSH-like activity in leopard geckos and is capable of stimulating spermatogenesis in these animals outside of their normal breeding period.
4.4.5. Histopathology of Testes and Epididymis

The histological findings of this study confirm that PMSG had an impact on spermatogenesis, but did not significantly impact steroidogenesis. The measurements collected in this study, including the diameters of the epididymis and seminiferous tubules, epididymal and seminiferous tubule epithelial heights, numbers of intraepithelial secretory granules and interstitial cells, and abundance of interstitial cell cytoplasm, cytoplasmic vacuoles, and germ cells, were based on previous histologic descriptions in other lizards (Jones, 1973; Sonar and Patil, 1994, Gribbins, 2011). Seminiferous tubule diameters were larger and the epithelial heights were taller in animals receiving PMSG compared with controls. Additionally, intraluminal spermatozoa were nearly twice as abundant in animals receiving PMSG (Figure 4.5) compared to saline (Figure 4.6). However, there were no significant differences in the interstitial cell numbers, nuclear diameters or epididymal epithelial heights between the saline and PMSG treatment groups, suggesting that no additional Leydig cell hyperplasia or hypertrophy took place in animals receiving PMSG. The findings of this study are in contrast to others in reptiles that directly measured testosterone concentrations and testicular histology following the administration of PMSG (Eyeson, 1971; Jones, 1973; Jalali et al., 1976; Arslan et al., 1977; Sonar and Patil, 1994). In these previous studies, significant increases in circulating testosterone were measured following PMSG administration, and histology of the testes and epididymides noted an increase in the interstitial cells, suggesting probable androgen production. However, in these studies, sampling was conducted during the quiescent phases of reproduction for each species. The breeding season of leopard geckos in the Northern Hemisphere begins as early as January and extends to late September (de Vosjoli et al., 2005). However, despite this study taking place firmly within the proposed non-breeding phase for this species, the animals used in
this study possessed high mean baseline testosterone concentrations averaged over the three months of the study (79.4 ng/mL). Based on these results, it is possible that the leopard gecko follows a three-phase reproductive cycle, similar to those exhibited in the Caspian bent-toed gecko (*Cyrtopodion caspium*) and the house gecko (*Hemidactylus flaviviridis*). However, it is also possible that, similar to the common gecko (*Hemidactylus brookii*), leopard geckos may be spermatogenically active throughout the year (Shanbhag *et al.*, 2020). The seasonally breeding Caspian bent-toed gecko was described as having three phases of spermatogenesis: the active, transitional, and inactive phases (Hojati *et al.*, 2013). Additionally, house geckos possess a three-phase reproductive cycle, characterized by quiescent, recrudescent, and active phases (Al-Amri *et al.*, 2013). The quiescent phase of the reproductive cycle in the house gecko was characterized by flaccid, small testes, no spermatogenic activity, and low plasma steroid concentrations, while the recrudescent phase, occurring in September-October, demonstrated increasing testicular mass, increased primary and secondary spermatids, a rise in plasma steroid concentrations, and increased steroidogenic factors in the Sertoli and Leydig cells. The active phase, occurring in November-May, exhibited large numbers of mature spermatozoa in all sections of the epididymis, peak plasma steroids, and fully developed ultrastructural steroidogenic features (Al-Amri *et al.*, 2013). Conversely, previous findings in common geckos from India determined that although there was significant variation in testicular mass between different months of the year, the testes were spermatogenically active throughout most of the year, with the exception between June and September during the wet season where few animals possessed abundant spermatozoa (Shanbhag *et al.*, 2000). Smaller sizes of Leydig cell nuclei were also observed from May-August in these geckos, suggesting reduced androgen output during low spermatogenic activity. Lizards in general exhibit prenuptial spermatogenesis (Shanbhag *et al.*, 2000), with spermatozoa
being produced prior to mating. Thus, PMSG likely did not further increase testosterone production during this period of early spermatogenesis since it was already elevated naturally during this phase, further supporting the idea that leopard geckos likely follow a prenuptial pattern of reproduction. Based on results obtained in the current study, the authors propose that the leopard geckos were either in the late stages of the recrudescent phase of spermatogenesis, or that they may exhibit more continuous spermatogenesis throughout the year, and that PMSG acted to further stimulate spermatogenesis in these animals. Ultimately, extending sample collection from January through September will be needed to confirm one of these theories.

Figure 4.5. Abundant mature spermatozoa (grade 3/3) in seminiferous tubule of gecko receiving PMSG

Figure 4.6. Few mature spermatozoa (grade 1/3) in seminiferous tubule of gecko receiving saline
4.4.6. Testosterone Concentrations

The mean testosterone concentrations measured in the leopard geckos during this study were higher than baseline testosterone concentrations recorded in other lizards, including veiled (12.93 ng/mL) and panther chameleons (11.64 ng/mL) (Perry, 2020), house geckos (15 ng/mL) (Al-Amri et al., 2013), and green iguanas during their reproductive season (29.7 ng mL⁻¹) (Moyano et al., 2020). Additionally, the range of testosterone concentrations observed in the leopard geckos (11.1-465.7 ng/mL) varied widely, and were not dissimilar to previously reported mean testosterone concentrations for this species (87.6-139.69 ng/mL) and Madagascar ground geckos (*Paroedura picta*) (3.30 to 144.22 ng/mL) (Crews et al., 1997; Golinski et al., 2014). Thus, some species of gecko may normally exhibit a high variability in testosterone concentrations between individuals, or it is possible that the breeding season of these animals is less well defined than previously thought in captive situations. For example, when in captivity, the Madagascar ground gecko has been found to breed continuously (Kubička and Kratochvíl, 2009; Kubička et al., 2012; Starostová et al., 2012) and male leopard geckos that have had previous sociosexual experiences were found to express higher circulating androgen concentrations than naïve males (Crews et al., 1997). Measuring testosterone concentrations over the course of the reproductive cycle will be necessary to determine if leopard geckos have a three-phase reproductive cycle, characterized by active, recrudescent, and quiescent phases, and to better understand comparisons with other species.

Other possibilities for the lack of testosterone stimulation in the leopard geckos receiving PMSG may be that a higher dose or more frequent dosing is required in these animals to elicit an effect, or that leopard geckos do not follow a one gonadotroph, two-cell theory of reproduction. The doses selected for this study, 20IU and 50IU, were standardized to animal rather than an
IU/kg basis. This was done because hormones tend to flood all available active sites at the level of the tissue, causing a ceiling effect. The dosages selected in this study were thought to be mid-range, with the aim to use the lowest effective dosage of hormone required to elicit an effect. Previous studies evaluating the effects of PMSG in lizards used more frequent dosing (daily or every other day) with shorter durations of administration (2 days to 21 days) (Eyeson, 1971; Jones, 1973; Jalali, 1976; Arslan, 1977; Sonar and Patil, 1994). However, in a recent study evaluating the effects of hCG administration in veiled chameleons, it was determined that weekly injections of hCG were sufficient to maintain elevated plasma testosterone concentrations over a month-long period (Perry, 2020). Additionally, in oriental garden lizards, spermatogenesis was not impacted when 5IU of PMSG, hCG, or a combination of the two hormones were given daily for 10 days, leading the authors to consider the need for higher doses or a longer period of administration (Sonar and Patil, 1994). Based on these results, weekly injections of PMSG were selected for use in leopard geckos with the aim of maintaining elevated plasma testosterone concentrations over a longer period of time to be able to more fully assess the impact on spermatogenesis, since it has been determined that spermatogenesis in reptiles may take between 5-8 weeks to complete (Gribbins, 2011). Future studies should consider administering higher dosages (e.g. 100IU/animal) or administering PMSG on a daily or every other day basis over shorter time periods to determine dosing efficacy. Additionally, blood was sampled 7 days following the most recent PMSG injection, which may have been too long of a time period to catch a peak increase in plasma testosterone concentrations following administration of PMSG. Lastly, the failure of PMSG to stimulate additional testosterone production may be due to the possibility that leopard geckos do not follow the one gonadotroph, two-cell theory of reproduction. Based on the results of this study, PMSG has demonstrated its FSH-like effect in
this species by stimulating spermatogenesis, however, testosterone concentrations did not increase concurrently as expected. Ultimately, PMSG administration at a time of quiescence and low baseline testosterone concentrations will be needed to more accurately determine the effect of exogenous PMSG on Leydig cell testosterone production to further characterize if leopard geckos are a species that follows the one gonadotroph, two-cell theory.

4.4.7. Limitations

There were several limitations associated with this study. Our lack of understanding of the leopard gecko reproductive cycle in captivity limited our ability to perform this study during a time of low testosterone production in order to evaluate the effect of PMSG on stimulating testosterone production in this species. Future studies may consider measuring study subject testosterone concentrations prior to recruitment into the study to ensure concentrations are truly associated with a quiescent phase of reproduction. Additionally, more frequent blood collection or sampling within 24 hours of PMSG treatment may help identify a peak in circulating levels. Limitations associated with the measurement of testicular volumes via ultrasound were also present in this study. The superimposition of the gastrointestinal tract, urinary bladder, liver, and intra-abdominal fat in the location of the testes made visualization of the testicle borders challenging. Measurement error could have also occurred with the ultrasound unit and caliper placement when measuring the small testes; however, measurements were all performed by the same two authors each time (AM and MM) to limit bias. Another limitation was associated with the suture material used to close the surgical incisions. A quarter (6/24) of the leopard geckos experienced suture reactions to the 4-0 Maxon and required subsequent repair. In all of these cases, the suture material was intact and extruded through the skin or body wall. Based on these findings, the authors do not recommend its use in leopard geckos. Other limitations of this study were
associated with the small ejaculate volumes, mechanical losses due to semen evaluation technique, sample contamination, and sperm clumping. The low volume may be attributed to the smaller relative size of the leopard geckos in comparison to the other lizards, in addition to the methodology used to evaluate and collect the samples. Ejaculates were obtained and placed directly on a slide with an overlying cover slip to confirm the presence or absence of spermatozoa and to characterize motility and any contamination present before being washed into a cryovial with 10% buffered formal saline. This step likely led to decreased sample volume for analysis, and potentially led to a reduction in spermatozoa concentrations. Additionally, the consistency of white ejaculates was more viscous than samples that were more clear and were found to clump to a higher degree; this also could contribute to falsely lowering concentrations in some samples. Samples were often contaminated with feces and urine, which could have yielded artificial decreases in sperm concentrations. Other studies evaluating lizard spermatozoa used an extender prior to evaluation rather than evaluating raw samples (Zimmerman et al., 2013; López Juri et al., 2018; Martínez-Torres et al., 2018). A semen extender was not used in this study due to concerns for over-diluting the small semen volumes; however, future studies should consider extenders to increase sample volume and allow for additional sample testing.
CHAPTER 5. CONCLUSIONS

This thesis was pursued to contribute to our current understanding of leopard gecko reproductive physiology. The hope is that the information obtained from performing these studies in a model species of gecko will have applicability for the development of ART in threatened and endangered geckos. The need for this type of information is ever increasing, as anthropogenic threats continue to decimate reptile populations globally. While the current studies have enabled us to take a small step forward in better understanding the complexities of leopard gecko reproduction, there is a strong need for more evidence-based research before we will be able to apply these techniques for the conservation of entire species.

The objective of the first study was to determine if a single dose of hCG could be used to increase circulating testosterone concentrations within 24 hours of administration in the leopard gecko. hCG administered at 50 IU or 100IU was ineffective at increase circulating testosterone concentrations during September in the Northern Hemisphere. However, testosterone concentrations were found to be elevated prior to the administration of hCG. One potential explanation for the leopard gecko’s lack of response was that the study was performed during the recrudescent phase rather than quiescent phase of the leopard gecko reproductive cycle. The study design was originally based on what has been published as the non-reproductive season (September-December) for leopard geckos in the Northern Hemisphere (de Vosjoli, 2005). Additionally, leopard geckos may need a larger or repeated dose of hCG to elicit a response. The possibility of leopard geckos following the one gonadotroph, two cell theory, was also discussed, whereby they may require an FSH-like substance for steroidogenesis in addition to spermatogenesis.
The second study had several objectives. The first objective was met but our hypothesis was rejected, as nine weekly doses of PMSG were not effective at increasing plasma testosterone concentrations over time. As was the case in the hCG study, baseline testosterone concentrations were high, and PMSG had no impact on further testosterone concentrations. Based on these findings, we propose that leopard geckos were not in the quiescent phase of their reproductive cycle as originally assumed during the months of October-December in the Northern hemisphere, and may instead follow a three-phase reproductive cycle with quiescent, recrudescent, and active phases. The second objective was also met, and our hypothesis accepted, as electrostimulation was a safe and effective tool of collecting repeated semen samples from the leopard gecko. The third objective and hypothesis were also met, as ultrasound was used to successfully measure testicle size over the course of the study, and the results positively correlated with gross testicle measurements. Finally, the fourth objective was met and the hypotheses accepted as PMSG was found to increase testicular volume, spermatozoa concentration and motility, and the proportion of morphologically normal sperm compared with saline controls. These results confirm that when the reproductive tract is primed with testosterone, PMSG has a gonadal effect in the male leopard gecko and that this hormone may be beneficial in developing ART protocols for geckos.

Ultimately, our goal of contributing to our collective understanding of leopard gecko reproductive physiology was met. At this point in time, we have determined that leopard geckos are not in the reproductive quiescent phase (de Vosjoli, 2005) during the months of October-December in the Northern hemisphere as evidenced by their elevated testosterone concentrations and ability to yield ejaculates containing mature spermatozoa in saline treated geckos. One dosage of hCG administered as a single agent to geckos already producing testosterone does not
have an impact on further increasing testosterone concentrations. It is possible that this exogenous hormone may be of limited use in developing ART for these animals. However, the ability of hCG to increase testosterone concentrations in geckos during the quiescent phase, or when administered concurrently with other exogenous hormones, still remains undetermined. The use of ultrasound to non-invasively monitor testicular volume was validated and confirmed to be accurate. Electrostimulation was determined to be an effective tool for safe and effective semen collection in a model species of gecko. Furthermore, PMSG was found to have a gonadal impact by contributing to increased spermatogenesis, and thus may be useful in the development of future ART. A small window of opportunity currently exists for humans to mitigate the loss of biodiversity that we have perpetuated, but only if we act quickly. Only by understanding the species we are hoping to conserve, can we develop approaches to protect them.
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