Can Assisted Reproductive Technologies Help Conserve 300 Million Years of Evolution? A First Attempt at Developing These Technologies for Male Reptiles

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CAN ASSISTED REPRODUCTIVE TECHNOLOGIES HELP CONSERVE 300 MILLION YEARS OF EVOLUTION? A FIRST ATTEMPT AT DEVELOPING THESE TECHNOLOGIES FOR MALE REPTILES.

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ABSTRACT

Biodiversity loss is the most critical environmental problem threatening ecosystem, animal, and human health today. Increases in extinction rates have been observed over the past 50 years, with reptile losses occurring twelve times faster than traditional extinction rates. This demonstrated biodiversity loss is secondary to climate change, habitat destruction, infectious disease, invasive species, poaching, and unsustainable trade. Approximately 20% of all reptiles are threatened with extinction and population declines are approaching rates similar to the current amphibian extinction crisis. Preventing the extinction of reptiles will require humans to acknowledge these losses and develop plans to preserve these evolutionary sentinel species.

Assisted reproductive technologies (ART) are well developed in a handful of species, and these technologies have become integral parts of conservation programs for threatened and endangered species. The creation of functional and sustainable reproductive assistance programs for reptiles using ART will strengthen our conservation capacity. Combining ART with an understanding of reproductive physiology will enable scientists to capture genetic material from different animals housed at different institutions, overcoming reproductive barriers. Subsequent gamete transport will reduce the need to transport animals from stressful or dangerous environments for breeding. Additionally, these gametes could be stored indefinitely to preserve genetic diversity.

The goal of this research was to systematically apply ART to male reptiles. Semen was safely and successfully collected from veiled (Chamaeleo calyptratus) and panther chameleons (Furcifer pardalis) using electroejaculation. The annual reproductive cycles of these two chameleons were characterized under captive conditions, and both species follow season breeding cycles. Human chorionic gonadotropin can be used to increase circulating plasma...
testosterone concentrations in veiled chameleons. Short-term cooled semen storage can be done in red-eared sliders (Trachemys scripta elegans) and diamondback water snakes (Nerodia fasciata) using Ham’s F10, INRA 96, and sperm washing buffer; green anole (Anolis carolinensis) semen could not be stored using these same extenders. Red-eared slider turtle spermatozoa motility was lost following cryopreservation, but plasma membrane integrity remained. Reptile survival is dependent on how we plan today. ART will help us develop programs to preserve the genetics of these sentinel animals.
CHAPTER 1. INTRODUCTION

Humans are responsible for transforming the Earth’s landscape and putting over 1 million plant and animal species at risk of extinction (Ceballos et al., 2015). This demonstrated biodiversity loss is the most critical environmental problem threatening ecosystem health and human well-being (Barnosky et al., 2011). Anthropogenic activities have directly led to a significant increase in extinction rates across all taxa and a reduction in reptile populations worldwide (Böhm et al., 2013; Böhm et al., 2016; Alroy, 2015). Approximately 20% of all reptiles are currently classified as threatened, with expectations that these numbers will rise dramatically without mitigation strategies (Böhm et al., 2013). Extinction model estimates that include expected climate changes and habitat losses suggest as many as 76% of reptiles will be committed to future extinction by 2050 (Thomas, 2004). Three-hundred million years of evolution. Gone. Lost in approximately 300 years of human industrialization (Steam engine, Thomas Newcome, 1712, https://learnodo-newtonic.com/industrial-revolution-dates).

The true biodiversity loss facing the earth is immeasurable; however, there is something we can do about it. Active in situ conservation efforts for reptiles are gaining momentum, although many ecological threats still persist. In spite of these efforts changes to the climate and habitat degradation will continue to make in situ work complicated for these ectothermic sentinels. Because of these challenges, the future survival of reptiles may be based on ex situ conservation efforts, such as captive species management. For captive breeding programs to become successful, it requires a detailed understanding of reproductive biology to ensure successful propagation. Unfortunately, our understanding of reptile reproduction is limited because of the diverse strategies these animals practice, and the sheer number of species (>10,000) (Clulow and Clulow, 2016). To truly conserve and preserve the 300 million years of evolution tied to the
genes of reptiles, it is important that we develop functional \textit{ex situ} conservation methodologies now that will help us navigate the future.

Applying assisted reproductive technologies (ART) to novel taxa or species for conservation is an adjunct effort to enhance our basic species knowledge and overcome reproductive barriers. Gamete collection and preservation, hormone analysis, and artificial insemination have all become integral components of \textit{in situ} and \textit{ex situ} conservation programs for threatened and endangered species of mammals, birds, amphibians, and fish; however, few studies have investigated these technologies for reptiles. (Mattson et al., 2008; Mattson et al., 2007; Fahrig et al., 2007; Zacariotti et al., 2007; Zimmerman et al., 2013; Zimmerman et al., 2017; Browne et al., 2011; Martínez-Torres et al., 2019, Martínez-Torres et al., 2019; Perry et al., 2019; Johnston et al., 2014; Johnston et al., 2014). The creation of functional and sustainable ART for reptiles could help prepare us for a future where captive propagation is necessary to conserve reptile genetics.

The overall goal of this dissertation was to develop an outline for scientists to follow when developing ART for any male reptile. To be successful, we need to first develop programs that are designed to answer the feasibility of applying standard ART to a reptile. For example, if electroejaculation is not safe, performing studies purely to collect ante-mortem samples for a single study will have limited value. The following represent the path chosen for this dissertation:

\textbf{Applying ART to Male Reptiles}

1) Can you collect semen repeatedly in a reptile without harming the individual?

2) Does understanding the normal reproductive physiology help us predict when we can successfully collect semen?
3) Can we exogenously (hormonally) stimulate a reptile to control its reproductive system, and thus when it produces semen?

4) When semen is successfully collected, can we preserve it? If so, what semen extenders should we use to handle the semen and how long can reptile semen be stored at refrigeration temperatures?

5) Can we preserve (biobank) reptile spermatozoa indefinitely?

The work presented here set to answer these questions using model species. For the first four chapters, chameleons were used. This group was selected because they are being heavily impacted by anthropogenic influences on their natural ecosystem. Based on the IUCN data, more than half (55%, 113/202) of all known chameleon species are categorized as threatened (i.e., critically endangered, endangered, vulnerable) or near-threatened (Jenkins et al., 2014, IUCN, 2019). To date, no reproductive research has been done evaluating any of these questions in male chameleons. The fifth and sixth chapters used temperate species. This was done to determine whether similar techniques could be used to preserve semen in animals evolved under similar climatic conditions. The specific biological hypotheses for these chapters were:

1. Electroejaculation can be used to collect semen from veiled chameleons, and that four weekly electroejaculations under anesthesia will have no effect on survival, physical examination findings, or hematological parameters.

2. An increase in plasma testosterone concentrations will either precede or be associated with increased testicular size (e.g., volume) and semen output/quality (e.g.,
electroejaculation success, ejaculate volume, spermatozoa concentration, and spermatozoa morphology).

3. Administration of exogenous hCG will increase circulating testosterone concentrations within 24 hours of administration. Repeated injections of hCG will maintain elevated testosterone concentrations, increase testicular size, and increase the likelihood of collecting a semen sample.

4. Spermatozoa motility (survival) will be higher with the commercial semen extenders compared with the crystalloids, and that spermatozoa motility will be highest in samples stored in test yolk buffer, INRA 96, and sperm wash media.

5. Chelonian spermatozoa can be cryopreserved.
CHAPTER 2. LITERATURE REVIEW

2.1. Is the Sixth Mass Extinction a Good Enough Reason to Develop Assisted Reproductive Technologies in Reptiles?

2.1.1. Global Decline in Biodiversity

Biodiversity is defined as the number, abundance, composition, spatial distribution, and interactions of genotypes, populations, species, functional types and traits, landscapes in a given system (Díaz, 2013). Ecosystem services provided by biodiversity make human life possible and worth living. These ecosystem processes produce ecosystem services valued by stakeholders or groups, depending on the context. Some ecosystem services involve direct provision of material and non-material goods and are associated directly with the presence of a species of plant or animal. Other services can arise indirectly from the continued function of the ecosystem processes. Human well-being (the human experience) includes the necessary materials for a good life, freedom of choice and action, health, social relationships, cultural identity, and security (Diaz et al., 2006). The assessment of well-being is specific due to cultural, geographical, and historical contexts and is based on cultural and socioeconomic factors and ecosystem services. Human society is built on the direct delivery of essential ecosystem services, such as the production of food, fuel, shelter, water, and protection of natural hazards. To preserve ecosystem services provided to humans, we need to focus on maintaining or restoring their biotic integrity in terms of species composition, abundance, organization, and species numbers, rather than maximizing the numbers of species present. Biodiversity has been shown to be a stabilizing force in ecosystems because it can buffer environmental change. Biodiversity and ecosystem stability have been found to be positively correlated. This relationship is likely applicable to many...
ecosystems, given the globally prevalent combination of intensive long-term land management and species loss (MacDougall, 2013). Unfortunately, at this time, biodiversity loss is the single most significant threat to human, animal, and ecosystem health and well-being. Biodiversity loss is not occurring at random; drivers such as climate change, biological invasions, and land use are leading to a decline in species (Diaz et al., 2006; Ballie, 2004; Mace, 2005; Kotiaho, 2005; McKinney, 2002). No level of biodiversity is safe from exploitation.

To measure biodiversity, we must take into consideration that biodiversity typically includes species loss (extinction) and species acquisition (speciation). Extinction is a common event that is often offset or balanced with speciation. The balance of speciation and extinction can waver back and forth like a pendulum within ecosystems; however, an equilibrium occurs. In fact, over the earth’s history, the balance between speciation and extinction has only wavered five times, with extinction being the predominant driving force leading to biodiversity loss. These five “events” have been qualified as “mass extinctions” and occurred at the end of the Ordovician, Devonian, Permian, Triassic, and Cretaceous periods (Barnosky et al., 2011). These mass extinctions have been associated with different events, including an asteroid impact (Cretaceous); elevated atmospheric CO$_2$ levels which increased global temperatures and led to a calcification crisis in the world oceans (Triassic); alternating glacial and interglacial episodes leading to CO$_2$ sequestration (Ordovician); global cooling, followed by global warming and the decrease in atmospheric CO$_2$ (Devonian); and ocean acidification associated with elevations of H$_2$S and CO$_2$ (Permian) (Barnosky et al., 2011). Debate also still continues as to whether an asteroid strike was responsible for the Permian and Devonian extinctions. In all of these events,
species extinction rates increased higher than any other geological interval over the last 540 million years, with an estimated loss of 75% of estimated species (Barnosky et al., 2011).

A modern extinction crisis, termed the Anthropocene or sixth mass extinction, has been documented by scientists as early as the mid-1990s. We are about to enter the point of no return, as current extinction rates are exceptionally high and increasing. Scientists have been and are now becoming more vocal about recognizing and describing species and populations extinctions; however, these estimates are an underestimate of the true risk because many species have yet to be formally described. The driving force behind the Anthropocene extinction is present humans, and for the first time in the earth’s history, a species from earth has been identified as the cause of a mass extinction. Humans are co-opting resources, fragmenting habitats, introducing non-native species, spreading pathogens, killing species, and changing the global climate.

This human-induced extinction crisis has led to an increase in species extinction rates that are higher than any from the pre-human background rate (Ceballos et al., 2015). In previous studies comparing modern versus background extinction rates, the background rate was assumed to be somewhere between 0.1 and 1 species extinction per 10,000 species per 100 years, which is equal to 0.1 to 1 species extinction per million species per year (E/MSY). Recently, Ceballos et al. 2015 estimated extinction rates using an overestimated background extinction estimate to be 2 E/MSY. The study used an extremely conservative model to estimate extinction; the rate was empirically determined using fossil records of mammals combined with extinction counts from paleontological databases, published literature of the fossil record, and historical records. Data for extant species was taken and evaluated from the ICUN. They determined current extinction

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rates have increased dramatically over the past 200 years and are considerably higher than background rates. Extinction rates vary among vertebrate groups; however, some of the groups (i.e., reptiles) have not been completely reviewed by the IUCN and losses are likely underestimated (Ceballos et al., 2015). In vertebrates, the extinction rate has increased since 1900 to 22-53 E/MSY based on the model chosen, while since the 1500, the rate is 8-15 E/MSY above the background rate of 2 E/MSY. These data are highly correlated to one single human event, industrialization.

Climate change over the past 30 years has led to major shifts to species abundance. Mathematical models incorporating species distribution and moderate changes in climate have shown that by 2050 approximately 15-37% of species and taxa will be extinct (Thomas, 2004). It has been suggested that humans need to return to pre-industrial revolution global temperatures to prevent the anticipated climate-related extinctions. A meta-analysis of 131 published papers evaluating the influence of climate change on extinction revealed that extinction risks would accelerate with future global temperatures, threatening one in six species with extinction. Endemic species with smaller ranges and certain taxonomic groups, such as amphibians and reptiles, are predicted to face higher extinction risks (Urban, 2015; Sinervo, 2010; Gibbon, 2000). Species in South America, Australia, and New Zealand appear to have the highest risks of extinction. We are reaching a point where extinctions from climate change are expected to increase with every 1° C rise in global temperatures. Climate-change induced extinctions will become increasingly apparent in the future, and the question today is whether recovery is even

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possible (Urban, 2015). Avoiding this sixth mass extinction will require rapid and intensified conservation efforts across all taxa.

2.1.2. Global Decline of Reptiles

Three hundred million years ago, reptiles diverged from amphibians to make the transition from an aquatic lifestyle to a terrestrial lifestyle (Pough et al., 1998). These taxa are inextricably linked within the scientific and popular literature despite their significant morphological and life history differences; however, they do maintain some similarities such as being ectothermic tetrapods, inhabiting similar habitats, and are equally vulnerable to environmental changes. Habitat loss and degradation, invasive species introduction, environmental pollution, infectious disease, unsustainable harvesting, illegal trade, and global climate change are all significant threats to reptile populations worldwide. Comparatively, amphibians are susceptible to the same threats and have received much of the attention related to the current extinction crisis.

The IUCN red list is the standard to assess global species risk of extinction based on quantitative criteria. Species are categorized into one of eight categories of extinction risk, and these eight categories are further subdivided into two categories, threatened and non-threatened. The categories that consider a species threatened with extinction are Critically Endangered, Endangered, or Vulnerable. The IUCN red list has evaluated 66.8% (5,424/8,110 species) of known amphibian species and 61% (6,680/10,793) of known reptiles. These findings suggest that risk assessments for both groups should be considered incomplete and an underestimate of current extinction estimates.

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Many studies evaluating extinction rates in reptiles use the IUCN red list for comparison. Current estimates for reptiles suggest that one in five reptile species is threatened with extinction; however, the same data acknowledges that one in five species is considered data deficient (Böhm et al., 2013). The first global analysis of the conservation status of reptiles, including distribution patterns with known threats affecting them, was only completed in 2013. This study highlighted conservation priorities and knowledge gaps that needed to be addressed to ensure the survival of the world's reptiles. According to Böhm et al. (2013), significant numbers of both amphibians (42%) and reptiles (20%) are threatened. A short-cut method of evaluating 1,500 species from the IUCN red list was performed in order to identify areas of conservation need. Results demonstrated that a higher proportion of threatened reptiles occur in the freshwater environments in tropical regions and oceanic islands. Data was too deficient in other tropical areas, such as central Africa and Southeast Asia, and with fossorial species to draw conclusions on their risk. This study promoted a need for research to be focused on tropical areas that are experiencing the most dramatic rates of habitat loss. Additionally, fossorial reptiles and specific taxa, such as snakes, are lacking appropriate data, indicating that their extinction risk is underestimated. This study subsequently called for specific conservation actions to mitigate the effects of human-induced habitat loss and over-harvesting as threats to extant reptile populations (Böhm et al., 2013). A subsequent study by the same investigators evaluated factors that correlate with higher extinction risks in squamate reptiles; these risk factors included range size, habitat specialization, species size (larger species are more likely to go extinct), and accessibility to species range by humans (Böhm et al., 2016). Some believe that smaller-scale analyses should be done to evaluate...
and test these predictors. Despite this global approach to evaluating species extinction risk using the IUCN red list data, the patterns described remained the same among geographic, taxonomic, and threat specific data (Böhm et al., 2016).

Extinction rates of reptiles and amphibians have been estimated using museum specimens combined with data from HerpNet, AmphibiaWeb, and the Reptile Database. Conservative Bayesian models have estimated that anurans and squamates will experience 66.1 extinctions in 100 years and 54 extinctions in 100 years, respectively. In squamates, losses are expected to vary based on geographic distribution, with Mesoamerica experiencing the highest number of extinction events (14.74 species) and Brazil (1.77) and Southern Europe (1.86) the lowest numbers of extinction events (Alroy, 2015). In Madagascar, one of the most biologically diverse hotspots in the world, the extinction risks of the island’s reptiles were estimated using data mined from the IUCN red list and known species spatial data. The main threats identified were deforestation and direct exploitation of species for food and the international pet trade. The results of the study were used to develop priority action plans, including efforts to reduce deforestation and habitat degradation by strengthening law enforcement and building the capacity of local communities to pursue sustainable economical opportunities in and around existing protected areas. Strategies should be developed to conserve the habitats of those threatened species occurring in the yet largely unprotected areas (Jenkins et al., 2014). Additionally, three research priorities were identified, including the exploration of taxonomy and species diversity, assessing the vulnerability of Malagasy reptiles to climate change, and remedying the surprising lack of studies on the effect of logging and forest degradation on
reptiles. From this, a collaborative approach to conservation is needed and called for by this study by all stakeholders (Jenkins et al., 2014).

All of the previously noted studies focused on physical threats to reptiles in their respective ranges; however, one major driving force behind the Anthropocene extinction that has not been fully evaluated in reptiles, despite Jenkins et al. 2014 calling for it, is climate change. Extinction estimates for reptiles that include climate change with habitat loss estimate in the most severe models that 76% of species will be committed to future extinction by 2050 (Thomas, 2004). This rate is similar to mammals and birds in the same models, but is higher than amphibians. Overall, in these same models, species and taxa extinction rates estimate that 15-37% of all species will be committed to extinction by 2050 (Thomas, 2004). Unfortunately, this is the harsh reality of our lifetime. Because of these environmental changes, the discipline of conservation biology was born back in the mid-1980's. Ultimately, the goal of conservation biology is the preservation of biological diversity (Soulé, 1985). This multidisciplinary science is a “mission-oriented crisis discipline” developed exclusively to address the loss of biodiversity (Soulé, 1986). No single applied discipline is comprehensive enough to tackle and address critical threats to biodiversity (Primrack, 1993). As a multidisciplinary science, conservation biology has relied on numerous disciplines to facilitate progress and conservation efforts. What methods and disciplines have been used to preserve and conserve reptilian biodiversity? Unfortunately, not enough. However, this story need not end here.

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2.1.3. Conservation Strategies to Preserve Endangered Reptile Species

The goal of conservation is to mitigate the loss of biodiversity and preserve ecosystem resources, including services, species, and genetic diversity for the future (Schwartz et al., 2017). In 2010, the 10th Convention on Biological Diversity identified Aichi targets to form guidelines for biodiversity conservation through 2020 (Schwartz et al., 2017). For example, strategic Goal C, Target 12, "By 2020 the extinction of known threatened species has been prevented and their conservation status, particularly of those most in decline, has been improved and sustained."(Schwartz et al., 2017) Of the 20 Aichi targets that were to be completed by 2020, good progress has only been made in individual components of four of the targets, moderate progress was made on some components of seven targets, and poor progress was made on all components of six targets. Unfortunately, there was insufficient information to assess the progress on some or all components of the remaining three targets (UN Report).

2.1.3.1. In-situ v. Ex-situ Conservation Strategies

Defined by the convention on biological diversity, in-situ conservation is the conservation of ecosystems and the maintenance and recovery of viable populations of species in their natural surroundings (Pacicco et al., 2018). Ex-situ conservation is defined in the same context but with the work being done outside of a species natural habitat. The IUCN adds, "Ex-situ collections include whole plant or animal collections, zoological parks and botanic gardens, wildlife research facilities, and germplasm collections of wild and domesticated taxa"(Braverman et al., 2014). Despite the history of having in-situ and ex-situ conservation strategies for species, ultimate success can only be achieved with collaboration at all levels by

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invested stakeholders. A movement to have a multi-disciplinary approach to conservation has started to integrate both *in-situ* and *ex-situ* conservation efforts to have a single or “One Plan Approach” to conserve a species.

### 2.1.3.2. *In-situ Conservation Strategies*

*In-situ* conservation preserves biological information within the species’ home range. These conservation efforts can be completed by habitat protection or restoration, education and training, individual species protections with stakeholders, and in-country research. *In situ* conservation offers advantages over *ex-situ* methods in terms of habitat protection, resource utilization, and economic sustainability. Education and training of locals and stakeholders allows for self-sufficient and efficient management of the species or environment at risk. Economic sustainability can be provided by *in situ* conservation efforts as the country maintains specific biodiversity stores for future economic benefit (e.g., ecotourism), which can directly benefit the local environment and species. Compared to *ex-situ* methods, *in-situ* methods are typically lower cost and more secure and efficient. Challenges using *in-situ* methods include the expanded vision of protected areas, developing new models for conservation, and expanding range options for economic development. Risks associated with *in-situ* efforts include uncertain events, such as demographics, natural catastrophes, and genetic uncertainty. These do not include anthropogenic uncertainties, although those hope to be mitigated in the attempt to preserve biodiversity (Braverman, 2014).

The primary strategies employed to preserve reptile biodiversity *in-situ* depend on the species of interest. Numerous *in-situ* conservation efforts for reptiles have been successful,
including providing habitat protection, in-situ captive breeding programs, head-starting facilities, and re-introduction programs. The Burmese star tortoise (Geochelone platynota) is one success story where in-situ conservation efforts were able to save a species (Platt et al., 2017). This species was functionally extinct in the wild, with only a few hundred individuals remaining. All of the tortoises were captured and housed in a captive breeding facility within their home range of Myanmar. Since then, and along with habitat protection, the captive breeding program has produced more than 14,000 individuals (2017) and ensured the repatriation of over 1,000 individuals into native habitat. This program is an excellent example of how in-situ conservation efforts can work with reptiles; however, it is only one of many that are needed.

2.1.3.2. Ex-situ Conservation Strategies

Ex-situ conservation strategies often involve zoological institutions for preserving a species outside of its native range. Zoos and aquaria are considered the “perfect” institutions for ex-situ conservation efforts, although this has not always been the case. In 1826, the Zoological Society of London, founded by Samford Raffles, was the first zoological institution to support scientific investigation (Wemmer and Thomson, 1995; Monfort, 2014). Since then, zoos have evolved from menageries of wild and exotic species to places that exhibit animals in their natural setting, changing the narrative that zoos now exist to preserve species and their habitats. It wasn’t until the 1960s that major zoological institutions established zoo-based research departments with broad research interests, including reproductive biology, genetics, behavior, animal health, and husbandry (Benirschke, 1984; Monfort, 2014). Now, many zoological institutions employ scientists and conservationists to advance these disciplines. Links between

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zoos and conservation were not well established until the 1980s, with the development of endangered species programs and species survival plans. Zoo personnel shifted from being exclusively animal managers and caretakers to include scientists and researchers; this has led to more evidence-based research for species kept in captive collections. Insight has been gained from these opportunities in numerous species-specific biological fields, including behavior, infectious diseases, endocrinology, reproductive management, nutrition, and genetics (Ryder, 1995). The modern-day zoo has developed into a true scientific institution.

One of the main conservation goals of *ex-situ* populations is education. Zoos and aquariums that are part of the World Association of Zoos and Aquariums reach over 700 million visitors per year (Fa et al., 2014). An estimated one in ten people visits a zoo each year, amounting to more than 600 million people worldwide (Braverman, 2014; West and Dickie, 2007:5). Zoos are an access point to educate a wide-ranging audience about the value of nature. Visitors can intimately encounter the marvels of nature and use this to inspire themselves to conserve the environment and the species encountered. The individual species maintained in captive collections at zoos or aquaria can be seen as ambassadors for the species and empower visitors to contribute to global biodiversity conservation by these experiences. Outreach and education are vital components of conservation. A zoo's ability to empower the public to learn to care about the species they encounter at zoos will inspire others, and promote conservation of these species in their native ranges.

*Ex-situ* conservation strategies have used zoos and aquaria to establish *ex-situ* assurance and breeding colonies. Zoos can provide resources to maintain a viable population of a species or
maintain genetic material for an insurance policy for species extinction. In addition to species survival, these managed breeding programs are vital to public education; however, these programs can be costly in both the short and long term. Additionally, successful programs can lead to limited resources, as \textit{ex-situ} populations can outgrow the space allocated to them.

 Genome resource banks are one of the classic methods in which \textit{ex-situ} conservation strategies have been developed. The idea behind utilizing assisted reproductive technologies, such as gamete, tissue, and stem cell cryopreservation in order to preserve and maintain current or extinct genetic diversity, is not novel. Collaborative efforts to develop genome resource banks to develop biodiverse frozen biorepositories is a widely adopted method to address conservation challenges. Many projects exist, including the Frozen Zoo at the San Diego Zoo, the Frozen Ark, and the Pan-Smithsonian Cryo-Initiative, which store hundreds of thousands of cryopreserved germplasm, embryo, blood product, tissue, DNA, and fecal/urine samples (Comizzoli and Holt, 2014). The proactive collection of these samples has helped sustain genetic diversity and maintain genotypes of rare species. Although these \textit{ex situ} methods are widely adopted, genome resource banks have to be more widely available to enable scientists to preserve samples from both wild and captive populations and create a link between \textit{in situ} and \textit{ex situ} conservation efforts through the collection and transport of these biomaterials.

\textit{Ex-situ} conservation strategies for reptiles have focused on the captive breeding of species in need within zoos and aquaria. Additionally, conservation organizations such as the Turtle Survival Alliance (TSA) have developed \textit{ex-situ} conservation centers that focus on selective breeding of endangered chelonians. Partnerships between individuals within the private

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sector and zoological institutions have allowed this program to flourish and expand over the past 20 years. However, at this point, ex-situ reptile populations have not contributed as significantly to in-situ populations as have other taxa, such as amphibians. As a wildlife conservation veterinarian, my hope is to use a practical foundation in reproductive science and apply current assisted reproductive technologies (ART) to an understudied taxa (reptiles) in an attempt to lay a foundation of new knowledge that addresses our knowledge gaps and moves the needle forward in advancing the application of ART to reptiles. It is my hope that further developing and applying ART to reptiles will further aid in both ex-situ and in-situ conservation efforts for these imperiled animals.

2.1.4. Assisted Reproductive Technologies (ART) History

Scientific progress in the reproductive sciences of a small subset of species over the past 100 years has led to the development of applied assisted reproductive technologies (ART). The techniques utilized in modern ART were first developed in domestic animals or for human reproduction. In 1784, the first documented case of artificial insemination was reported in a dog (Foote, 2002). Artificial insemination has subsequently been used for decades in livestock management to select for specific genes to improve the maternal or paternal characteristics of different breeds (Foote, 2002; Dresser, 1988). In 1949, it was discovered that rooster sperm could be frozen and stored at low temperatures using a cryoprotectant (Polge, 1951). The development of cryopreservation revolutionized ART and the management of genetics in the agriculture industry, making genes easy to store and transport long distances. Application of ART to wildlife and zoological species became common in the late 20th century. While these

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techniques are far from perfect, they are being used with increased frequency to preserve threatened and endangered wildlife. In captivity, it is often difficult to fully replicate the perfect environment for a species because of there is a dearth of information regarding its biology and there are few species accounts to gain information. This is often the case with reptiles and makes ART even more of a challenge. A recent review of academic databases evaluating ART in mammals showed 104,754 publications with the terms sperm, spermatozoa, oocyte, oocytes, embryo, or embryo in the title. Humans and mice were included in titles of >13,000 papers. Cow, rats, and pigs were found in >4000 papers. Hamsters, rabbits, sheep, and horses were each found in >1000 papers. These nine species accounted for 45.3% of all citations (Herrick, 2019). Research is clearly focused on only a handful of species.

2.1.5. Historical Use of ART in Captive Collections/Zoological Institutions

Zoos and aquariums have evolved dramatically since their origin from private animal collections to modern conservation centers. Many large zoos now have departments specifically focused on the development of ART for endangered species. While ART has a history in these institutions dating back the past 40 years, it has not lived up to the potential to enhance breeding management and enhance the sustainability of small populations (Wildt et al., 2004; Holt and Loyd, 2009; Monfort, 2014). The main hurdle remains our ignorance regarding fundamental species biology (Monfort, 2014). Overcoming this hurdle can take time and investigation and requires us to develop a basic understanding of spermatogenesis, ovulation, follicular development, ovulatory mechanisms, seasonality, pregnancy/gravidity, and infertility for a

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species. Once we understand the basics of a species’ reproductive biology, we can start to enhance, augment, manipulate or overcome blocks to reproduction.

ART is often required in zoological institutions because captive breeding can be challenging to manage naturally. Combining ART with a thorough understanding of reproductive physiology allows for external stimuli to be bypassed and natural hormone cycles to be controlled exogenously (Marcec, 2016; Kouba and Vance, 2009; Kouba et al., 2008; Roth and Obringer, 2003; Watson and Holt, 2001; Wildt and Wemmer, 1999; Wildt, 1991). It also allows scientists to capture and share genetic material from different animals being housed in different institutions. Gamete transport limits an animal's need to undergo stressful and dangerous transport for breeding (Clulow et al., 2014; Kouba et al., 2009; Wildt, 1991). Using ART, gametes can also be stored indefinitely to ensure genetic preservation from animals that die unexpectedly or that die of natural causes, to maintain genetic diversity (Clulow et al., 2014; Kouba et al., 2009; Watson and Holt, 2001; Wildt, 1991).

2.1.6. ART in Reptiles?

While ART is being done more routinely in zoos, few are applying these techniques to reptiles. The lack of work in reptiles is primarily associated with out limited understanding of their reproductive biology. However, the time is now to begin to investigate the biology of these animals if we hope to preserve them. The data suggests many reptiles will not survive the Anthropocene era unless proactive measures are taken. This dissertation will attempt to expand our knowledge of ART in reptiles by taking a standardized approach to better understanding the reproductive biology of reptiles, including characterizing the hormone cycles of lizards and

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controlling them using exogenous hormones, characterizing lizard, snake and chelonian semen, and assessing different methods to extend and cryopreserve semen in reptiles.

2.2. Squamate and Chelonian Reproductive Anatomy and Physiology

2.2.1. Squamate and Chelonian Gonadal Development and Sex Determination

Gonadal development in reptiles is fairly consistent across species; however, the timing within the egg varies by taxa/species. Three broad stages exist: genital ridge formation, the period of the gonad being bipotential, and then differentiation. Genital ridge formation occurs at stages 24-30 of development in squamates; the gonad first develops as a thickening of undifferentiated mesenchymal tissue underlying the coelomic epithelium and extending down the ventromedial surface of the mesonephros (Jones, 2011). This forms the genital ridge, which grows and bulges into the coelom. Germ cells migrate from the posterior germinal crescent to the genital ridge at the end of gastrulation. This migration is thought to occur through the vascular system as in birds since germ cells lie at the surface of the coelomic epithelium in both groups (Kumar et al., 2011). After ridge formation, the gonad is bipotential and contains both medullary and cortical elements. The gonad in lizards remains bipotential for a shorter period of time compared to chelonians and crocodilians. At this point, depending on the species' reproductive strategy, temperature sex determination or genetic sex determination (GSD) occurs. Primordial germ cells migrate to the developing gonad and multiply by mitosis (Jones, 2011). Gonadal primordia, the medullary region of the gonad, is invaded by epithelial cells from the external epithelium of Bowman's capsule and the coelomic epithelium bordering the mesonephric kidneys on the lateral side of each gonad and the germinal epithelium of the gonad. Cells from the

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Malpighian corpuscles and lateral coelomic epithelium become organized in the dorsal part of the gonadal primordium to form the rete cords (Kumar et al., 2011). Germinal epithelium cells give rise to thin sex cords that penetrate into the underlying initial mesenchyme. In animals that are determined to be male, sex cords envelop the germ cells and differentiate into typical testis cords; otherwise, they degenerate. In females, differentiation of the ovary beings as the primordial germ cells congregate in the cortex and begin to differentiate into oogonia at stages 31-32. By stages 33-35, gonadal sex can be recognized; the ovary contains a prominent cortex with one or two layers of oogonia, the tunica albuginea begins to develop, and medullary oogonia become scarce (Warner, 2011). The oocytes begin to appear in the rete and cortex, particularly in the anterodorsally region of the gonads; these regions may later form the germinal beds. During the final stages of embryonic development, stages 36-40, the ovarian cortex becomes well-developed, containing six to ten layers of oogonia and oocytes, the medulla degrades and becomes filled with stromal tissue, and the rete cords are absent or degenerating. Folliculogenesis begins after hatching or birth. Ovaries of neonatal lizards contain clearly defined germinal beds and some primordial follicles, consisting of an oocyte surrounded by a single layer of monomorphic granulosa cells; these are apparent about ten days after birth (Warner, 2011). The morphological stages noted are similar in turtles and crocodilians; however, differentiation occurs during the last third or quarter of embryonic life as the gonads enter a rapid development phase (Warner, 2011).

Many conserved genes are involved in sex differentiation and gonadal development in reptiles. Steroidogenic factor 1 (Sf1) and Wilms tumor 1(Wt1) genes are important for the initial
formation of the bipotent gonad. Double sex and mab-3-relates transcription factor (Dmrt1) is an important testis determining gene, as its expression increases during the temperature-sensitive period and remains high during testicular development. Action of Dmrt1 in testis development is downstream to temperature and upstream to another important agent, anti-Mullerian hormone (AMH). SOX9 does not regulate the upregulation of AMH in reptiles as in mammals, SOX9 expression is observed later than AMH expression. Downstream molecular events in testis differentiation need to be explored in a large number of reptiles in order to develop a conceptual knowledge of the molecular regulation of sex (Kumar et al., 2011).

**Sex Determination in Reptiles.** Sex determination in reptiles has been investigated from ecological and evolutionary standpoints to elucidate the proximate mechanisms of sex determination. The class Reptilia has the most diverse sex determination mechanisms in vertebrates. Mechanisms range from full genetic control of sexual differentiation (GSD) to temperature-dependent mechanisms (TSD) that influence gonadal development in the embryo. Sex differentiation in reptiles exists on a continuum, not a dichotomy, and includes species with true GSD that exhibit highly differentiated sex chromosomes, to species with no sex chromosomes and a sex ratio based solely due to temperature. Intermediates of both strategies have also been documented in nature. Some genera, such as *Pogona*, have differentiated sex chromosomes; however, a thermal influence can still affect the sex ratios. *Apalone* have no thermal influence on the primary sex ratios, but the expression of the sex-determining genes is thermally sensitive. A full description of sex determination in reptiles is beyond the scope of this article; however, a basic understanding of sex determination in reptiles is required in order to

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comprehend the implications of anthropogenic pressures, such as climate change, on the long-term success of reptiles.

Temperature sex determination in reptiles is differentiated into three general patterns. In pattern Ia, males are produced at low incubation temperatures and females at high incubation temperatures. This pattern occurs in some chelonians and lizards. In pattern Ib, males are produced at high incubation temperatures and females are produced at low incubation temperatures. This pattern is found in tuatara and some lizards. In pattern II, males are produced at intermediate incubation temperatures and females are produced at thermal extremes (low and high temperatures). This pattern is found in crocodilians, several lizards, and some chelonians. Although these general patterns have been established since the 1980s, further work has been completed demonstrating an exact thermal-sensitive period. This thermal sensitive period coincides with the time in which gonadal differentiation occurs; this has more significant implications in crocodilians and chelonians because they tend to oviposit at earlier stages of embryo development while squamates tend to oviposit at later stages of development after their gonads have already differentiated. This pattern may lead to a maternal influence or maternal regulation of gonad development.

GSD occurs secondary to inherited factors from the parents (Warner, 2011). GSD is well defined in both birds and mammals. Comparatively, GSD in reptiles can follow several patterns, including male and female heterogamety using both the XY and ZW systems. Females in the ZW system are heterogametic and males are homogametic, while in the XY system males are

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heterogametic and females are homogametic. Additional sex chromosomes can also occur. XXY is found in Gekkonidae and Iguanidae, while ZZW is found in Lactertidae (Warner, 2011).

Squamate Sex Determination. TSD has been documented in over 37 lizards but has yet to be documented in a snake. Our current understanding is that snakes are entirely under the control of GSD. Additionally, until 2017, it was believed that all snakes only had sex chromosomes Z and W. However, it was recently documented that snakes in Pythonidae and Boidae possess the X and Y chromosome system (Emerson, 2017). There is much we don’t know about snakes, but based on the limited number of species investigated it is possible that a species does practice TSD. Based on phylogenetic evidence, snakes are derived lizards, and TSD appears to be an ancestral trait in lizards, so more investigation is needed. This is especially important with the current issues with climate change, as TSD reptiles will be more severely impacted.

Lizards have demonstrated both TSD and GSD strategies. In lizards, TSD is confined to only a few lineages Agamidae, Gekkota, and Scincidae, although some of them to possess temperature overriding GSD mechanisms. Recent advances in comparative genetics have documented a number of genes in mammalian gonadal development that have homologs in TSD reptiles; these include DMRT1, SOX9, WT-1, SF1, and DAX1. In the inland bearded dragon (Pogona vitticeps), sex reversal can occur at extreme temperatures despite being GSD. Recently, a study identified two transcripts of a Jumonji chromatin modifier gene, JARID2/JMJD3, that occurs in high-temperature female dragons that have been sex-reversed by temperature. JARID2 is a component of the master chromatin modifier Polycomb Repressive Complex 2, and the mammalian sex-determining factor SRY is regulated by a closely related Jumonji family member.

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(Deveson et al., 2017). This alters the epigenetics and overrides chromosomal sex-determining cues, triggering sex reversal at extreme temperatures. Sex reversal may then facilitate a transition from GSD to TSD, with JARID2/JMJD3 intron retention preserved as the regulatory signal (Deveson et al., 2017). These same JARID2/JMJD3 transcripts were observed in the embryonic gonads of TSD alligators and turtles (Deveson et al., 2017).

*Chelonian Sex Determination.* A majority of chelonians are considered to have TSD. Of the 149 species that have been karyotyped, GSD was only identified in 8 species. Incubation temperatures and specific sex ratios are species-specific. Recently, advances have been made in examining the genomic implications of temperature and gonad development. A candidate protein called cold-inducible RNA-binding protein was identified in the common snapping turtle (*Chelydra serpentina*). This protein mediates the temperature effects on the developing gonads (Schroeder et al., 2016). In the red-eared slider (*Trachemys scripta elegans*), histone H3 lysine 27 (H3K27) demethylase KDM6B exhibits temperature-dependent sexually dimorphic expression in early *T. scripta* embryos before the gonad is distinct. A knockdown of KDM6B incubated at a temperature in which it should develop into a male, induced a male to female sex reversal. This study was able to demonstrate a link between epigenetic mechanisms and temperature-dependent sex determination (Ge et al., 2018). A subsequent study compared the embryonic gonadal development of epigenetic machinery in chelonians. This study evaluated noncoding RNAs involved in DNA/histone acetylation, methylation, ubiquitination, phosphorylation, and RNAi in TSD and GSD species. Differential expression of methylation and histone acetylation genes responded strongest, suggesting that TSD may be mediated by
epigenetic controlled hormone pathways or hormonally controlled epigenetic processes, suggesting that epigenetic events may be responsible for the differences between TSD and GSD (Radhakrishnan et al., 2018). In the Chinese soft-shelled turtle (*Pelodiscus sinensis*), it was determined that DMRT1 expression is required for testicular development (Sun et al., 2017). Further research is needed on this candidate protein to determine if a genetic marker can be developed to accurately sex animals based on its expression (Perry and Mitchell, 2017).

### 2.2.2. Squamate and Chelonian Reproductive Anatomy

**Male squamate reproductive anatomy.** Compared to mammals, reptiles are unique in that the testes are intracoelomic and develop embryologically closely associated with the kidney. Testes are located dorsally in the middle to caudal coelomic cavity, just cranial to the kidney, and adjacent to the adrenal glands, (Stahl and DeNardo, 2019). In certain lizard species with intrapelvic kidneys, such as green iguanas, the testes are not cranial to the kidney and remain in the middle to caudal coelomic cavity. Testes are typically elongated and cylindrical in snakes, and ovoid to round in lizards. Squamate testes are cream to white in color, except in some species (e.g., chameleon) where the tunica albuginea is pigmented. In chelonians, the testes are yellow to white and oval to elongated. The teste is encapsulated with a thin tunica albuginea, which is connected to the body wall with the mesorchorium. Within the mesorchorium lies the adrenal gland and the testicular artery and veins. The veins drain into the post cava, while the artery arises from the dorsal aorta. In chelonians, the mesorchorium is bound to the kidneys. The seminiferous tubules can be visualized grossly when reproductively active. Testes are composed of two compartments, similar to mammals. A coiled mass of seminiferous tubules, the location

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of spermatogenesis, and an interstitial compartment that dissects between the seminiferous
tubules and contains the Leydig cells, lymphatics, blood vessels, leukocytes, myofibroblasts, and
multiple forms of collagen fibers. The spermatogonia develop within the testes into spermatids,
which then drain into the rete testis and ductuli efferentes before draining into the ductus
epididymis/epididymis. Rete testis do not have ciliated epithelium, whereas the ductuli efferentes
possess both ciliated and non-ciliated cells, making the two regions easy to distinguish (Trauth
and Sever, 2011).

*Ductus Epididymis/Epididymides.* 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 transition from the ductuli efferents into the epididymis is sharply delineated as the
epithelium changes from a cuboidal to ciliated epithelium to tall columnar secretory epithelium.
There is a large amount of secretory material during the reproductive season. Studies have shown
that the rete testis and ductuli efferents appear to be highly conserved in squamates, while the
epididymis is highly variable between taxa. The primary role of the epididymis is the secretion of
cellular products into the lumen. These secretions are modulated by testosterone and other
androgens. The role of the secretory material in the epididymis of lizards is unknown; however,
it likely functions in the final maturation of spermatozoa and has an effect on motility and
fertilization. In lizards, this site is thought to be where spermatozoa are stored, whereas the
ductus deferens is thought to serve this role for snakes. Snakes do not have a well-defined
epididymis, but instead have a ductus epididymis. The ducts leading away from the seminiferous
tubules of snakes are called ductuli efferents, and they connect to a ductuli epididymides which

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empties into the ductus deferens for sperm storage. In chelonians, the ductuli epididymides join the long, convoluted ductus epididymidis, the site for spermatozoa storage in this group.

**Ductus Deferens.** The ductus deferens is a uniform structure that connects the ductus epididymis/epididymis and the cloaca, this is known as the primary location for sperm storage in male squamates. It is formed into short tight loops as it passes caudally in the coelom. The differentiation between epididymis and ductus deferens is progressive, and the lumen of the ductus deferens is wider and the epithelium lower than the epididymis (Trauth and Sever, 2011). Grossly, the duct straightens caudally from the ductus epididymis/epididymis and courses within a mesentery until reaching the dorsal surface of the kidney, where the serosae of the kidney and ductus deferens are joined but different. In chelonians, this structure is extremely short. Caudal to the kidney, the ductus deferens is suspended by the same mesentery as the ureter until reaching the cloaca. Depending on the taxa/species, the ampullae ductus deferentia, ampullae ureters, or ampullae urogenital papillae are the location in which the ductus deferens and urogenital tract drain into the cloaca (Trauth and Sever, 2011).

**Cloaca.** The cloacal is composed of three different segments, the coprodeum, urodeum, and proctodeum. Folds separate these segments within a shared chamber; these are known as the coprourodeal fold and proctourodeal fold. The contents of the reproductive tract drain in conjunction with the urinary tract and sexual segment of the kidney into the urodeum through common pores associated with either a single, medial urogenital papilla or through pores of the bilateral urogenital papillae.

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Hemipenes. All squamates share the same feature of paired copulatory organs, known as hemipenes. Morphological diversity across species is remarkable, and has been studied for evolutionary purposes; however, an in-depth discussion of this is beyond the scope of this article. Typically, the hemipenes are located laterally within the ventral tail base and are maintained in position by retractor muscles. Hemipenes are only for copulation and serve no purpose with regards to excretory function. Additionally, no erectile tissue exists in squamate copulatory organs, unlike chelonians and crocodilians. The hemipenes have no direct connection to the ductus deferens or urogenital papilla, although each hemipenis directs the ejaculate from the urodeum by capillary action into the sulcus spermaticus to facilitate spermatozoa transfer during copulation.

Chelonian Phallus. The single phallus is formed from two longitudinal ridges forming a groove in the ventral floor of the cloaca. It remains in the cloacal in its relaxed state. Each ridge is composed of a coelomic canal and erectile tissue, the corpus cavernosum and corpus spongiosum. Erection is caused by tumescence. The corpus cavernosa fills with blood and becomes engorged, and the phallus is extruded through the vent. With full erection, the phallus curves downward and slightly forward. The engorged semination ridges close the groove into a tube that guides the semen from the opening of the vas deferentia at the phallus base into the receptive female at copulation (Kuchling, 1999). Additionally, support and rigidity is provided by the corpus fibrosum this lies ventral to the groove and extends to the phallus tip, the glans penis. The gland has distinct folds: the plica externa, plica media, and plica interna. The seminal groove

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terminates between the plicae interna. A retractor muscle assists in pulling the phallus back into the cloaca after the corpus cavernosa is no longer engorged (Kuchling, 1999).

**Sexual Segment of the Kidney.** The sexual segment in the kidney (SSK) has been primarily described in snakes and some lizard species, although there may be evidence of its existence in chelonians. The first documented evidence of an SSK was described by an association of renal hypertrophy and androgen secretion in some skink species (Gist., 2011). The secretory activity of the male skinks switched from a mucoid secretion to a granular secretion during the reproductive season. The portion of the kidney in which the SSK resides is different based on taxa. The SSK is not limited to only males. SSK can be found within the intermediate segment, distal convoluted tubule, collecting duct, ureter, or a combination of these regions. In snakes, the secretory process is controversial. It has been suggested that the secretion from the SSK has a merocrine origin, although evidence suggests it could be apocrine in origin. Studies in lizards described both holocrine and apocrine secretions. The impact of the SSK on reproduction in reptiles can only be speculated at this time. Below are the currently proposed hypotheses for the SSK in reptiles:

1) Secretions secure the separation of the semen from the urine by temporarily blocking the renal tubules or the ureter during coitus (Blackburn and Stewart, 2011).

2) Secretions are expelled after the semen, thereby securing the total emptying of the ampulla and seminal grooves (Blackburn and Stewart, 2011).

3) Secretions form a plug which blocks the female's cloaca or oviduct to insure retention of the semen (Blackburn and Stewart, 2011).

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4) Secretions dissolve in the urine; the secretion might give this excretion a characteristic scent, thus enabling the male to leave a trail (Blackburn and Stewart, 2011).

5) Secretions serve as a nutrient medium for sperm (Blackburn and Stewart, 2011).

6) Presence of acid phosphatase in the secretion suggests an activating function analogous to that of the prostatic secretions in mammals (Blackburn and Stewart, 2011).

7) Secretions increase the viscosity of the seminal fluids, thus enabling it to remain in the sulcus spermaticus during mating (Blackburn and Stewart, 2011).

8) Secretions serve as a lubricant. This assists the flow of seminal fluid through the sulci of the hemipenes.

**Female Reproductive Anatomy**

*Ovary.* The ovaries of female squamates are located in a similar position within the coelomic cavity as the testes. In lizards, the ovaries are located in the dorsal mid to caudal coelomic cavity, adjacent to the adrenal gland, and cranial to the kidney in most species. In snakes, the ovaries can be found between from 60-80% the distance from the snout. In squamates, the right ovary is cranially to the left ovary.

The ovaries are supported by the mesovarium, which attaches to the dorsal coelomic wall. In squamates, the adrenal glands are within the mesovarium, while in chelonians and crocodilians they are retroperitoneal and dorsal to the gonads. The dorsal aorta gives rise to the ovarian arteries, while the ovarian vein drains into the renal vein and post-cava (Stahl and DeNardo, 2019). Ovarian morphology and physiology are only well known from a few model species of lizards, including the European common lizard (*Zooteca vivipara*), Italian wall lizards

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(Podarcis siculus), common garden lizards (Calotes versicolor), and the green anole (Anolis carolinensis). Ovarian morphology is apparently well conserved. The ovaries are hollow sacs consisting of a cortex that is covered by a thin wall of surface ovarian epithelium and tunica albuginea (Guraya, 1989). This epithelium is simple squamous and the tunica albuginea is a dense connective tissue. Oocytes are produced within germinal beds (GB), located on the dorsal side of the ovary near the ovarian hilum at the mesovarium attachment. This region consists of proliferating oogonia, undifferentiated somatic cells, oocytes, and primordial follicles. Growing follicles undergo recruitment from the GB and are located in the ovarian cortex, along with the corpus leuta, and atretic follicles. The ovarian stroma is composed of highly vascular connective tissue that surrounds ovarian follicles. The gross appearance of the ovary can vary based on several factors, including maturity and season. Immature ovaries are flat and granular in appearance; however, once the animal matures, different follicular stages can be seen grossly.

The number and location of GB are variable based on species, and no association with the number of eggs per ovary or clutch size has been made. It is thought that GB may allow for growing follicles to develop separately at multiple locations rather than groups; this is supported by snakes as their GB are scattered as irregular patches over the outer border of ovarian stroma (Lance and Lofts, 1978; Jones, 2011).

Squamate Oviduct. The oviduct connects the ovary to the urodeum; it contains and transports the ovulated ovum and facilitates its transformation into either an egg or a fetus. In juveniles, the oviducts appear as homogenously straight organs that are highly translucent. In non-vitellogenic adults, the infundibulum and glandular uterus become coiled and the oviduct appears more...
opaque. In vitellogenic adults, the entire oviduct is highly coiled and opaque. As gravid adults, the "functional regions" of the oviducts become obvious: the region cranial to the eggs in the glandular uterus is the infundibulum and the region caudal to the eggs in the glandular uterus is the non-glandular uterus (Blackburn and Stewart, 2011).

*Squamate Ostium/Infundibulum-cranial/caudal.* The infundibulum is the most cranial portion of the oviduct and is delineated by a very thin mucosa and muscularis. It is the site where ova are ovulated into the oviduct. The infundibulum can be separated into two regions, the cranial region which terminates in the ostium, and the caudal region where sperm storage occurs in some taxa. The main difference between these regions is the presence of distinct infundibular glands; the caudal region has a higher concentration of glands. The entire infundibulum is folded and lined by primary ciliated epithelium with occasional non-ciliated/microvillus cells. In addition to the glands, another significant difference between the cranial and caudal portions of the infundibulum is the presence of glandular-like invaginations or crypts. The crypts are the sites for sperm storage in some female species and are more common in the cranial segment.

*Squamate Glandular Uterus.* The glandular uterus is considered the middle section of the oviduct and is the site where eggs receive their shell in oviparous species. This region is demarcated by dense aggregation of uterine glands; the density of the glands is usually higher in oviparous species than viviparous species. The glandular uterus is encompassed by a muscularis composed of an inner circular layer and outer longitudinal layer. The epithelial lining of the mucosa, which is highly obscured by mucosal glands, is comprised of a simple/pseudostratified cuboidal to columnar epithelium with secretory (microvillus) cells and ciliated cells (Blackburn and Stewart,

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2011). The epithelium lining of the uterus is primarily secretory/microvillus, although numerous authors have described changes in the epithelial surface in lizard taxa based on parity mode and seasonal reproductive cycles (Blackburn and Stewart, 2011). The glandular uterus is highly vascularized, with vascularization increasing during reproductive activity (Guillette and Jones, 1985; Masson and Guillette, 1987; Picariello et al. 1989; Blackburn and Stewart, 2011). Gland abundance does occur between taxa. During reproductive activity, the epithelium lining of the lumen of the glandular uterus increases in height (Picariello et al., 1989), with a concurrent increase in the height of epithelial cells lining the mucosal glands (Picariello et al., 1989). In oviparous species, gland hypertrophy is so significant that the lamina propria is obscured (Picariello et al., 1989). The glands of the glandular uterus are well known for the production of proteins that are involved in shell membrane formation (Blackburn and Stewart, 2011).

Squamate Non-Glandular Uterus/Vagina. The non-glandular uterus (NGU) is the most caudal portion of the oviduct that enters into the oviductal papilla. This may not be the most appropriate term for squamates, as some lizards have ciliated crypts within this portion of the oviduct to allow for sperm storage. Minimal variation is observed in this portion of the reproductive tract across reptiles. A thick muscularis can be found at the junction of the cloaca and highly folded mucosa. Most of the epithelial lining of the NGU is ciliated, with occasional secretory cells. Secretory cells of the NGU epithelium produce mucous and/or neutral carbohydrates (Blackburn and Stewart, 2011). Little variation is observed throughout the reproductive and non-reproductive season in this region; the only reported variation has been an increase in epithelial height during the reproductive season. The NGU may act as a sphincter since there is highly

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developed circular smooth muscle in the muscularis externa, and tightening during egg
development in the glandular uterus allows from the correct positioning of eggs in the glandular
uterus (Blackburn and Stewart, 2011).

**Chelonian Oviduct.** The adult chelonian oviduct has oviductal glands and the epithelium height
differs between segments (Sen and Maiti, 1990; Kuchling, 1999). Five oviductal segments are
generally described in chelonians. The first segment is the proximal portion is the infundibulum
and contains the ostium. The second segment is the convoluted tuba uterina or glandular segment
or the pars albuminifera. The isthmus or intermediate segment is where the shell membrane is
formed. The caudal segment is where the hard outer eggshell is produced. The final segment of
the chelonian reproductive tract is the short vagina or cervix, which empties into the urodeum of
the cloaca (Kuchling, 1999).

**Cloaca.** The cloaca of female chelonians is similar to squamates. The oviduct connects to the
cloaca via the oviductal papilla; there are two in chelonians.

### 2.2.3. Squamate and Chelonian Sex Identification

**Squamate Sex Identification.**

**Snake Sex Identification.** Sexual dimorphism is not consistent in snakes. With a trained eye, one
may see a thickened and longer tail base in males; however, there is much variation between
species. In some species, females are larger than the males, whereas in other species the males
are larger. Some male lizards may have larger spurs adjacent to the vent. In smaller snake
species, eversion of the hemipenes can be attempted with digital pressure. e. In some larger

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species of snakes, this is not an option and probing is required. In females, the openings to the scent glands can be seen in the cloaca. (Stahl and DeNardo, 2019).

Cloacal probing can be used to sex snakes and some lizards. Probing the cloaca is performed with a slender, blunt instrument and can be used to confirm the presence of the hemipenes. Commerical snake probes are available for purchase. Probes can be lubricated with non-spermicidal lube or water prior to sexing an individual. With an appropriately restrained reptile, the probe is inserted into the vent and directed caudo-lateral. As the probe enters an inverted hemipene, it is advanced the length of the sulcus spermaticus. In males, the probe can be inserted 2-3 times the depth of a female.

**Lizard Sex Identification.** Sexual dimorphism is commonly observed in lizard species. Males tend to have a more robust appearance with a larger head and jowl. Male lizards may also have additional ornamentation, such as spikes, scales, crests, or horns. Some male lizards (e.g., green iguana) have femoral or (e.g., leopard gecko) pre-anal pores that secrete a waxy substance used for territory marking. Probing can be performed in some lizards as described previously in snakes; however, this method is less reliable in lizards than in snakes. Everting hemipenes can also be done in lizards; however, it is more challenging unless anesthesia is used.

**Transillumination.** In small lizards, transilluminating the tail base can be done to visualize the hemipenes. A focal light source that does not produce heat should be used. In males, transillumination will outline the hemipenal structures, which have increased tissue and vascular presence compared with the female hemiclitores (Stahl and DeNardo, 2019).

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**Radiography.** Larger lizards, especially monitors, often have mineralized hemibacula within their hemipenes. These structures can be visualized on radiographs. This technique may only be valuable in older animals, as mineralization may not occur until an animal matures. Introduction of contrast media into the hemipenal/hemiclitoral sac using a tom-cat catheter can also be done. Male skinks were found to have radio-opacities that were wider and spiral in shape compared to females with straight and horizontal radio-opacities. This method was 100% sensitive in skink species to predict sex in animals with snout vent lengths >15 cm (Stahl and DeNardo, 2019).

**Ultrasonography.** Ultrasonography has historically been limited to identifying sexually mature females. Ovarian follicles can be visualized easily compared to testes, acyclic females, and immature animals. No studies have evaluated the diagnostic sensitivity or specificity of ultrasound for sexing reptiles. The only study to date evaluated hemipenes and determined that the accuracy of ultrasound to confirm sex was poor at 64.3% (Stahl and DeNardo, 2019).

**Endoscopy.** Endoscopic sex determination is infrequently used in squamates. No studies exist evaluating its accuracy in predicting sex, although it would be expected to be a definitive method of sexing squamates.

**Molecular DNA Sexing.** In species where sex is determined genetically, sex can be determined secondary to heterogametic chromosomes. Unfortunately, an understanding of the exact mechanism for the species is required. A general marker for reptiles is not likely possible because of the wide variety of species and reproductive strategies. PCR has been used to sex Komodo dragons (*Varanus komodoensis*) because males had consistently longer read lengths. (Stahl and DeNardo, 2019)
Plasma sex steroids/Hormonal. In squamates, plasma sex steroids are often not used to confirm sex due to the other methodologies available. These methods would be similar to the methods used in chelonians, with testosterone being higher in immature juvenile males than females.

Chelonian Sex Identification.

External Sexual Characteristics. Tail length and plastron conformation (tortoises only) are typically used to sex chelonians. In males, the tail is longer and the vent more caudal (beyond carapacial rim) than in females. Additionally, male tortoises tend to have a more concave plastron; however, this may be altered in animals with shell pyramiding because of abnormal shell growth. The plastron concavity and longer tail are evolutionary adaptations to help facilitate mating behaviors such as mounting and intromission. Species-specific secondary sexual characteristics may also be useful for determining the sex of chelonians, including skin color, eye color, toe-nail length, scute size, and adult size. Examples of species-specific sexual dimorphism in chelonians include: the red iris color of male eastern box turtles (*Terrapene carolina carolina*), elongated toenails of the forelimbs of male red-eared slider turtles, painted turtles (*Chrysemys picta*) and map turtles (*Graptemys spp.*), and enlarged gular scutes of male sulcatta tortoises (*Centrochelys sulcata*). Body size may also help differentiate the sexes. For example, in diamondback terrapins (*Malaclemys terrapin*) and map turtles, females are generally larger; however, for desert tortoises (*Gopherus agassizii*), males are, on average, larger. It is important for veterinarians to learn these species-specific characteristics to assist their clients with sexing their mature animals (Perry and Mitchell, 2017; Innis and Boyer, 2002; Sykes, 2010).
Chelonian Coelioscopy for Sex Identification. Coelioscopy can be used to determine sex in juvenile animals or those without overt dimorphic characteristics (Perry and Mitchell, 2017; Rostal et al., 1994; Mitchell et al., 2009; Perpiñán et al., 2013). Ideally, endoscopic examination should be performed under general anesthesia, although the technique can be performed using local anesthetics. However, one study did observe higher pain scores in Chinese box turtles (*Cuora flavomarginata*) endoscopically sexed under local anesthetics versus general anesthesia (Perry and Mitchell, 2017; Hernandez-Divers et al., 2009).

Chelonian Cloacoscopy and Cystoscopy for Sex Identification. Endoscopic examination of gonads may also be attempted through the cloaca/urinary bladder (Martínez-Silvestre et al., 2015; Pronécá, 2016). One advantage of this technique is that it doesn't typically require surgical anesthesia. A disadvantage to this technique is that uric acid sediment can hinder visualization. Additionally, the accessory vesicle can be highly vascularized and impede visualization. Unfortunately, the sensitivity of this method may not be high. One study attempted to identify sex in red-eared sliders by the presence of the phallus/clitoris; however, males were misdiagnosed as females (100%) and females as males (38%). Additionally, a recent study performed in 30 immature (36–90 g) red-eared sliders showed that only 10% (3/30) of the animals had their sex accurately identified by cystoscopy and 23% (7/30) animals experienced bladder or cloacal rupture (Pronécá, 2016).

Measuring Hormone Concentrations for Sex Identification. In juveniles, the measurement of plasma testosterone concentrations is also a useful method to determine sex (Rostral et al. 1994). showed that animals that were endoscopically sexed to be male had a significantly higher plasma...
testosterone concentration compared to females. There was 98% agreement observed between
the two methods. A follicle stimulating hormone (FSH) test has also been shown to be useful in
confirming sex. FSH can be injected intracoelomically and blood drawn 4 hours later to measure
plasma testosterone. Male concentrations will be > 0.5 ng/ml, while females will be < 0.2 mg/ml
(Perry and Mitchell, 2017; Innis and Boyer, 2002).

2.2.4. Squamate and Chelonian Reproductive Hormones and Reproductive Cyclicity

Hypothalamic-Pituitary-Gonadal Axis (HPG).

Hypothalamus. The HPG axis is the primary anatomic and physiological controller within the
body that links the brain to the gonads and accessory reproductive organs in reptiles. This system
is conserved across taxa with minor variations. In reptiles, gonadotropin-releasing hormones
(GnRH) initiate reproduction by the hypothalamus (Kumar et al., 2011; Licht, 1979; Bona-Gallo
et al., 1980; Van-Dyke, 2015). GnRH secretion in reptiles is likely pulsatile as in other species;
however, this has only been demonstrated in chelonians (Tsai and Licht, 1993). In reptiles, many
different forms of GnRH have been demonstrated, including cGnRH-I and cGnRH-II in
crocodilians and chelonians (Kumar et al., 2011; Lovejoy et al., 1991, Millar and King, 1994;
Sherwood and Whittier, 1998; Tsai and Licht, 1993a), and cGnRH-II and salmon sGnRH in
lizards (Powell et al. 1985; Powell et al., 1986). The significance of these different GnRH forms
in reptiles is unknown. Synthetic cGnRH-II has been demonstrated to stimulate pituitary
gonadotrophs, while cGnRH-I does not always stimulate lutenizing hormone (LH) secretion in vivo, although it has been demonstrated to work in-vitro (Licth and Porter, 1985; Tsai and Licth,
1993c; Licth et al., 1987). GnRH receptors (GnRH-R) were first documented in reptiles in

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leopard gecko (*Eublepharis macularis*) when phylogenetic analysis showed a cloned GnRH-R belongs to type-2/non-mammalian I (Ikemoto et al., 2004). Subsequent studies showed three separate GnRH-R that responded to GnRH ligand, cGnRH-I, and cGnRH-II. Only one of these receptors was isolated to the anterior pituitary, while the other two were located in the intermediate and posterior pituitary (Ikemoto and Park, 2007). In female iguanas, administration of cGnRH-II with an osmotic pump induced a five-fold increase in plasma estrogens and elicited reproductive behaviors (Jones, 2011; Phillips et al., 1987). Gonad inhibitory hormone (GnIH) has been documented in avian species, which could play a role in the inhibition of GnRH or gonadotropin release. GnRH release stimulates the production of gonadotropins from the anterior pituitary. The primary gonadotropins thought to occur in reptiles are LH and FSH, although both of these hormones have not been well documented across reptile species.

**Anterior Pituitary.**

**Gonadotropins.** Gonadotropins in mammals are well documented, and there are defined roles for LH and FSH in this group. In females, FSH is the primary regulator for oogonia proliferation, oocyte growth, and oocyte differentiation, while LH stimulates ovulation and steroidogenesis (Jones, 2011). In males, FSH regulates Sertoli cell function and LH regulates androgen synthesis. Unfortunately, in reptiles these roles are not well defined. Two distinct gonadotropins have been documented in chelonians and crocodilians, while squamates are thought to only have one gonadotropin that is not FSH or LH specific. In chelonians, this was documented first by fractionation studies (Licht et al., 1976; Papkoff et al., 1976; Licht and Papkoff, 1985).

Additionally, in chelonians, partial cDNA sequence for the β-subunit of FSH and the full cDNA
sequence for the β-subunit of LH were described from the pituitary of the Reeves’ turtle
(Geoclemys reevesii) (Aizawa and Ishii, 2003). LH is thought to not exist in squamates (Licht,
1974; Licht et al., 1974; Licht and Crews, 1975), and FSH appears to be the only functional
gonadotropin in this group (Licht and Crews, 1975; Licht, 1979). There is, however, much we
don’t know and that needs further investigation. For example, the lizard genome contains
sequences that are homologous to those that code for the β-subunit of mammalian FSH and LH
(Borrelli et al., 1997), while an additional study of jararacas (Bothrops jararaca) could not
demonstrate evidence for an LH receptor but was able to clone cDNA for the FSH receptor and
showed it is expressed in the ovary and testis (Jones, 2011; Bluhm et al., 2004). In the Italian
wall lizard, two gonadotropins have been confirmed immunochemically in the pars distalis with
human LH and FSH β subunit antibodies (Desantis et al., 1998). Additional southern blot
analysis in these lizards has demonstrated nucleotide sequences coding for the β-subunit for both
LH and FSH. Currently, evidence only supports a one gonadotropin-two function strategy
compared to the two-cell two gonadotropin theory that drives the reproductive cycle of higher
vertebrates.

**Posterior Pituitary (Neurohypophysis)**

Arginine vasotocin (AVT)/Oxytocin. In reptiles, debate still occurs on which hormones are
released from the neurohypophysis. Arginine vasotocin and oxytocin are typically thought of as
the hormones secreted from the neurohypophysis in higher vertebrates; however, in lower
vertebrates, a duplication of the gene encoding the precursor molecules and subsequent
mutations has led to the production of vasopressin-like and oxytocin-like lineages (Krohmer and

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In reptiles, birds, amphibians, and marsupials, the oxytocin-like peptide is mesotocin. However, conflicting information exists in reptiles. In snakes, hypophysial extracts from Indian cobras (Naja naja) and grass snakes (Tropidonotus natrix) showed the presence of oxytocin (Follett, 1967; Pickering, 1967). In viperine water snakes (Natrix maura), oxytocin-like immunoreactivity was observed in the hypothalamus and neurohypophysis, although most evidence supports that mesotocin is the oxytocin-like peptide in snakes. (Krohmer and Luitterschmidt, 2011; Follett, 1967; Pickering, 1967; Perez-Figares et al., 1995; Silveira et al., 2002; Lazari et al., 2006; Acher et al., 1969; Pickering, 1967; Acher et al., 1969; Fernández-Llebrez et al., 1988; Mancera et al., 1991; Andrades et al., 1994; Acher et al., 1968; Acher et al., 1969).

AVT appears to play a significant role in the processes of oviposition or parturition. In oviparous and viviparous species, AVT induces oviductal contractions in vitro (Girling, 2011; La Pointe, 1969; La Pointe, 1977; Guillette and Jones, 1980; Cree and Guillette, 1991; Fergusson and Bradshaw, 1992; Guillette et al., 1992) to induce oviposition or parturition (Guillette and Jones, 1982; Guillette, 1979; Guillette and Jones, 1982; Cree and Guillette, 1991). Maximal sensitivity of the oviducts to AVT has been reported in reptiles at approximately the same temperature as the preferred optimal body temperature (Girling, 2011; La Pointe, 1977). In the olive Ridley turtles (Lepidochelys olivacea) and loggerhead sea turtles (Caretta caretta), AVT and neurophysin (prohormone) concentrations are low in animals until oviposition (Girling, 2011; Figler et al., 1989). Values in these turtles are consistent with the hypotheses that the AVT-neurophysin complex is stimulated during nesting and that AVT is the physiological regulator of oviductal

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contractions in sea turtles. Similarly, in the tuatara (*Sphenodon punctatus*), high levels of AVT were recorded during oviposition (Guillette et al., 1991d). In a viviparous species, the short-tailed skink (*Tiliqua rugosa*), plasma AVT rose approximately 30 days prior to parturition (Girling, 2011; Fergusson and Bradshaw, 1991). Elevations in AVT were also observed in these skinks along with a decrease in plasma progesterone from the high levels measured during midpregnancy to basal levels at parturition as the corpus luteum degenerated (Girling, 2011; Fergusson and Bradshaw, 1991).

The actions of AVT may also be attenuated or enhanced by sex steroids. In the skinks, uterine contraction strength in vitro was reduced by pretreatment in vivo with progesterone or estradiol, although the frequency of AVT-induced contractions was enhanced by estradiol pretreatment (Fergusson and Bradshaw, 1992). Contraction strength was not significantly different between pregnant and non-pregnant females. Spontaneous rhythmic contractions were present only in pregnant females. Ovariectomy did not affect the spontaneous or the AVT-induced contractions in the skinks (Girling, 2011; Fergusson and Bradshaw, 1992). If these results are valid, AVT may have additional functions in reptiles, especially viviparous species.

**Gonads.**

**Testicles.** Mammalian GnRH (mGnRH) and its agonists do not affect plasma LH or gonadal steroids in male green sea turtles (*Chelonia mydas*), musk turtles (*Sternotherus odoratus*), or olive Ridley turtles (Kumar et al., 2011; Licth et al., 1982, Licth et al., 1984). cGnRH-I is also ineffective in musk turtles. On the other hand, mGnRH does stimulate testosterone secretion in male alligators (Kumar et al., 2011; Lance et al., 1985). These studies used highly variable doses,
frequencies of treatment, and multiple GnRH variants. In Italian wall lizards, a single dose of mGnRH, cGnRH-II, or sGnRH increased plasma testosterone, although mGnRH and cGnRH-II were more potent than sGnRH. Prolonged administration of GnRH inhibits testicular and epididymal activity due to desensitization at the pituitary (Kumar et al., 2011; Ciarcia et al., 1989). This has also been demonstrated in female red-eared slider turtles as prolonged administration of cGnRH-I or cGnRH-II attenuated LH release from the pituitary.

In mammals, intratesticular activity of FSH normally regulates Sertoli cell function, while LH controls Leydig cell androgen synthesis. The exact role of FSH and LH in the regulation of testicular function in chelonians and crocodilians is not well understood. mFSH (mammalian) has been reported to be a more potent regulator of steroidogenesis in chelonians and alligators compared to mLH (Kumar et al., 2011; Callard and Ryan, 1977; Tsui and Licht 1977; Lance and Vliet, 1987) although in alligators in-vitro incubation of interstitial cells with alligator LH increased androgen production, not FSH (Tsui and Licht, 1977). Crocodilians and chelonians are the two reptilian taxa in which evidence exists for a two-cell, two gonadotropin theory of reproduction. Although some evidence exists for a two-cell, two gonadotropin theory for squamates, it is predominantly viewed that a two-cell, one gonadotropin theory regulates reproduction in this group. A single FSH-like molecule is a predominant gonadotropin that regulates testicular function in squamates. In western whiptails (Cnemidophorus tigris), a high-affinity binding site for mFSH is present within both the interstitial and tubular compartments of the testicle. An in-vitro study evaluating ovine FSH has provided evidence of activity in the Sertoli cells and Leydig cells of Indian wall lizards (Kahn and Rai, 2005).

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Ovaries. Reproductive cyclicity in female reptiles is associated with changes in circulating plasma concentrations of the sex steroids: 17β-estradiol (E₂), testosterone (T), and progesterone (P₄). The ovary is the primary endocrine gland responsible for the secretion of sex steroids in female reptiles, although P₄ has been documented to be synthesized in the adrenal glands and uterus/oviduct/placenta (Highfill and Mead, 1975; Painter et al., 2002; Painter and Moore, 2005; Girling and Jones, 2003). Estrogens and progesterone are assumed to be the primary hormones regulating reproduction in female reptiles, although some evidence exists that other metabolites may also be critical in reptile reproduction. In the eastern bearded dragons (Pogona barbata), E₂ concentrations were low during vitellogenesis, which had never been documented before, and led to speculation that E₂ may not be necessary in this species and that a metabolite of E₂ may be more critical for reproduction (Amey and Whittier, 2000). Other species have also been documented to have alternatives to E₂, including blotched blue-tongue skinks (Tiliqua nigrolutea) and common garter snakes (Thamnophis sirtalis parietalis) (Edwards et al., 2002; Whittier and Hess, 1992). Alternative metabolites to P₄ could also be important in regulating reptile behavior. The reptile ovary produces T, but it has been found to vary throughout the reproductive cycle (Callard et al., 1978; Edwards and Jones, 2001); this too suggests that it may have a role in the female reproductive cycle. Non-steroidal hormones, such as prostaglandins, have also been shown to be produced by the reptile follicle and corpus luteum, which suggest it too likely plays a role in ovulation (Guillette et al., 1988; Guillette et al., 1991; Gobbetti et al., 1993a). GnRH-1, bradykinin, and their respective receptors have been identified on the ovary.
of oriental garden lizard (*Calotes versicolor*) (Singh et al., 2007); however, the role of these hormones in this animal are unknown.

**Pineal gland.**

The pineal gland functions as one of the primary neuroendocrine converters of environmental stimuli into cellular and endocrine functions. The primary secretory product of the pineal gland is melatonin (N-acetyl-5-methoxytryptamine). Melatonin production is directly influenced by environmental cues such as photoperiod and temperature, and the subsequent pattern of melatonin secretion from the pineal gland is the interface between extrinsic cues and physiologic mechanisms and behaviors. Melatonin concentrations increase secondary to darkness. Pinealectomized desert iguanas (*Dipsosaurus dorsalis*) and ruin lizards (*Podarcis sicula*) had abolished circadian rhythms of plasma melatonin, although melatonin can be synthesized by the hardarian gland, retina, and intestine. In common garter snakes, plasma melatonin levels did not differ between pinealectomized and sham-operated animals regardless of whether the sample was collected during the light or dark cycle. These findings suggest that extra-pineal production of melatonin contributes to baseline concentrations. Plasma melatonin concentrations are modulated by a number of extrinsic cues, including photoperiod phase and duration, light intensity, and temperature. Melatonin provides a neuroendocrine index for the time of day, and changes in the duration of melatonin signal reflect annual changes in the light: dark ratio and seasonal changes. An increase in the duration of melatonin synthesis has been implicated in the reproductive cycles of some species (Krohmer and Luitterschmidt, 2011; Bittman et al., 1983 and Carter Coldmand, 1983). Photoperiod influences the phase and duration
of the melatonin cycle, while environmental temperatures modulate the amplitude. In diamondback water snakes (*Nerodia rhombifer*), cold and warm temperature extremes decreased the amplitude of the melatonin cycle (Krohmer and Luitterschmidt, 2011; Tilden and Hutchison, 1993). This pattern has also been observed in other reptiles, including three toed box turtles (*Terrapene carolina*), marbled geckos (*Christinus marmoratus*), and green anoles (*Anolis carolinensis*) (Krohmer and Luitterschmidt, 2011; Vivien-Roels et al., 1988; Rawding Hutchison, 1992; Tilden and Hutchison 1993; Moyer et al., 1995; Garcia-Allegue et al., 2001). In common garter snakes, elevated hibernation temperatures significantly increased overall melatonin concentrations (Luitterschmidt and Mason, 2009). Environmental temperature, in the absence of a changing photoperiod, is sufficient in modulating melatonin rhythms and androgen concentrations during hibernation. Additionally, cold temperature exposure has a lasting influence on melatonin rhythms, as animals hibernated at 5°C and 10°C persisted even after being acclimated to 10°C (Luitterschmidt and Mason, 2009). The amplitude of the melatonin cycle also appeared to increase in response to prolonged low temperatures.

In green anoles, pinealectomized male and females experienced testicular growth, spermatogenesis, ovarian weights, oviductal weights and follicular development (Mayer et al., 1997; Levey, 1973; Underwood, 1985). Administration of melatonin abolished the effects in female anoles (Levey, 1973). This evidence supports the theory that the pineal gland and melatonin are inhibitory to reproduction. The anti-gonadotropic effects of melatonin have also been observed in other reptiles, including oriental garden lizards and zebra tailed lizards (*Callisaurus draconoides*). In male Hermann’s tortoises (*Testudo hermanni*), melatonin implants

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decreased the gonadosomatic index and testosterone concentrations (Krohmer and Lutterschmidt, 2011; Vivien-Roels and Arendt, 1983; Vivien Roels and Pévet, 1983). In green anoles and Western fence lizards (*Sceloporus occidentalis*), removal of the pineal gland blocked reproductive responsiveness to photoperiod (Underwood, 1981a; Underwood, 1985 a,b), which allowed animals to become reproductively active in the non-breeding season when exposed to inhibitory photoperiods (Underwood 1985a; Lutterschmidt et al. 2004). Pinealectomy in Indian chequered water snakes (*Natrix piscitor*) had an effect on testicular size based on environmental humidity. Testicular size decreased in pinealectomized snakes housed in high humidity, and increased in both low and moderate humidity (Lutterschmidt et al. 2004; Haldar and Pandey, 1989 a,b). Administration of melatonin or 5-methoxytryptamine in these snakes suppressed testicular function (Haldar and Pandey, 1988) and significantly inhibited courtship behavior of the snake during the spring. The pineal gland is an important coordinator of the synchrony of the environment and reproduction in reptiles, and it should be considered when developed ART.

**Hypothalamic Pituitary Adrenal Axis (HPA).**

*Corticotropin-releasing factor (CRF).* CRF has been identified in the brains of green anoles, red-eared sliders, and viperine water snakes. The distribution of CRF within the brain of reptiles is similar to mammals, including localization within the paraventricular neurons. In water snakes, CRF is co-localized with AVT. CRF receptor antagonists have been shown to block stress related effects on ovarian recrudescence (Ganesh and Yajurvedi, 2002b). Negative feedback also occurs with CRF production as demonstrated in red-eared sliders. CRF stimulates the cells within the adenohypopsis that produce ACTH. Pituitary corticotropins synthesize and release

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ACTH, which has been demonstrated to stimulate adrenal corticosteroid synthesis and release in several lizards, including Eastern fence lizards, marine iguanas (Amblyrhynchus cristatus), New Zealand common geckos (Hoplodactylus maculatus), White’s skinks (Egernia whitii), and a crocodilian, spectacled caiman (Caiman crocodilus). This has yet to be confirmed in a chelonian (Sanford and Stephens, 1988). Conflicting evidence exists regarding the reptilian adenohypophysis-adrenal axis; mammalian ACTH failed to stimulate adrenal steroid secretion in the turtle, crocodilian, snake, and lizard (Sanford and Stephens, 1988; Nothstine et al.1971; Macchi and Phillips, 1966; Wright and Chester Jones, 1957; Phillips et al., 1962; Gist and deRoos,1966). Instead, other mediators, such as angiotensin II, norepinephrine, and epinephrine, may be responsible for stimulating the release of corticosterone in reptiles, or ACTH may not be the same structure in reptiles as it is in mammals.

**Hypothalamic Pituitary Thyroid Axis (HPT).**

The thyroid has been found to influence the male reptile’s reproductive cycle, particularly with regards to temperature-induced reproductive activity. Thyroid hormones regulate metabolic function in reptiles; however, thyroid hormones are sensitive to thermal cues and likely play a major role in reproduction. In the oriental garden lizard, lizards were thyroidectomized, the testicular cycle during the reproductive season was inhibited, and then rescued in individuals administered 0.02 µg/g L-thyroxine. Additionally, administration of L-thyroxine to non-thyroidectomized lizards suppressed spermatogenesis and induced atrophy of the sexual segment of the kidney, indicating that excessive thyroid levels can inhibit reproduction in male lizards.

The thyroid is essential for male reproduction, especially in a seasonal species, as there appears
to be an intermediated concentration of plasma thyroid that promotes reproduction (Haldar-Misra and Thapliyal, 1981). Treatment of hypophysectomized male glossy snakes (Arizona elegans) with thyroid stimulating hormone (TSH) stimulated and restored spermatogenesis, although the effect of TSH in this case may have resulted from cross-reactivity of the hormone with gonadotropin receptors (Chiu and Lynn, 1971). Further investigation is needed to fully elucidate the role of the thyroid gland in reptile reproduction.

**Prolactin (PRL).** Prolactin has been found to originate from the pars distalis of the adenohypophysis in squamates and chelonians and has been identified as exerting an effect on reproductive activity and behavior (Licht, 1969; Mazzi and Vellano, 1987). A hypothalamic prolactin-releasing hormone has been documented in red-eared sliders. Additionally, an endothelin 3-like substance is co-localized in secretory granules containing gonadotropins and thyrotropin in the pituitary of Chinese soft-shelled turtles. The expression of the endothelin 3-like substance is more pronounced during the breeding season and in males than females.

Endothelin 3-like substance stimulates the release of prolactin and growth hormone in vitro (Suzuki et al., 1997; Kuchling, 1999). In other species, prolactin secretion has been found to be mediated by the action of dopamine; dopamine inhibits prolactin secretion and prolactin stimulates dopamine production. In Northern house geckos (Hemidactylus flaviviridis), exogenous administration of pimozide (50 µg/kg Q 24 hrs), a dopamine receptor antagonist, resulted in increased growth rate in tail regeneration in animals exposed to a 24-hour dark cycle, where previous studies showed that the tails would not re-grow. In those studies, re-growth was initiated with porcine prolactin administration. This suggest that prolactin secretion in reptiles is

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mediated, as in other species, through dopamine (Ndukuba Ramachandran, 1989). In green anoles, ovine prolactin depressed oviductal weights in vitellogenic animals but had zero effect on non-reproductive animals. Prolactin treatment also had no influence on the ability of estradiol to stimulate oviductal growth. Ovine FSH, when administered with prolactin, depressed ovarian and oviductal growth, only in vitellogenic animals (Hensgen et al., 1980). In the desert iguana, administration of ovine prolactin with gonadotropin decreased follicular size but did not decrease follicular numbers. Following hypophysectomies, animals were administered a combination of growth hormone, pregnant mare serum gonadotropin/equine chorionic gonadotropin (PMSG/eCG), and ovine prolactin. Prolactin only inhibited PMSG induced ovarian growth in the presence of growth hormone (Callard, 1969). Prolactin does have other physiological effects, including the induction of hypercalcemia. In yellow monitors (Varanus flavescens), daily prolactin administration of 10IU/100g body weight for seven days resulted in an elevation in serum calcium concentrations (Swarup et al., 1985). The same effect was seen in checkered keelback water snakes (Natrix piscator) following injections of 10 IU/100g body weight, as well as an increase in phosphorus. These changes were attributed to bone resorption.

Melanocyte-stimulating hormone (MSH). α-MSH has been documented and localized to the intermediated lobe of the pituitary gland in one species of reptile, the common wall lizard (Lacerta muralis). α-MSH precursors have also been documented in green anoles. The effect of α-MSH secretion on reproduction in reptiles is unknown.

Somatotropin (Growth Hormone). In reptiles, limited information exists on the process of puberty. Somatotropin releasing cells have been characterized in the anterior pituitary of at least Portions of this section was previously published as:
one chelonian species, the pond slider (*Pseudemys scripta*). In both male and female reptiles, puberty is defined based on the ability of the gonad to respond to stimulation. It is generally believed that puberty in reptiles is associated with a threshold body size and mass. Once a reptile becomes sexually mature and is reproductively competent, the gonads respond to hormonal stimulation.

*Leptin.* Reptile leptin localized from the *Anolis* genome has a similar structure, size, and location to mammalian leptin (Boorse and Libbon, 2010). Its function has been demonstrated in a few lizard species, with physiological effects as associated with temperature regulation and food intake. (French et al., 2011; Sciarrillo et al., 2005, Niewarowski et al., 2000, Putti et al., 2009). Serum leptin concentrations are highest in the spring prior to vitellogenesis, when adipose tissue mass is greatest, and fall as adipose tissue mass decreases during vitellogenesis (Paolucci et al., 2001). Circulating serum leptin concentrations are typically correlated with adipose tissue mass. Leptin is remarkably lowest in the autumn, when adipose tissue mass is greatest (Spanovich et al., 2006). In female ornate tree lizards (*Urosaurus ornata*), murine leptin administered to vitellogenic females with or without food was implicated in suppressing immune function in reproductive females but not in non-reproductive females (French et al., 2007a, 2007c). French et al. 2011, demonstrated that reproductive animals undergoing food restriction exhibited decreased fat stores, suppressed wound-healing, and slowed follicular growth. Leptin treatment reversed the immunosuppressive effects as well as attenuated the reproductive effects. This was the first example of leptin being immunomodulatory. Since then, green anoles that experience increased activity, via exercise training, have a marked decrease in immune function, as well as
reproduction, when calorically restricted. Leptin treatment rescued immune function in endurance training and calorie restriction; however, reproduction was not rescued. These results suggest that immune function and reproduction have different sensitivities to leptin or that reproduction can be prioritized when energy is available (Wang et al., 2019). In Italian wall lizards, leptin administration was shown to play both a direct and indirect regulatory role on reproduction and testicular function. The presence of leptin in a seasonal breeder may delay testicular regression through either PPARα or 17β-HSD function at the level of the germ cells (Putti et al., 2009).

Furthermore, leptin receptors have been documented in the thyroid glands of Italian wall lizards and exogenous administration of leptin may stimulate the thyroid gland; T3 and T4 release were observed following administration, but TSH concentrations decreased (Sciarrillo et al., 2005). It is thought that a functional receptor of leptin exists in the pituitary based on experimental studies. Recombinant human leptin was administered to female Italian wall lizards at four different concentrations, and there appeared to be a dose-dependent effect on FSH release at the level of the pituitary (Ferrandino et al., 2015). This pattern is consistent with what occurs in nature. Female reptiles appear to adjust clutch and litter size depending on resource availability. Clutch/litter size are determined at two points during the reproductive cycle, either females recruit a given number of ovarian follicles for primary vitellogenesis at the start of a reproductive cycle or females selectively allocate yolk and ovulate a fraction of the "committed" follicles, while the rest undergo atresia (Aldridge, 1982; Aldridge and Semlitsch, 1992). Selective follicular atresia appears widespread in squamates (Shine, 1977; Jones et al., 1978;
Trauth, 1978; Etches and Petitte, 1990; Mendez-De La Cruz et al., 1993). Exogenous FSH has been shown to increase clutch size in several species (Sinervo and Licht, 1991; Jones and Swain, 2000). The correlation of body condition/fat body mass and clutch size, along with the observation that FSH stimulation increases clutch size, suggest hormones signal resource abundance within the brain and act in a dose-dependent manner. Leptin appears to be that link. Selection could be expected to act strongly on this mechanism because overcommitting resources to reproduction could lead to starvation before oviposition/parturition, while under committing could prevent females from realizing their actual reproductive value (Fisher, 1930; Lack, 1954).

**Kisspeptin.** The kisspeptin system plays a vital role in regulating seasonal changes in reproductive physiology, metabolic control of reproduction, and the development of reproductive capability in mammals (Neuman-Lee et al. 2017; Greives et al., 2008, Castellano et al., 2009; Ball and Wade, 2013). Kisspeptin has been documented to regulate the hypothalamic-pituitary-gonadal axis; however, in lower vertebrates, this system has been poorly investigated. *Kiss1* and *Kiss1R*, which are called *Kiss2* and *Kiss2R*, in lower vertebrates, have been documented in zebrafish (*Danio rerio*), African clawed frogs (*Xenopus laevis*), and grass lizards (*Takydromus tachydromoides*). Grass lizards possess the gene for *Kiss2* but not *Kiss1*. Immunohistochemical staining of the brains of green anoles using human kisspeptin antiserum revealed the presence of kisspeptin-like immunoreactivity in the preoptic area (Dunham et al., 2009), which is vital in the activation of the HPG axis. This is evidence that a functional *Kiss 2* peptide exists, suggesting that the kisspeptin system may regulate reproductive function in reptiles, as in mammals (Neuman-Lee et al., 2017). One study in reptiles examined if kisspeptin administration can...
activate the HPG axis. Administration of 50 µM Kiss1 in PBS intracoelomically to side blotched lizards (Uta stansburiana) during the reproductive season led to an increase in plasma testosterone concentrations, while there was no effect on plasma corticosterone concentrations. There was no observable change in behavior with the kisspeptin treatment; however, a correlation was seen with testosterone and push-ups. Castration effectively eliminated the elevation of plasma testosterone, thus proving that the effects were centrally mediated and not at the level of the gonad.

**Ghrelin.** Ghrelin, a peptide hormone, is typically secreted by the stomach in response to filling and emptying, creating a neurohormonal feedback loop that regulates appetite at the hypothalamus. In some lower vertebrates, such as fish, ghrelin has been linked to gonadotropin production; thus, this would likely influence reproduction based on environmental resource (food) availability (Rousseau et al., 2007). Ghrelin has been identified in red-eared sliders and is similar in structure to other vertebrates (Kaiya et al., 2004). Ghrelin has not been observed in squamates; however, this may be a way to link diet/food intake to reproduction in reptiles.

**Opioids.** Opioids may play a role in the regulation of seasonality in lizards. In Italian wall lizards, treatment with an opioid antagonist, naltrexone, increased androgen levels in both plasma and the testicles (Ciarcia et al., 1994)

**Other neurotransmitters worth investigating.** In reptiles, norepinephrine, dopamine, and serotonin (5-HT receptors) have been shown to influence gonadal function (Jones et al., 1990l; Jones et al., 1997; Desan et al., 1992). Stress likely induces changes in these neurotransmitters systems, which then leads to a negative influence on reproduction. In green anoles, stress from

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social interactions diminished telecephalic serotonergic and dopaminergic activities but increased brainstem serotonergic activity. This may indicate a negative feedback in the 5-HT system (Summers et al., 1997). Courtship behavior and ovarian cycles of socially stressed subordinate females were also reduced in controls (Summers et al., 1995). Combined treatment with corticosterone and a 5-HT receptor antagonist reduced male courtship behavior in garter snakes (Lutterschmidt et al., 2004).

*IGF-1.* IGF-1 has been documented to be influential in reproduction. IGF-1 has a role in maturation via gonadotropins, but it also directly stimulates gonads to facilitate sex steroid secretion, gamete (sperm and egg) development, follicle selection, luteal maintenance, embryonic implantation, and maternal transfer of nutrients into the embryo (Sparkman et al., 2011; Kagawa et al., 1994; Maestro et al., 1999; Macpherson et al., 2002; Ginther et al., 2004; Wuertz et al., 2007; Silva et al., 2009). In placental mammals, IGF-1 rises during gestation and peaks during mid-gestation (Sparkman et al., 2010; Baxter and Martin, 1989; Hess-Dudan et al., 1994; Moyes et al., 2003, Sparkman et al., 2015). In reptiles, the role of IGF-1 in reproduction has not been investigated in detail, although current evidence shows it plays a role in reproduction. IGF-1 is found in the reproductive tracts of female American alligators during vitellogenesis. Plasma IGF-1 has been found to be elevated in both reproductive alligators and loggerhead sea turtles (Cox and Guillette, 1993; Crain et al., 1995b; Guillette et al., 1996). Additionally, gravid free-ranging garter snakes characterized by high reproduction have, on average, higher concentrations of IGF-1 than those from populations with low reproduction (Sparkman et al., 2009, Sparkman et al., 2015). It has been hypothesized that IGF-1 may provide

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a mechanistic link between environmental food availability and diverse aspects of reproduction from studies in wild populations of garter snakes. IGF-1 may also be involved in the development of eggs and sperm. In African house snakes (*Lamprophis fuliginosus*), a peak of IGF-1 was observed post-mating, and it was thought to be mating-induced. If this is the case, IGF-1 may induce initiation of reproductive activity following mating. Following mating, IGF-1 concentrations decreased between 0-9 days, indicating this may play a role in ovulation in snakes (Sparkman et al., 2010). In contrast, IGF-1 peaks mid-gravidity in alligators when luteal activity and progesterone secretion are elevated; IGF-1 is known to play a role in luteal function in other species too (Sparkman et al., 2011; Guillette et al., 1996).

**Reproductive Cyclicity in Reptiles.**

Reproductive cyclicity in reptiles is commonly reported within the literature; however, its description varies from other species because of the approach biologists/herpetologists use in describing reproduction and reproductive strategies. In many cases, these descriptions are simply focused on whether a reptile reproduces during a specific season or is aseasonal. The low level of specificity associated with this terminology can be attributed to our limited understanding of the reproductive physiology of these animals. In other disciplines, such as veterinary theriogenology, reproductive cycles are often referred to using professional or colloquial terms that are focused on the reproductive physiology of the individual, such as estrus/oestrus and estrous/oestrous cycle. For example, the mare and queen are typically referred to as seasonally polyestrous, which effectively communicates that these particular species undergo a reproductive cycle (follicular and luteal phases) multiple times during a specific season. Estrous/Oestrus cycle
refers to the estrous cycle, while estrus/oestrus refers to what is seen behaviorally but is ultimately sexual receptivity. Understanding the terminology used for mammals is important, as it provides more specifics regarding the reproductive cycles of the individual. To better understand reproduction in reptiles, it is essential that we pursue this level of information so we can meet the specific needs of these animals. Herein I describe a nomenclature methodology that can be applied to all invertebrate/vertebrate taxa and could effectively eliminate confusion when communicating or evaluating species across and within taxa if adopted by the field.

**Seasonal v. Aseasonal.**

Reptile reproductive strategies can be grouped into two broad categories, seasonal reproduction (discontinuous reproduction) or aseasonal reproduction (continuous reproduction). In reptiles, these patterns are fairly well-documented across taxa, but are often times based on geographic distribution. The general thought is that species that live in temperate environments tend to have seasonal reproduction (discontinuous reproduction), while reptiles closer to the equator and in the tropics tend to be aseasonal. Seasonality is based on the Summer Solstice, Autumnal Equinox, Winter Solstice, and Vernal Equinox, which are all based on the earth's position to the sun. Following this theory suggests that the primary cue for reproductive activity is photoperiod; however, other factors can be directly or indirectly tied to these seasonal changes, including temperature, humidity, rainfall, barometric pressure, and resource availability. To simply assume photoperiod is the key factor would underestimate the complexity of reproduction. It is for this reason that scientists need to evaluate these different variables to

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develop a better understanding of species-specific requirements. This will be important if we hope to develop ART and preserve these animals.

In reptiles, herpetologists often refer to female sexual receptivity rather than estrus. In some species, there are behavioral and pheromonal cues that males use to detect the receptivity of a female and confirm she undergoing estrus. In some cases, scientists may be unaware of these specific cues or are unable to detect them because of limitations in our diagnostic methods; additionally, some species may undergo silent estrus. Female reptiles do indeed experience estrus, as they have a period of sexual receptivity for copulation. However, unlike mammals, ovulation may not be directly linked to copulation, as reptiles have the capacity to store sperm.

Reptiles that are seasonal reproducers effectively undergo a period of ovarian or testicular quiescence if gonadal growth doesn't occur outside the reproductive season. If a species continuously develops follicles throughout the non-reproductive season, this would be considered a continuous reproductive cycle, although it may take a full year. A reproductive cycle in a female reptile should be defined from the initiation of folliculogenesis to oviposition/parturition. An additional factor that complicates this definition is that some reptiles do not reproduce yearly (e.g., sea turtles); thus, if a female reptile’s reproductive cycle (folliculogenesis) requires more than two years to occur, defining the animal as seasonally monocyclic would be incorrect. Annually monocyclic would be a better description for a species that reproduces annually, whereas bi-annually or tri-annually monocyclic would describe a species that reproduces every two or three years. Seasonally monocyclic should be reserved for a species that undergoes the reproductive cycle during a defined season and experiences a level

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of gonadal quiescence outside the reproductive season. Seasonally polycyclic implies follicular growth, ovulation, and oviposition/parturition occur multiple times in a single defined season. Species that experience an aseasonal (continuous) reproductive strategy during a single year should be considered polycyclic.

**Prenuptial and Postnuptial Reproductive Cycles (Associated and Dissociated).**

Historical descriptions of the reptile gonadal cycles fall into two categories: prenuptial (associated) or postnuptial (dissociated). These characterizations were first described in the common European viper (*Vipera beris*) (Volsoe, 1944), and the patterns were associated with the timing of spermatogenesis and ovulation.

**Prenuptial spermatogenesis/follicular growth.** The prenuptial pattern is defined by synchronized development of the ovary and testicle (gonadal recrudescence, sex steroid production, and gametogenesis) in a defined time-period before sexual receptivity or mating; this occurs during the winter months in some species and the spring in other species.

**Postnuptial spermatogenesis/follicular growth.** The postnuptial pattern is defined by gonadal growth, in both males and females, following oviposition in the late summer, early fall. At that time, synchronized development of the ovary and testicle (gonadal recrudescence, sex steroid production, and gametogenesis) occur, in conjunction with sexual receptivity/breeding. Subsequently, climactic conditions drive hibernation, brumation, or aestivation for several months. Following the climactic change(s), the gonads recrudesce and complete/continue development while sexual receptivity/breeding may occur again. Subsequent ovulation and oviposition occur in the early spring.

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Mixed. A mixed reproductive cycle is associated with species undergoing a postnuptial reproductive strategy but allows for spermatogenesis/vitellogenesis (folliculogenesis/follicular recruitment) to occur. Gamete maturation/development (spermiation, preovulatory follicles) is completed in the spring of the following year. Ultimately, this a form of gonad development arrest during hibernation/brumation/estivation.

Pre-postnuptial. A pre-postnuptial reproductive pattern has been reported in chelonians, especially from temperate climates. The male’s testicular cycles undergo both pre- and postnuptial spermatogenesis, while females are sexually receptive in both the Fall and Spring (Licht, 1984).

So what information should be included to characterize and classify a reptile’s reproductive strategy? How can we concisely describe a reptile's reproductive strategy without confusing herpetologists, reproductive physiologists, theriogenologists, and other professionals with all this varying nomenclature? Combining a description that encompasses the reproductive cycle in relation to when gonad development (spermatogenesis peek/oocyte ovulation) is at its peak, when copulation likely occurs, and the timing/frequency of the reproductive cycle would be the most effective way to communicate information. In species with associated reproductive strategies, all the information can be effectively communicated.

- **Green anole (Anolis carolinensis):** Prenuptial seasonally polycyclic
- **Common side-blotched lizard (Uta stansburiana):** Prenuptial seasonally polycyclic
Green iguana (*Iguana iguana)*:

Prenuptial seasonally (Winter) monocyclic

Rhinoceros iguana (*Cyclura cornuta)*:

Prenuptial seasonally (April-June) monocyclic

In cases where the male and female gonadal cycles are disassociated, the effective method to describe them would be to place the male reproductive strategy first, followed by a dot or hyphen, and then following with seasonality and estrous cyclicity. The word disassociated could be used to describe the strategy; however, it does not specify if the male or female is pre-nuptial or post-nuptial.

Male gonad strategy-Female gonad strategy

- Seasonality with regards to the female reproductive cycle (define if season is known)
- Reproductive cycle strategy

Banded water snake (*Nerodia fasciata)*

- Postnuptial-Prenuptial seasonally monocyclic

Red-eared slider (*Trachemys scripta elegans)*

- Postnuptial-Prenuptial seasonally monocyclic
- Postnuptial-Prenuptial seasonally monocyclic
- Disassociated seasonally monocyclic

Green sea turtle (*Chelonia mydas)*:

- Prenuptial-Postnuptial bi-triannually monocyclic

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Arrau turtle (*Podocnemis expansa*):

Prenuptial-Postnuptial bi-triannually monocyclic

Unfortunately, this descriptive terminology does not appropriately communicate when copulation occurs in the species. While copulation is often associated with ovulation, it is not the case when sperm storage is used in the reproductive strategy.

**2.2.5. Squamate and Chelonian Spermatogenesis and Sperm Structure.**

Reptiles were the first group to transition from anamniotes to amniotes. This evolutionary transition was characterized by the move from external fertilization to internal fertilization, the development of the amniotic egg, and a morphological change associated with the testicles. Anamniotes were characterized by a cystic form of spermatogenesis, while amniotes evolved to have a distinct two-compartment system in the testis, the tubular compartment and the interstitial compartment. As described previously, the testicles in some species undergo a seasonal change in size, while in others this does not occur.

**Testicular microscopic anatomy.**

*Tubular Compartment/Seminiferous Tubules.* Testicles are composed of an aggregation of highly convoluted tubules that are continuous with the testicular ducts; these are the seminiferous tubules. Seminiferous tubule diameter can change with season in some species. Spermatozoa develop within the seminiferous epithelium, enter into the tubules, and progress into the epididymis or ductile epididymis until ejaculation occurs. Sertoli cells reside permanently within the germinal epithelium, irrespective of the season, and are considered the "nurturing cells" for

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the developing germ cells. These cells synthesize and store lipids to provide nutritional provisions to the germ cells during development. Changes within the Sertoli cells are linked to seasonality in some species, including observations of lipid accumulation and loss. Sertoli cells have been documented to contact spermatids in rainbow whiptails (*Cnemidophorus lemniscatus*), green anoles, and Northern house geckos (Khan and Rai, 2004; Del Conte, 1976; Clark, 1967). The Sertoli cells envelop the spermatids with laminae, increasing the surface area contact between these cells. These connections in reptiles are thought to be desmosome mediated. Tight junctions between adjacent Sertoli cells are seen in the germinal epithelium. A blood-testis barrier has been documented in the following squamates: green anoles, Italian wall lizards, Bibron's tree iguana (*Liolaemus bibroni*), Ruibal’s tree rguana (*L. ruibali*), high mountain lizards (*Phymaturus palluma*), Japanese rat snakes (*Elaphe climacophora*); and cottonmouths (*Agkistrodon piscivorus*) (Gribbins and Rheubert, 2011; Bergman et al., 1984, Gribbins et al., 2015; Baccetti et al., 1983; Cavicchia and Miranda 1988; Hondo et al., 1996; Bacetti et al., 1983). The blood testis barrier is maintained throughout the year, irrespective of reproductive status.

*Interstitial Compartment.* The interstitial compartment is comprised of two layers, the compact boundary area and the inner layer that consists of loose connective tissue. The interstitium is composed of Leydig cells, fibroblasts, blood and lymphatic vessels, and nerves. During the reproductive season, the interstitium can be challenging to visualize from the boundary layer. Interstitium morphology is consistent across species. Gross differences are observed in myoid-like and fibroblast-like cells during the spermatogenically active and inactive months. Leydig
cells show a cyclic accumulation of lipid (Gribbins, 2015; Lofts, 1968; Guraya, 1973; Unsicker and Burnstock, 1975; Callard et al., 1978; Leceta et al., 1982; Kan and Rai, 2004; Boretto et al., 2010). Asynchrony can be observed between Leydig cell morphology and Sertoli cell activity, but Leydig cells undergo seasonal hypertrophy during the progression of spermatogenesis. As spermatogenesis climaxes and spermiation occurs, there is a loss of lipid content and cellular atrophy of Leydig cells in lizards. Secondary sex characteristics often develop during spermiation, with a rapid lipid loss in Leydig cells (Leceta et al., 1982; Aranha et al., 2006; Villgrán-Santa Cruz et al., 2013). The loss of the lipid content in the Leydig cells, parallels the development of secondary sex characteristics, the onset of breeding, and elevated androgen production in the Leydig cells.

**Spermatogenesis.** As described previously, the seasonal cycles that exist for spermatogenesis include both postnuptial and prenuptial strategies. This seasonality highly influences germ cell development and the process of spermatogenesis. The spermatogenic cycle has only been studied histologically in a few species of reptiles, although the studies that have been performed maintain the same temporal germ cell development strategy (Gribbins and Gist 2003, Gribbins et al., 2003, 2005, 2006, 2008, 2009; Rhuebert et al., 2009, 2011; Gribbins and Rheubert, 2011).

The seminiferous epithelial compartment of reptiles can contain up to 8 or 9 generations of germ cells within the germinal epithelium, which is more than other amniotes. At this time, we have not uncovered whether germ cells enter the spermatogenic cell cycle continually in seasonal reptiles like mammals and birds, or whether spermatogenesis is an episodic event. In reptiles, the same temporal germ cell development strategy is used, independent of reproductive

Portions of this section was previously published as:
cyclicity (pre- or postnuptial, biannual, or continuous) (Gribbins and Gist, 2003; Gribbins et al., 2003, 2005, 2007, 2008, 2009; Rheubert et al., 2009a; Rheubert et al., 2009b; Gribbins and Rheubert, 2011). The three types of cells that are found in all vertebrate testes are: spermatogonia, spermatocytes, and spermatids.

The seminiferous epithelium contains two morphologies of pre-meiotic cells, spermatogonia A and B. Morphologically, the two types of spermatogonia are different, with the A type being ovoid and containing one large nucleolus and the B type being round and lacking a prominent nucleolus (Gribbins and Rheubert, 2011). Both spermatogonia are found near the basement membrane away from the lumen, and associated with the basal compartments formed by the Sertoli cells. Both spermatogonia types undergo mitosis to maintain spermatogonial populations, and many of the B spermatogonia divide to form pre-leptotene spermatocytes. These two types of spermatogonia are typically most active mitotically immediately before the onset of meiosis and spermiogenesis (Gribbins and Rheubert, 2011).

Meiotic cells are characterized by increasing nuclear and cytoplasmic size and condensation of nuclear chromatin into distinct chromosomes. Spermatogonia B undergo mitotic divisions at different points of the reproductive cycle to produce pre-leptotene cells. Pre-leptotene spermatocytes have nuclei with prominent nucleoli and fine granular chromatin. Pre-leptotene cells and step one spermatids are the smallest germ cells (Gribbins and Rheubert, 2011). Leptotene and zygotene spermatocytes are slightly larger, stain more intensely, and have chromatin fibers that pack their nuclei. Zygotene cells have more condensed globular chromatin compared to the filamentous chromatin found in leptotene cells (Gribbins and Rheubert, 2011).
Pachytene spermatocytes typically constitute a large proportion of the primary spermatocyte population found in the seminiferous epithelium.

Pachytene cells remain in the seminiferous tubules throughout spermatogenesis and are the most common spermatocyte within the reptilian testis. These germ cells undergo a substantial size increase over their development, and their nuclei contain very thick chromatin fibers that are interspersed with areas of open nucleoplasm (Gribbins and Rheubert, 2011). Diakinesis, metaphase I and II, and secondary spermatocytes are transitional germ cells usually found together within the seminiferous epithelium. Diakinesis cells are characterized by thick, fully condensed chromosomal fibers that are interspersed with large open areas of nucleoplasm (Gribbins and Rheubert, 2011). Metaphase I and II cells contain a condensed grouping of chromosomes located on the equatorial plate without nuclear boundaries. Metaphase II cells are smaller than metaphase I cells and have half the amount of chromatin of metaphase I cells. Secondary spermatocytes are dispersed between metaphase I and II cells. Secondary spermatocytes have lightly stained centrally located nuclei and are typically 15% larger than subsequent step 1 spermatids in the reptilian testes (Gribbins and Rheubert, 2011).

**Spermiogenesis.** Relatively few studies exist describing spermiogenesis in reptiles, with most reports being in lizards. Spermatid differentiation in the described reptiles is similar to other eutherian species: the spermatid undergoes development of the acrosome complex, elongation of the nucleus, and condensation of chromatin material. Spermiogenesis is the longest phase of germ cell development.

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Round spermatids are the first haploid cells that develop into what we identify as the specialized mature sperm. Spermiogenesis includes the development of the acrosome system, including the development of the acrosome vesicle and granule that sit on the apex of the developing sperm nucleus. One to two pro-acrosomal vesicles have been observed in reptiles. Squamates share the same structures within the acrosome, including an actin-rich extranuclear perforatorium that helps propel the acrosome forward during fertilization (Ferreira et al., 2006). Once the acrosome system is fully developed, round spermatids undergo a transitional step where the apex of the nucleus begins to elongate (Gribbins and Rheubert, 2011). This elongation continues until nuclei are rod-shaped. During their development, a prominent flagellum is often seen protruding from these spermatids into the lumen of the seminiferous tubules. Once elongation is completed, chromatin material is condensed and cytoplasmic material is removed to form an aerodynamic cell that is better suited to move through the female reproductive tract. In squamates, chromatin condensation occurs in the elongation phase and results in filamentous chromatin fibers within the nucleus during the mid to late stages of spermiogenesis (Clark, 1967; Ferreira and Dolder, 2002; Vieria et al., 2004; Rheubert et al., 2011; Rheubert et al., 2012; Gribbins and Rheubert, 2011). The manchette can be visualized in elongating spermatids and is thought to aid in the elongation of the nucleus (Russell et al., 1990, Fawcett et al., 1971; Cole et al., 1988). There are two arrays of microtubule scaffolding that make up the manchette in squamates. Upon condensation, the nuclei become slightly curved, which leads to the characteristic reptilian spermatozoa filiform shape. These elongating spermatids are often found in bundles within large columns of seminiferous epithelium and develop together as cohorts of
cells. Once spermiogenesis is complete, mature spermatozoa are shed from the seminiferous epithelium and enter the lumen of the seminiferous tubules (Gribbins and Rheubert, 2011).

Spermatozoa. Mature reptile spermatozoa follow the general structure of other amniotes: a highly filiform shape with a head (containing the acrosome and nucleus), midpiece, and flagellum. These regions will be discussed in detail.

Acrosome. The acrosome is an elongated structure in the cranial region of the sperm that contains the proteins and enzymes responsible for the degradation of the oocyte extracellular layers during fertilization. The acrosome complex of reptiles is a cone-shaped structure that can appear flattened laterally. The outer portion of the acrosome can be divided into the acrosome cortex and medulla; this can only be differentiated with electron microscopy. Within the acrosome medulla, a single perforatorium is present. The outer acrosome vesicle is separated from the inner subacrosome cone and is referred to as the subacrosome space.

Nucleus. The reptile spermatozoa nucleus is an elongated, electron-dense structure composed of condensed chromatin. The apical region of the nucleus is tapered and extends into the acrosome complex. In some species, a lacuna can be observed within the nucleus. The nucleus is indented at the distal end, forming the nuclear fossa where the proximal and distal centrioles of the flagellum axoneme attach.

Midpiece/Flagellum. The reptile spermatozoa flagellum can be divided into four distinct regions: the neck, the midpiece, the principal piece, and the endpiece. The neck is composed of the proximal centriole and the anterior portion of the distal centriole. The proximal centriole is located within the nuclear fossa, and projections can be seen off this centriole depending on the

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species. The midpiece of the flagellum is easily identified and is the region surrounded by mitochondria. Within the centrioles are nine sets of triplet microtubules surrounding a central pair of microtubules, which then has the typical amniotic organization of the 9+2 microtubule arrangement within the axoneme. Peripheral fibers that are associated with microtubule doubles 3 and 8 are enlarged in all species studied to date, except the Brazilian bush anole (*Polychrus acutirostris*) (Teixeira et al., 1999b). The axoneme is enveloped by an electron-dense fibrous sheath located shortly after the neck region in the spermatozoa; the starting location of this is variable between species. Surrounding the fibrous sheath are mitochondria aligned in a linear/trapezoid or irregular shape; the organization of the mitochondria can be variable between species. Lizards tend to have short midpieces compared to snakes. Snakes tend to have longer midpieces because they hold 12 tiers of mitochondria (Furieri, 1970; Oliver et al., 1996; Gribbins., 2011; Gribbins and Rheubert, 2011). The end of the midpiece is demarcated by the end of the electron-dense annulus. The principal piece does not contain mitochondria but the fibrous sheath is still present. The flagellum's terminal portion, the endpiece, consists of a 9+2 microtubule arrangement. No mitochondria or fibrous sheath is found in this region.

### 2.2.6. Squamate and Chelonian Oogenesis, Follicular Recruitment, Follicular Development, Vitellogenesis, Ovulation, Corpus Luteum Development, and Oviposition

#### Ovarian dynamics.

**Follicular recruitment.** Oogenesis and folliculogenesis continue throughout the female reptile’s reproductive lifespan. Oogonial proliferation and differentiation start with the embryo and continues into adulthood. Oogonia undergo mitosis in the GB and produce oocytes that enter meiotic prophase. The oocytes become surrounded by a single layer of granulosa cells and form

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the primordial follicle. Recruitment occurs seasonally in seasonal breeding species or can occur continuously in continuously breeding species (Jones, 2011).

**Follicular Development.** Follicles develop on the dorsal surface of the ovary (Miller, 1963). In lizards, each ovary usually contains two GB, although some species have only one germinal bed (Jones et al., 1982; Jones, 2011; Guraya, 1989). In snakes, multiple GB are located throughout the ovary in scattered patches of the ovarian stroma (Lance and Lofts, 1978). Oogonia in the GB undergo mitosis first and then enter a meiotic prophase to form oocytes. Since many reptiles are telolecithal, follicular development is associated with extensive vitellogenesis. Follicular recruitment is typically associated with stimulation of both gonadotropins and ovarian factors, but these are often induced by environmental factors that act on the HPG. These cues are often species-specific. In seasonal reptiles, the ovary may have a refractory period, despite stimulation with the appropriate environmental cues. Ovarian tissue demonstrates differential responsiveness based on a multitude of factors. Prefollicular oocytes showed differential response to exogenous FSH in Italian wall lizards based on the season. The oocytes were most responsive during the time of year in which peak ovarian activity was naturally observed for the species. In green anoles, ovaries became unresponsive despite controlled environmental cues indicating breeding season (long-photoperiod, higher temperatures); this was thought to be attributed to the corpora atretica (Crews and Licht, 1974).

Environmental factors have been shown to inhibit ovarian recrudescence. In green anoles, ovarian recrudescence was inhibited in animals exposed to chronic low humidity. The presence or absence of a male may also influence ovarian activity. In green anoles, the presence of a male

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may be necessary for gonadotropin secretion (DeNardo and Autum, 2001; Crews et al., 1974; Jones, 2011). Mating is a facultative and sometimes obligatory stimulus for vitellogenesis in some snake species. β-endorphin, which is released in response to stress, might mediate ovarian recrudescence. β-endorphin inhibits both seasonal and FSH induced recrudescence in the golden skink (Mabuya carinata)(Jones, 2011; Ganesh and Yajurvedi, 2003). In blood pythons (Python curtus) and the common garter snake, mating has been shown to induce vitellogenesis, which is a reproductive strategy in which females only commit resources to reproduction to ensure fertilization. Autocrine and paracrine factors also likely influence folliculogenesis in reptiles, although information about this is unavailable. During folliculogenesis, follicles not only differentiate and grow but also acquire the ability to respond to gonadotropins.

**Follicular Atresia.** Follicular atresia is thought to be the primary mechanism that regulates clutch size in reptiles. Atresia can be induced at any stage of follicular development. In common side-blotched lizards, exogenous FSH induced follicular development but resulted in atretic follicles once FSH was discontinued; this suggests that gonadotropins are required to maintain follicular maturation (Ferguson, 1966). In gravid animals, a large number of atretic follicles are commonly observed; this may be due to the presence of progesterone inhibiting gonadotropin production and thus inhibiting follicular development (Fox and Guillette, 1987). It is unclear if corpora atretica (CA) in reptiles are steroidogenic, although evidence exists that the CA suppresses ovarian sensitivities to gonadotropins. In green anoles, surgical removal of the CA increased the response of the ovary to exogenous gonadotropins (Crews Licht, 1974). Local factors could also be inhibiting vitellogenesis or steroidogenesis.

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Vitellogenesis. During follicular growth and maturation in reptiles, a large amount of yolk is deposited into the oocyte. Vitellogenin (VTG) is a large molecular weight lipophosphoglycoprotein protein that is synthesized by the liver and subsequently transported to the oocyte (Ho, 1991; Jones, 2011). VTG provides the developing embryo with amino acids, lipids, phosphate, and multivalent ions; however, it only provides about 12-15% of the lipid content for the developing embryo. Very-low-density lipoproteins (VLDL) also provide an additional source of yolk lipid. Finally, e albumin, vitamin -binding proteins, and immunoglobulins (IgGs) constitute the remaining proteins deposited into the yolk (Thompson and Speake, 2004; Jones, 2011). The degree in which vitellogenesis occurs in a reptile depends on the reproductive strategy. Oviparous embryos rely solely on the yolk for nutrients, while some viviparous species require less yolk prior to ovulation. Liver hypertrophy occurs secondary to an elevation in plasma estrogen concentrations, which signals protein production and VTG biosynthesis. VTG is produced and delivered into the bloodstream where it is bound by calcium. Plasma calcium concentrations are often used as a predictor of vitellogenesis in reptiles (Rostral et al., 1998). VTG is acquired by the oocytes through receptor-mediated endocytosis and then subsequently stored in membrane-bound compartments. Exogeneous yolk precursors are delivered by micropinocytosis and accumulate in the yolk globules. Once in the oocyte, VTG is broken down into phosvitins and lipovitellin as energy for the developing embryo (Polzonetti-Magni, 2004). In order to meet the metabolic needs of reproduction and VTG synthesis, a significant amount of maternal reserves and body condition are required. Poor body condition precludes the physiological process that induces vitellogenesis. The exact physiological...
mechanism for this is unknown; however, it is believed that leptin activates the HPG axis to divert resources for reproduction.

Ovarian development and vitellogenesis are associated with elevated plasma concentrations of E₂, and upregulation of ER’s in the liver may signal the readiness for vitellogenesis. Administration of E₂ into non-reproductive females resulted in the mobilization of energy reserves seen in vitellogenic females, including hypercalcemia, hyperphosphatemia, elevated phospholipids, hypercholesterolemia, hyperlipidemia, and hyperproteinemia. In pond turtles, VTG appears in the plasma 6-8 hours following administration of E₂. In loggerhead sea turtles, plasma E₂ concentrations rise significantly at the time the ovaries contain hundreds of vitellogenic follicles but drop at the time of final nesting for the season (Wibbels et al., 1992). The low concentrations of E₂ in Eastern bearded dragons and green sea turtles during the presumed time of vitellogenesis suggest that other estrogens may play a role in this activity in these species. Estrogens are not the only hormones that have been associated with vitellogenesis. P₄ is thought to inhibit vitellogenesis with direct action on the liver. P₄ and T have been demonstrated to inhibit estradiol-induced vitellogenesis in pond turtles (Ho et al., 1981). Elevations in P₄ and T observed in the periovulatory period may be the signal to complete vitellogenesis (Ho, 1991). A progesterone response element may be closely associated with the estrogen response element of the VTG gene. Oriental garden lizards produce vitellogenic follicles while being gravid, and show an elevation in E₂ and a low P₄ concentration. This may be the reason why we see differential concentrations of P₄ throughout the gravidity/pregnancy period in reptiles; additionally, in some species, high P₄ is not needed during late
gravidity/pregnancy to maintain pregnancy. P₄ may also have a pro-vitellogenic effect; this could be because of different progesterone receptors within the liver. In pond turtles, PRA has been documented as a transcription inhibitor, while PRB is a gene-specific positive regulator that is only expressed during those times follicular growth occurs (autumn and spring); this may be under estrogen control. P₄ and the P:E ratio could modulate estrogenic induction of vitellogenesis (Custodia-Lora et al., 2004).

Pituitary hormones have also been implicated in the regulation of vitellogenesis in reptiles. Yolk uptake in the follicle has been demonstrated to be mediated by FSH in the Italian wall lizard. Hypophysectomys in pond turtles abolished the liver's ER concentrations during the reproductive season (Ho, 1989), suggesting that pituitary hormones, such as growth hormone or prolactin, may mediate their expression; however, limited work has been done in this field and more is needed to evaluate this theory. Additionally, the presence of GnRH receptors and bradykinin in the ovary of reptiles suggests that GnRH and bradykinin may play a role in ovarian and follicular development.

Ovulation. Ovulation is the release of the oocyte from the ovary; this occurs secondary to the culmination of vitellogenesis and follicular/oocyte maturation. During folliculogenesis, the deposition of VTG occurs secondary to elevations of estrogens, which rise significantly during the later stages of vitellogenesis as the follicle matures. Once ovulation occurs, estrogen concentrations fall and the post-ovulatory follicle transforms into the corpus luteum. At the time of ovulation, oocytes are arrested in metaphase II (Cuellar, 1971).

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Ovulation occurs secondary to enzymatic degradation, rupture, and expulsion of follicular wall to expel the oocyte. Follicular rupture occurs at the stigma, which is a pale avascular region. This avascular region ruptures due to ischemia, resulting in necrosis rather than an inflammatory response as in mammals (Jones, 2011; Jones, 1987). Prior to stigma rupture, short microvilli of the granulosa lose their connections with the oocyte surface (Laughran et al., 1981). Collagen fibers dissociate and an accumulation of extracellular fluid occurs within the theca between the tunica albuginea and the theca externa. Additionally, extracellular fluid accumulates between the granulosa cells and their basal lamina, causing a subsequent reduction in blood flow immediately before rupture (Jones et al., 1988). Final follicular numbers ovulated per clutch are variable based on several factors, including the number of follicles maturing together in a group, the number of vitellogenic follicles produced from this group, and the number of follicles that become atretic. Ultimately, the number of follicles ovulated is determined by circulating hormones, peptides (Jones et al., 1982; Jones, 2011, Ramírez-Pinilla et al., 2015), and atresia (Jones, 1978; Méndez-de la Cruz et al., 1993, Ramírez-Pinilla et al., 2015). Follicle numbers in each group are related to the number of follicles recruited in the GB and are linked to the number of oogonia per GB. This number can increase with gonadotropin levels, as FSH can stimulate oogonia division (Jones et al., 1975). These follicular groups then appear to inhibit other groups from maturing. An exception to this is seen with polycyclic reptiles. In these cases, follicular development/vitellogenesis occurs prior to the previous clutch being laid. Hormonal regulation differs in these species. In oriental garden lizards, the vitellogenic follicles are produced under high concentrations of estrogens while the animal is gravid with eggs. In this group,

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progesterone may not be required to maintain gravidity/pregnancy late in gestation (Radder et al., 2001).

Hormonal control of ovulation in reptiles has not been well-studied; however, we can use mammals as a guide to understanding this process. In mammals, the follicular phase is characterized by high estrogen concentrations and transitions to the luteal phase, which is dominated by high progesterone concentrations. The high E₂ concentrations that precede ovulation provide positive feedback to the hypothalamus before any ovulatory hormone is released from the hypothalamus. In reptiles, there is little evidence of feedback control over the gonadotropins. In female blue spiny lizards (Sceloporus cyanogenys), estrogen implants into the hypothalamus inhibited ovulation and depressed ovarian steroidogenesis (Callard, 1972a; Jones, 2011), while P₄ hypothalamic implants inhibited ovarian growth and allowed for ovulation. P₄ inhibited gonadotropins to prevent folliculogenesis. In chelonians, P₄ is responsible for positive feedback regulation of the ovulatory LH surge (Etches and Petitte, 1990; Jones, 2011). Ovulation is also associated with testosterone, and it has been found to increase in female chelonians, crocodilians, and some lizard species at the time of ovulation. The precise roles of gonadotropins in reptile ovulation remain unclear, primarily due to the uncertain characterization of gonadotropins in reptiles. To date, most of the information available is associated with sea turtles. In sea turtles, plasma FSH concentrations are low except for a transient peak associated with the onset of oviposition; LH exhibits a surge during nesting (Owens and Morris, 1985). FSH may have a priming effect for the ovary to prepare for LH. In loggerhead sea turtles, concurrent surges of LH and FSH were documented in the periovulatory period, and may

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represent non-specific action of GnRH on the gonadotropins. Peak LH preceded or was coincident with peak P₄ concentration in plasma; this is evidence that LH stimulated ovarian production of P₄ by the post-ovulatory follicle (CL) (Wibbels et al., 1992; Jones, 2011). In green anoles treated with FSH, follicles demonstrated a reduction in blood flow immediately before rupture. This was confirmed histologically because dramatic changes in the stigma included marked accumulation of extracellular fluid and dissociation of collagen bundles to form "lacework of fibrils" without any evidence of an inflammatory response in the interstitial space (Jones et al., 1988). Follicles from green anoles undergo spontaneous rhythmic contractions that may allow for follicular rupture and ovulation through the stigma (Jones et al., 1984). Indomethacin, a non-steroidal anti-inflammatory agent, was used in green anoles to suppress the total ovarian PGE secretion in-vitro, which inhibited pre-ovulatory histologic changes to the follicular wall and delayed ovulation in response to exogenous FSH (Jones, 1990). PGF₂α and PGE₂ have been found in the ovary of Italian wall lizards. PGE₂ was observed during the early stages of vitellogenesis, suggesting this has a stimulatory effect on estradiol release by growing follicles, while pre-ovulatory follicles produced PGF₂α, indicating that PGF₂α likely has a role in ovulation (Gobbetti et al., 1993). Ovulation has also been correlated with plasma testosterone concentration in the blotched blue-tongued skink (Edwards and Jones, 2001). There is still much to do to determine the exact mechanisms associated with ovulation in reptiles. Oocyte maturation involves the completion of the first meiotic division, and until this is complete the oocyte cannot be fertilized. No studies have been done to describe oocyte maturation in reptiles.

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Corpus Luteum. All reptiles develop a true secretory corpus luteum (CL) following ovulation. The CL is formed from the ovarian granulosa cells that hypertrophy to form luteal cells and are surrounded by thecal cell layers. The CL undergoes three stages, 1) luteogenesis, 2) luteal maturity, and 3) luteolysis/luteal regression.

Luteogenesis. Luteogenesis occurs following ovulation. After ovulation, the collapsed follicle appears flaccid and sac-like, with a large opening where the oocyte ovulated. Post-ovulatory follicles shrink and the walls become thickened and folded to produce a multilayered granulosa. Granulosa cells become luteinized and hypertrophy. The granulosa involutes and hypertrophies to fill the central cavity of the collapsed follicle and forms the luteal cell mass, while the oocyte ovulation site disappears in most species other than crocodilians and tuatara (Jones, 2011; Guillette and Cree, 1997). The thecal cells proliferate to form two defined cell layers, the theca interna and theca externa. This then forms three distinct zones in the CL: the theca externa, theca interna, and luteal cell mass. All the luteal cells in the central portion are exclusively from granulosa cells. Although three different types of CLs have been documented in reptiles based on morphology, the functional differences between these structures is unknown.

Luteal maturity. The mechanisms associated with luteal maintenance in reptiles are unknown; however, in most reptiles, luteal function persists through gravidity/pregnancy. Species-specific periods of time have been documented for the CL, as not all species are entirely dependent on the CL to maintain gravidity/pregnancy because other organs (e.g., adrenal gland, placenta, oviduct/shell gland) can take over and produce P₄ at high enough concentrations to maintain gravidity/pregnancy. In the tuatara, the CL has been documented to persist for 8 months.
(Guillette and Cree, 1997). P₄ is the primary secretory product of the mature CL in the reptile ovary. Expression of 3β-HSD has been documented in reptile lutenized granulosa cells, and plasma concentrations of P₄ have been correlated with expression of 3β-HSD activity in oviparous and viviparous reptiles. Two patterns of elevated P₄ have been documented in reptiles, one that is a pre-ovulatory surge (chelonians) and the second occurs following ovulation (squamates). Luteolysis, ovariectomy, and luteectomy have all been associated with a drop in progesterone. Relaxin production from the CL has not been documented in reptiles to date.

**Luteolysis.** The CL degenerates as luteolysis occurs. In this process, the nuclei become pyknotic, vacuoles and lipid droplets appear, the theca becomes less vascular, fibrous connective tissue invade, and the thecal layers merge (Jones, 2011). Degenerative CL in reptiles resemble mammalian corpus albicans. The mechanism in which luteolysis occurs is unclear; however, there is evidence it is mediated by prostaglandins. A single injection of PGF₂α in green anoles and snapping turtles reduces P₄ concentrations. It is unclear if the initiation of luteolysis is associated with the withdrawal of pituitary prolactin (luteotropin) in reptiles.

**Oviposition.** Estrogens and progestogens have an antagonistic relationship in vertebrates. E₂ has been demonstrated to stimulate the oviductal myometrium, while P₄ inhibits oviductal contractions in pond turtles (Callard and Hirsch, 1976; Gist, 2011). Thus, the presence of P₄ inhibits the effectiveness of AVT in stimulating uterine contractions in pond turtles; this is likely due to an upregulation of AVT receptors in the oviductal myometrium once P₄ is gone (Callard et al., 1992). P₄ has also been shown to delay parturition in a viviparous species, Yarrow’s spiny lizard (Scleoporus jarrovi) (Guillette et al., 1991). Obliteration of the CL reduces the time that

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eggs are retained in the oviduct and P₄ administration delays oviposition (Roth et al., 1973; Klicka and Mahmoud, 1977; Cuellar, 1979; Gist, 2011). Viviparous species maintain higher P₄ concentrations for longer times than oviparous species. Evidence supports that a functional CL produces a progesterone blockade, preventing oviposition. Additional locations in which progesterone can be produced are the adrenal gland and the oviduct.

In Yarrow’s skinks, oviductal contractions could induced with PGF₂α. There may be some clinical value for PGF₂α in clinical reptile dystocia cases. Administration of a prostaglandin inhibitor, indomethacin, can delay parturition (Guillette et al., 1992). Plasma concentrations of PGF₂α were shown to be elevated at the time of parturition in sea turtles, the tuatara, and blue-tongued skinks (Guillette et al., 1991c; Fergusson and Bradshaw, 1991). In Yarrow’s skinks, AVT stimulated PGF₂α synthesis at the oviduct in vitro; an in vivo model reiterated this finding by demonstrating that AVT could be used to increase PGF₂α concentrations in plasma and indomethacin inhibited PGF₂α. AVT’s effectiveness varies throughout pregnancy, with animals being more responsive to it at the end of pregnancy and less responsive earlier in pregnancy. In green anoles, AVT administration alone did not result in oviposition; however, oviposition was induced with AVT administration once the anoles were pre-treated with β-adrenergic antagonist, a dichloroisoproterenol (Jones et al., 1983). Additional evidence suggests that β-adrenergic blockade in New Zealand common geckos enhances PGF₂α.

2.2.7. Oviparity v. Viviparity

Portions of this section was previously published as:
Reptiles demonstrate two different types of reproduction: oviparity, or egg-laying, and viviparity, or live-bearing. The term ovoviviparity has been used in the past to describe live-bearing species whose embryos are supplemented from materials deposited within the yolk before ovulation. At the same time, viviparity was restricted to species where the maternal-fetal interface provided nutrients after ovulation and there was some form of placentation. Further studies have shown live-bearng reptiles to have a spectrum of lecithotrophy and placentotrophy, so it is more appropriate to consider all live-bearing reptiles to be viviparous. Viviparity has over 100 independent origins, and about 20% of squamate species are viviparous (Lee and Shine, 1998; Pough, 1998).

**Oviparity.** Oviparity is the ancestral reproductive modality for vertebrates and reptiles. Most reptiles, including crocodilians, tuataras, chelonians, and some squamate species, are considered oviparous. The reptilian egg consists of an eggshell, extraembryonic membranes, albumen, and the yolk. Reptile eggs are characterized by a shell that provides mechanical protection for the embryo and makes eggs resistant to water loss while allowing passage of water vapor and respiratory gasses. The eggshell is comprised of two layers, an outside mineral layer composed of calcium carbonate and an inner organic layer. The calcium layer in chelonians is aragonite, while in squamates and crocodilians it is calcite. The inner organic layer is formed by proteinaceous fibers. Shell thickness varies between different groups of reptiles and different mineral layer structures, which ultimately form shell units. Squamates tend to have a thick inner layer and a thin outer layer without organized shell units; this allows their shell to be flexible. Squamate eggs differ from crocodilian and chelonian eggs because they need to be supplemented

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with water throughout incubation, at oviposition water content is high, and there is little water movement during development. Regardless of shell structure (hard or soft-shelled), the porous nature of the shell allows for gas and water exchange (Keller, 2019; Miller and Dinkelacker, 2008; Andrews et al., 2013). Softer-shelled eggs have a higher rate of oxygen exchange compared to more solid-shelled eggs; however, this does not affect the metabolism of the developing embryo, and mechanisms of how this occurs are unknown at this time (Keller, 2019; Andrews et al., 2013). Reptile embryos derive 20-25% of their calcium from the eggshell. The eggshell is the boundary between the developing embryo and the external environment.

The albumen is a transparent viscous proteinaceous material that is secreted around the yolk as the ovum passes through the oviduct. The proportion of albumen deposited within an egg can vary based on species. Albumen provides support to the developing embryo and assists in water storage. Antimicrobial peptides have been found in the albumen and serve to protect the developing embryo from infection. The extraembryonic membranes within the egg are the amnion, allantois, and chorion. The amnion directly surrounds the embryo. At hatching, smooth muscle within the amnion contracts around the neonate and is responsible for the internalization of the yolk sac. The allantois originates at the body stalk, is paired with the vitelline duct and vascular structures, and is a continuation of the urachus to the embryo (Keller, 2019). The allantois is responsible for collecting the nitrogenous wastes of the embryo, such as urea and uric acid. The chorion is the outermost membrane and encloses the embryo, amnion, allantois, yolk, and yolk sacs. The yolk and yolk sac are attached to the developing embryo at the body stalk and the vitelline duct, also known as omphalomesenteric duct. The vitelline duct is continuous with

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the developing gastrointestinal tract and allows for nutrients to be transferred from the yolk to the developing embryo. The yolk is the primary energy source for the developing embryo and the hatchling in the initial stages of life. The yolk can transfer retinol (the storage form of retinoids in reptiles), hormones, fatty acids, proteins, and minerals, such as calcium, phosphorus, and magnesium, to the embryo (Keller, 2019). The maternal diet influences the yolk fatty acid content and yolk size, thus affecting offspring nutrition. Infectious agents and persistent environmental toxins can also be transferred vertically through the yolk (Keller, 2019; Craven et al., 2008; Warner and Lovern, 2014).

**Viviparity.** Viviparity has not been documented in chelonians, crocodilians, or tuatara. In viviparous lizards and snakes, non-shelled eggs develop entirely within the oviduct, with the offspring being born fully developed. In contrast, in oviparous squamates, shelled eggs are oviposited 2-5 weeks following fertilization and the embryos are approximately 30% developed (Shine, 1983a; Greene, 1997; Clark ,1970a; Saint Girons,1964; Tinkle,1967; Blackburn,1995; Blackburn and Stewart, 2011). In viviparous snakes and lizards, placentation is formed out of fetal membranes and the maternal oviduct. Multiple forms of placentation have been reported in squamates. Viviparous squamates have a similar oviparous ancestry; however, much of the research in placentation is focused on lizards. There has been a significant amount of study in snakes on the evolution behind the cost of reproduction, selective pressures, and viviparity as a reproductive strategy; however, this is beyond the scope of this literature review and is summarized in Blackburn and Stewart (2015). Instead, I will briefly discuss the proposed sequence for placental evolution. One important evolutionary sequence is reported for the

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development of viviparity in squamates. The sequence is proposed in the following evolutionary trend: 1) increase in oviductal egg retention with egg oviposition occurring at a later stage developmentally; 2) retention of the embryos to term by the female associated with viviparous reproduction; 3) evolution of the placenta that functioned in gas exchange; 4) incipient placentotrophy where placenta supplies small quantities of nutrients to the embryo; and 5) substantial placentotrophy where the placental membranes supply most nutrients for the developing embryo (Blackburn and Stewart, 2011). This proposed evolutionary sequence describes the transition from lecithotrophy, where the yolk provides most of the nutrients to the developing embryo, to placentotrophy, where the placenta provides most nutrients to the developing embryo.

Placentation is defined as an organ formed through the apposition of embryonic and maternal tissues that functions in physiological exchange. These physiological exchanges include respiratory gases as well as inorganic and organic nutrients. The maternal component of the reptilian placenta is the lining of the oviduct, while the embryonic portion consists of the fetal membranes, chorion, chorioallantois, and yolk sac that line the eggshell (Stewart, 1997). In viviparous species, a significantly reduced shell membrane is present and contact between the oviductal lining and the fetal membranes form the placental structure that sustain the embryo during gestation (Blackburn, 1992; Stewart, 1997; Blackburn and Stewart, 2011).

Fetal membrane development studies in reptiles are limited. A total of four distinct fetal membranes are formed: two membranes persist until the end of development, one is the chorioallantois, and the other is the omphallantois. Early in development, extraembryonic
ectoderm, mesoderm, and endoderm spread peripherally from the embryo to cover the dorsal and lateral surfaces of the yolk mass. These germ layers comprise the choriovitelline membrane. The ectoderm and endoderm continue to spread as they encompass the entire yolk. The expanding mesoderm is diverted into the body of the yolk and a band of intravitelline mesoderm (Blackburn and Stewart, 2011). The mesoderm penetrates and separates the yolk material and forms an isolated yolk mass and the vitellus proper. The isolated yolk mass is bound by an avascular "bilaminal omphalopleure" that is composed of ectoderm and endoderm. An exocoelom develops in the extraembryonic mesoderm, splitting the choriovitelline membrane. As this exocoelom develops and expands, the choriovitelline membrane disappears and the allantois penetrates the exocoelom and contacts the external chorion forming a chorioallantois. The chorioallantois lines the entire embryonic hemisphere and the inner yolk mass becomes separated from the vitellus through the formation of an exocoelom lined by the intra-vitelline mesoderm. The allantois expands into the yolk cleft and lines the inside of the inner yolk mass (Blackburn and Stewart, 2011). The allantois is often loosely associated with the yolk sac omphalopleure. Unlike the other two fetal membrane complexes, the omphalallantoic membrane and the chorioallantois persist until gestation (Blackburn and Stewart, 2011).

All four fetal membranes contribute to the corresponding placenta through the apposition of the fetal membranes to the uterine lining. The choriovitelline placenta and omphaloplacenta are temporary structures, while the chorioallantoic and omphalallantoic placentas persists until the end of development. The choriovitelline placenta consists of a trilaminar yolk sac in apposition with the uterine lining. This form of placentation has rarely been described in

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squamates (Stewart and Thompson, 2000). The choriovitelline placenta is thought to form as a transitory structure in all viviparous squamates (Steward and Blackburn, 1988; Stewart, 1993; Blackburn and Stewart, 2011). A thin epithelium overlays the vascular mesoderm and yolk sac endoderm. The maternal interface consists of flattened epithelial cells over uterine capillaries. At the interface there is a thin shell membrane that separates the chorionic and uterine epithelia. This type of placentation is the first vascularized placenta to form and facilitates maternal-fetal gas exchange early in development before the formation of the chorioallantois (Stewart, 1993; Blackburn and Stewart, 2011).

The chorioallantoic placenta consists of the chorioallantois in apposition with the uterine lining. This is the only vascularized placenta to persist in viviparous squamates. The structure suggests a role in nutrient transfer, but compared to mammals it does not develop from an early, extraembryonic trophoblast layer. In squamates, placentas typically show a simple interdigitation of the chorioallantois within the uterine epithelium; however, in skinks the interface is more intimate (Stewart, 2015; Roberts et al., 2016). In one skink species, Trachylepis ivensi, invasion and implantation have been observed with direct contact between the chorionic projections and the maternal capillary endothelium (Blackburn and Flemming, 2012; Roberts et al., 2016). Evidence exists for the expression of active transporters and cytokines at the site of the maternal-fetal interface in some species, as well as steroid hormone synthesis (Murphy et al., 2011; Romagnoli et al., 2003; Painter and Moore, 2005). Placentation in squamates is superficially similar to epitheliochorial placentation in eutherian mammals, although its embryonic origin is not from trophoblasts, which is unique to placental mammals (Roberts, 2016).

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In the early developmental stages, omphaloplacenta consists of an avascular bilaminar yolk sac lined by ectoderm, endoderm, and isolated yolk mass. Apposition of the omphalopleure to the uterus forms the omphaloplacenta, and once the allantois invades the yolk cleft the complex is called the omphalallantoic placenta.

Although placentation has been thought to be a derived character and has shown up in multiple lineages of squamates, there is evidence that some lizards have undergone a facultative change in parity and transitioned from viviparous to oviparous. This breaks Dollo's law of irreversibility with complex traits and deserves further review (Laird et al., 2019).

2.2.8. Squamate and Chelonian Reproductive Disorders and Therapeutics

Reproductive Tract Disorders in Squamates-Males.

Infertility. Assessing fertility in squamates requires an understanding of the basic biology and husbandry of the species in question. To truly determine if an individual is infertile, a thorough evaluation of the animal's general health should be performed to determine there are no other underlying co-morbidities that may be leading to decreased fertility. Reptiles that are not kept in the appropriate environment or have a concurrent disease, will shift metabolic resources away from reproduction. The hemipenes should be evaluated to confirm there is no injury. Semen should be collected using either coelomic massage or electroejaculation and evaluated microscopically. This should be performed during the reproductive season. The testicles should be ultrasounded and screened for any gross abnormalities such as an abnormal shape or size. If repeated semen collection attempts are unsuccessful, then an endoscopic examination of the reproductive tract should be done, including a gross evaluation of the testicles, epididymis, and

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ductus deferens; biopsies should be collected from any abnormal tissues. Ovotestes have been described in squamates.

**Hemipenal cast.** Hemipenal casts consist of desquamated cornified epithelial cells and are usually shed over time; however, they can be retained and cause infection or discomfort. Plugs can become large and hard, leading to complications. Under manual restraint, the cast can be successfully removed by gently pulling on the cast. For firmly attached casts, lubricating the site with a lubricant jelly is recommended. Surgical treatment is often not needed; however, in severe cases may be indicated.

**Hemipenal prolapse.** Hemipenal prolapse is a common problem in captive reptiles. During copulation, the male everts the hemipenis to transfer gametes to the female. If the hemipenis is injured during this process, the tissue becomes inflamed and can be difficult to reduce. Initial medical management for this includes cleansing and disinfection, application of osmotic agents, lubrication, and reduction. Systemic anti-inflammatories should also be considered. Sedation or anesthesia may be required to relax the tissues for reduction. Once the hemipenis is reduced, two interrupted sutures should be placed in the vent to prevent re-prolapse. Sutures should remain in place for 5-7 days, and fecal and urine output monitored to ensure that the sutures aren’t limiting waste evacuation.

**Hemipenal amputation.** Necrotic or diseased hemipenes can be successfully amputated. Hemipenal amputation should be done under anesthesia and with appropriate analgesics. Local, regional analgesia can be performed simultaneously. Amputation is accomplished by placing two transfixation ligatures at the base of the hemipenis and transecting the tissue distal to the sutures.

Portions of this section was previously published as:
Absorbable sutures should be used. The squamate will no longer copulate with from this side, but can still be used as a breeder because of the contralateral hemipenis.

Orchidectomy. Orchidectomy can be performed in male reptiles to manage testicular diseases, such as cysts or neoplasia, or to electively control reproduction. Secondary sexual characteristics and testosterone responsive sequela may regress once the testicles are removed. Surgical removal of the testicles in lizards poses a challenge because they are intracoelomic and require a coeliotomy or endoscopic procedure. Additionally, because of the anatomy, the right and left testicles are in close apposition with the vena cava and adrenal gland, respectively, making them subject to surgical injury.

Reproductive Tract Disorders in Squamates- Females.

Cloacal prolapse. Cloacal prolapse is more commonly observed in females with reproductive disease than males. Females with dystocia commonly develop a prolapsed cloaca. This may be a primary condition related to the reproductive tract itself or related to a co-morbidity. It is important to identify the structure that is prolapsed in order to develop an appropriate treatment plan. Colon, oviduct, and even a kidney can prolapse in reptiles.

Preovulatory follicular stasis (POFS). POFS occurs when follicles fail to ovulate under normal physiological conditions. This is a common finding with captive reptiles, especially lizards. The follicles arrest after VTG deposition within the oocyte. Many of the female lizards presented for POFS are in a negative energy balance, housed alone, and have no contact with a male. Affected animals typically present for an enlarged coelomic cavity and anorexia. Care must be taken on palpation to prevent oocyte rupture and an egg yolk coelomitis. Medical management with
corrective husbandry and appropriate nutrition can reverse this condition; however, surgery is often required and preferred by most owners to prevent recurrence.

**Obstructive v. Non-Obstructive Dystocia.** Numerous conditions can lead to dystocia in female lizards and snakes. Anatomic abnormalities such as a narrowed pelvis (lizards), abnormally shaped eggs, masses, oviductal stricture, oviductal torsion, granulomas, and neoplasia can all lead to obstructive dystocia (Knotek et al., 2017). Differentiating between an obstructive and non-obstructive dystocia is vital for developing a therapeutic plan. An obstructive dystocia requires immediate attention. For eggs in the distal oviduct or cloaca, lubrication and ovocentesis may be attempted. Obstructions cranial to these sites require surgical intervention. Post-ovulatory uterine inertia (POUI) can occur secondary to obesity, endocrine disease, hypocalcemia, salpingitis, and inappropriate husbandry. In long term cases of uterine inertia or salpingitis, eggs can become adhered to the oviductal walls limiting the success of medical therapy. Medical management of non-obstructive dystocia includes changing the husbandry to promote oviposition, re-hydrating the patient, and the administration of calcium before one of the following: PGF, oxytocin, or AVT. Ideally, plasma concentrations of progesterone should be measured to determine if an animal would benefit from PGF treatment to induce leutolysis. In snakes, percutaneous ovocentesis can be performed in order to decompress eggs and make it easier to remove them non-surgically, care should be taken to prevent leakage into the coelomic cavity.

**Ovariectomy.** Ovariectomies are recommended for female reptiles to prevent reproduction and to control ovarian diseases such as cysts and neoplasia. The preferred time to perform this surgery

Portions of this section was previously published as:
is during vitellogenesis, as the ovary and mesovarium are under the influence of estrogen and are large and amenable to manipulation. Active ovaries are easily grasped and exteriorized from the coelomic cavity. Once exteriorization is complete and all follicles are visible, the artery and veins in the mesovarium are ligated with absorbable suture material or hemoclips. The mesovarium should be transected distal to the ligatures. Examination of the mesovarian stump is performed to verify hemostasis and confirm complete excision of the ovarian tissue (Knotek et al., 2017). If ovarian tissue remains in the coelomic cavity, follicles can dramatically enlarge with the next spike in estrogen and require another surgery. Multiple incisions may be needed to perform and ovariectomy on a snake. Animals undergoing ovariectomies do not require salpingectomy.

_Salpingotomy/Ovariosalpingectomy._ Salpingotomy or an ovariosalpingectomy is often performed in reptiles that are experiencing POUI. The breeding function of the animal is typically what determines which procedure is done. A salpingotomy can be performed to remove the eggs using a standardized coelomic approach for a lizard or snake. An incision should be made in an avascular site of the oviduct that is large enough to extract the egg. The eggs can be “milked” through the oviduct and extracted through the incision. Following copious flushing, the oviduct is closed in a simple continuous pattern with fine absorbable monofilament (3-0 to 5-0, size dependent). If the reproductive tract is to be removed, the ovaries must be removed too. Care must be taken when removing the ovaries in this case as they are under the influence of progesterone and are small compared to animals in POFS. Once the ovaries are successfully removed, the vessels within the mesosalpinx must be ligated and the distal portion of the salpinx

Portions of this section was previously published as:
ligated with the eggs en bloc at the salpinx's connection to the cloaca. This site can be closed with two absorbable (3-0 to 5-0, size dependent) transfixion ligatures. The procedure should be repeated on the contralateral side. The coelomic cavity should be flushed and closed in a routine fashion, including an everting horizontal suture pattern for the skin.

**Reproductive Tract Disorders in Chelonians-Males.**

*Infertility.* Infertility in males can occur for numerous reasons. A full evaluation of each animal that is intended for breeding should be performed. Individuals may exhibit or lack the drive or are unable to mate for several reasons. Husbandry related causes of infertility are common in reptiles because of an incomplete understanding of life histories. Husbandry related causes include no provision for brumation, insufficient humidity (precipitation), and incorrect sized animals or social structure. Co-habitation long term can suppress mating behaviors in certain animals; however, these animals may reproduce if separated and then re-introduced. Non-reproductive co-morbidities can also lead to infertility; thus, it is important to rule out any co-morbidities by performing a full workup. Finally, disease within the reproductive tract can also lead to infertility (e.g., phallic infection or trauma) (Sykes, 2010; Innis, 2004). To confirm infertility, evaluation of semen is necessary. Electroejaculation has been found to be successful in a variety of chelonians for collecting semen (Mitchell, 2009; Wood et al., 1982; Platz et al., 1980). Gross abnormalities in sperm can be evaluated and confirmed microscopically. Additionally, congenital testicular atresia or orchitis can be associated with infertility and can be diagnosed with endoscopy and testicular biopsy.

Portions of this section was previously published as:
Phallus Prolapse. Phallus prolapse may occur due to a variety of causes, including debilitation, neurologic dysfunction, excessive libido, urogenital or gastrointestinal disease, trauma, tenesmus, constipation, gastrointestinal foreign bodies, and cystic calculi (Innis and Boyer, 2002; Sykes, 2010; Barten, 2006). A normal phallus should only be exposed for a few minutes. A prolapsed phallus should be cleaned with warmed saline and returned to the cloaca if the tissue appears viable. Hypertonic solution, such as 50% dextrose, should be immediately applied to the phallus to properly reduce edema and swelling. Lubricating jelly is also useful for reducing the prolapse. In some cases, sedation is required to limit straining. Once the phallus is reduced, a purse-string suture or simple continuous sutures should be placed in the vent to maintain the reduction. It is important that the clinician evaluate the sutures to ensure they are loose enough to allow the chelonian to urinate and defecate. Sutures should remain for 7-14 days.

If the tissue is not viable, a phallus amputation is indicated. An appreciation of chelonian phallus anatomy is required to successfully perform a proper amputation. A detailed review of this anatomy is described earlier in this article. In small chelonians the base of the phallus may be ligated without clamping and by using encircling or vertical mattress sutures. Absorbable suture material should be used for this procedure. In larger chelonians the surgical procedure is more complicated, and a description can be found below (Innis and Boyer, 2002; Sykes, 2010; Rivera et al., 2011).

1. Surgical area draped and aseptically prepared

2. Phallus is retracted caudally

Portions of this section was previously published as:
3. Blood supply to each longitudinal ridge is identified and double ligated using absorbable sutures.

4. The main body of each longitudinal ridge, the corpus cavernosa, are separately clamped and double ligated.

5. The phallus can then be dissected free of the cloaca and transected.

6. Cloacal tissue remaining after dissection can then be closed over the stump of the phallus in a simple continuous pattern.

7. Post-operative antibiotic and analgesic medications should be provided.

8. Confirm underlying cause of prolapse.

**Reproductive Tract Disorders in Chelonians-Females.**

True fertility issues can also occur in females, although they are uncommonly reported. As with males, husbandry related causes and co-morbidities are common reasons female chelonians do not reproduce. In addition to those mentioned for males, inappropriate nesting material and poor nutrition are other contributors. Ultrasonography may be used to determine if folliculogenesis is occurring and to follow the progression of follicular development. In some cases, eggs are produced but fail to develop. This may suggest male infertility or improper egg management (e.g., excessive handling, incorrect incubation parameters). It has been suggested that repeated radiography of free-ranging chelonians during sensitive stages of gamete and embryo development may cause damage to the germlines and/or embryos, increasing the risk of decreased fecundity; however, this has not been proven experimentally (Innis and Boyer, 2002). Although no evidence exists, it may be prudent to evaluate reproductively active individuals with...
ultrasound rather than radiography. Additional causes of infertility in females include salpingitis, cloacaitis, oophoritis, neoplasia, and follicular stasis. If suspected, these should be worked up using bloodwork, diagnostic imaging, endoscopy, and biopsy.

*Dystocia/Egg Retention.* Dystocia is defined as the failure to oviposit eggs within the appropriate time for a species; however, this is often referred to egg retention. It is often difficult to differentiate between pathologic and normal egg retention because gravid females can retain eggs in the uterus for an extended period of time. Eggs have been documented to remain within the chelonian uterus well beyond the time they should normally be deposited without any pathological consequence. Females can elect to not lay eggs when husbandry parameters and social factors are not appropriate. Failure to provide an appropriate nesting site/substrate and inappropriate temperature and humidity may lead to egg retention. Social factors such as competition for nest sites and intraspecific aggression can also lead to failure to oviposit in breeding colonies (Gross et al., 1992; McArther, 2004; DeNardo, 2006). Chronically retained eggs can lead to infectious salpingitis, rupture of the oviduct with a resulting egg yolk coelomitis, and urinary/colonic obstruction. True egg retention is often incidental or determined based on the knowledge of the species, and is not associated with any clinical signs. In many cases, these animals are past their due date. Individuals may pass part of a clutch or only 1-2 eggs, but not the entire clutch. These cases can be managed conservatively without any intervention or with suggestions to improve husbandry (McArther, 2004; DeNardo, 2006). When making suggestions regarding nesting sites/substrates, a good rule of thumb is that it should be at

Portions of this section was previously published as:
least 1-2 times the length of the carapace and loosening the substrate (e.g., playsand) may be helpful (Innis and Boyer, 2002).

Diagnosing a dystocia can be done based on the knowledge of the species normal egg-retention time, owner/institution's previous experience, and the clinical examination of the chelonian. Educating clients on how to maintain breeding records can be quite helpful in assessing these cases. Eggs can often be palpated in the prefemoral fossa, and radiographs can be used to help identify the number, position, shell quality and integrity of the eggs; these images can also be used to help identify if obstructive causes of dystocia are present (e.g., pelvic canal stenosis or fractures) (Cheng et al., 2009). Large, misshaped eggs with thick walls typically indicate prolonged retention and a need to pursue the case. Ectopic eggs may be identified within the bladder or coelomic cavity from the radiographs or ultrasound (Cheng et al., 2009; Stetter, 2006; Knotek, 2009; Thomas, 2001). Blood work should be performed to rule out underlying conditions such as infection or hypocalcemia (ionized calcium <1 mmol/L), which is typically indicative of a pre-existing nutritional deficiency.

Treatment of dystocia in chelonians is rarely an emergency, unlike other reptiles. In chelonians, dystocia can often be resolved with conservative management/husbandry changes or medical therapy (Innis and Boyer, 2002; Sykes, 2010; McArthur, 2004; DeNardo, 2006; Johnson, 2006). Healthy individuals with normal radiographically appearing eggs can often oviposit with an appropriate nest site and removal of any social stressors. Oviposition can be medically induced in chelonians with a combination of oxytocin, β-blockers, fluid therapy, and calcium supplementation. Clinically dehydrated patients should have their dehydration and electrolyte

Portions of this section were previously published as:
abnormalities corrected first because medical management will not be successful without correction. Parenteral fluids should be employed to correct any deficits. Inducing oviposition in a dehydrated or unstable patient may lead to a worsening of a metabolic disorder or rupturing the oviduct (Tucker et al., 2007; Feldman, 2007; Innis, 2004; McArther 2004). In hypocalcemic animals, parenteral calcium supplementation is needed to assist in oviductal contractions; this can be done with either calcium glubionate or calcium gluconate. Oxytocin can be administered intramuscularly or continuously through an intravenous or intraosseous catheter. In chelonians, the low end of the dose range is often effective. If the animal does not respond to the oxytocin, the dose can be repeated. Various recommendations exist for oxytocin treatment, including administering three doses at 90-minute intervals and increasing the dose with each treatment. Another protocol recommends administering 50% to 100% of the original dose 1 to 12 hours later (DeNardo, 2006; McArther, 2004). Arginine vasotocin is reported to be more effective in reptiles than oxytocin; however, chelonians are one group of reptiles that respond well to oxytocin (DeNardo, 2006). Adjunct therapies and medications may also be beneficial for dystocia cases in chelonians; however, they are not well studied. Prostaglandins are one such adjunct. A combination of oxytocin (7.5 U/kg) and prostaglandin F<sub>2</sub>α (1.5 mg/kg subcutaneously) has been effective in inducing oviposition in red-eared sliders; however, this may be less effective in turtles weighing >5 kg (Sykes, 2010). Topical application of prostaglandin E<sub>2</sub> gel on the cloaca has been recommended by Innis 2002; however, no adverse or beneficial effects have been reported. β-blockers are thought to potentiate the effects of oxytocin in chelonians, and β-blockers that have been used include atenolol and propranolol (McArthur, 2004).
Propanolol has been successfully used in lizards, and may translate to chelonians (Gross et al., 1992). When conservative management fails, more aggressive therapies are indicated; this is especially true for animals showing signs of debilitation, tenesmus, or abnormally shaped/sized eggs on radiographs. Salpingotomy can be performed via a plastronotomy or a prefemoral approach. A plastronotomy is the preferred approach when a large field of vision is required; however, the prefemoral approach is less invasive. These procedures are both well described in the literature (Mader, 2006; McArthur, 2004; Minter et al., 2008; Nutter et al., 2000). A coelioscopy-assisted approach for ovariectomy and salpingectomy has also been described for species with a small prefemoral area (Innis et al., 2013; Innis et al., 2007; Proenca and Divers, 2015). This technique can also be used to approach a cystotomy to extract eggs found in the urinary bladder. Ovocentesis can be performed via the cloaca on eggs that can be visualized or palpated. A speculum aids in visualization and a large gauge need should be used to aspirate the contents. Egg's tend to fracture post-aspiration, but fragments usually pass on their own or an be removed carefully with forceps (Innis and Boyer, 2002; DeNardo, 2006). A technique exists for punctured eggs that do not pass. The tip of a Foley catheter is cut so the balloon is at the end of the catheter, the amount of air needed to inflate the balloon to the appropriate size for the egg to be removed is determined, and the infusion port is filled with water and placed in the freezer to improve the rigidity of the catheter. The catheter is then placed into the egg via the centesis hole, the balloon is inflated, and traction is applied to remove the egg. Care must be taken not to overinflate the balloon or tear the oviduct during this process (Sykes, 2010; McArthur, 2004). The authors' recommend irrigating the cloaca and oviduct after

Portions of this section was previously published as:
the procedure. McArthur additionally recommends continuing oxytocin and β- blockers to assist in expulsion of the egg remnants. When eggs are adhered to the uterus, a salpingotomy/salpingectomy may be required.

Follicular Stasis. Follicular stasis (i.e., preovulatory egg-binding or retained follicles) is commonly reported in lizards; however, it does also occur in chelonians. Inappropriate nutrition or environmental conditions are common causes of follicular stasis. Follicles that neither ovulate nor regress can become inspissated or necrotic, rupture, and lead to egg yolk coelomitis. These follicles may remain static for months. Affected chelonians often present for anorexia and lethargy. Clinical pathologic findings in affected animals include elevated concentrations of calcium, albumin, total protein, and alkaline phosphatase, with anemia, leukopenia and heteropenia (McArthur 2001; McArthur, 2006). A diagnosis is typically made using ultrasound to show the presence of persistent non-ovulated follicles. Ovariectomy is the preferred treatment. A technique for coelioscopy-assisted ovariectomy has been described and used successfully to treat follicular stasis in chelonians (Innis et al., 2013). Briefly, an endoscope is inserted through a prefemoral incision to visualize and gently grasp and retract the ovary out of the prefemoral incision; oocentesis can be done on larger follicles to facilitate further exteriorization of the ovary and the ovarian vessels ligated to complete the ovariectomy (Knafo et al., 2011).

Egg Yolk Coelomitis. Egg yolk coelomitis can occur secondary to retained follicles, oophoritis, salpingitis, or dystocia. Inflammation, degenerative changes, and infection can all affect the ovaries of chelonians. Follicular stasis and dystocia often predispose chelonians to developing
egg yolk coelomitis (DeNardo, 2006; McArthur, 2001; McArthur, 2004). During a stasis event, retained follicles undergo follicular necrosis, which can lead to the coelomitis. In a dystocia, rupture of the oviduct can lead to egg contents spilling into the coelomic cavity. Clinical signs are often non-specific, including anorexia, lethargy, inactivity, diarrhea, and/or decreased fecal/urate production. Clinical pathologic findings include hypercalcemia, hyperproteinemia, and anemia. Many of these animals develop multi-organ dysfunction syndrome. Ultrasonography and radiography can be used to assist in identifying follicular stasis or dystocia. Ultrasound can also be used to confirm the presence of free-fluid within the coelomic cavity and aid with coelomocentesis. Visualization of the coelomic cavity either surgically or with endoscopy is the definitive method of diagnosing egg yolk coelomitis because the clinician can visualize degenerative, hyperemic, brown or purple follicles and see free fluid or adhesions within the coelom. Chelonians with retained eggs and a ruptured oviduct must be diagnosed quickly because they tend to decline rapidly. The source of the egg material should always be removed, as well as strong consideration given towards performing an ovariectomy and salpingectomy to permanently correct the issue (McArthur, 2001; McArthur, 2004; Mans and Sladky, 2012) Samples collected during surgery should be submitted for histopathology and both bacterial and fungal cultures. The coelomic cavity should be flushed thoroughly with warmed saline to remove all possible yolk material. Post-operative care should include supportive care (e.g., fluid therapy, enteric nutrition), analgesics, anti-inflammatory drugs, and antimicrobials. Egg yolk coelomitis in chelonians carries a grave prognosis.

Portions of this section was previously published as:
Neoplasia of the Reproductive System. Reproductive cancer in chelonians is rare. A retrospective study evaluating 3,500 reptile necropsies from a zoo over a period of 100 years found a prevalence of cancer of 2.3% for all reptiles and 1.2% in chelonians. An additional study compiling the results from more than 5,000 biopsy specimens submitted to a laboratory found a prevalence of cancer of 9.8% for all reptiles and 2.7% in chelonians (Garner et al., 2004; Hernandez-Divers and Garner, 2003). Neither report listed reproductive tract tumors in chelonians. Case reports in male chelonians include a testicular interstitial cell adenoma and a seminoma. In the interstitial cell adenoma case report the testes were grossly unremarkable and rafts of tumor cells were found within the testicular blood vessels; this testicle did not produce any viable sperm within the seminiferous tubules. The seminoma was reported in a 13-year-old male spur-thighed tortoise (Testudo graeca) that presented for anorexia, apathy, and prolapse of penile tissue. It was initially suspected to be a seminoma antemortem based on ultrasonography and magnetic resonance imaging. The appearance and signal intensities were similar to those reported in testicular neoplasms in humans, especially seminomas. Necropsy results and histopathological findings were consistent with a seminoma (Pees et al., 2015). Reports of cancer in female chelonians are also limited and include oviductal leiomyoma in a desert tortoise (Gopherus agassizii), ovarian dysgerminomas in two unrelated red-ear sliders, and ovarian teratoma (Frye et al., 1988; Frye, 1994; Newman, 2003). In the cases of the ovarian dysgerminomas, each ovary was effaced by soft white tissue masses, one which was large enough to prevent the turtle from retracting her head and neck into the shell. Cloacal polyps have also been reported in a box turtle (Frye et al. 1994).

Portions of this section was previously published as:
2.3. Current Status of Assisted Reproductive Technologies (ART) in Reptiles

2.3.1. Gamete Collection Techniques in Reptiles

2.3.1.1. Spermatozoa.

Post-mortem Collection.

For post mortem spermatozoa recovery, a focus should be made on spermatozoa present in the epididymis and ductus deferens due to \textit{in vivo} maturation. This method yields higher spermatozoa numbers and uncontaminated samples compared to ante-mortem semen collection. During the reproductive season, both the epididymis and ductus deferens distend with spermatozoa because these are the sites spermatozoa undergo maturation and storage prior to ejaculation. Once the male reproductive tract is collected, spermatozoa can be collected by expressing the ductus deferens and/or macerating the epididymis under a dissection scope. In the viviparous lizard (\textit{Lacerta vivipara}), spermatozoa pass through the epididymis into the ductus deferens and gain motility, with maximum motility found in the distal segment where they accumulate (Depeiges and Dacheux, 1985). A sperm coating protein is proposed to be secreted in the extracurrent ducts (Depeiges and Dufaure, 1983). Samples should not be collected directly from the testicles, as testicular spermatozoa have poor motility (1%); even after being exposed to a phosphodiesterase inhibitor (caffeine), motility only increased to 7% (Depeiges and Dacheux, 1985). Post-mortem collection can be used for endangered species that die in zoological settings and from animals subject to mass-mortality events (Clulow and Clulow et al.,

Portions of this section was previously published as:
Successful artificial insemination has been performed using post-mortem recovered spermatozoa in crocodilians (Larsen et al., 1984). Unfortunately, this is likely the best method to perform actual experimental studies to further develop ART for reptiles because of the large sample volumes that can be collected, so having model reptile species is necessary to develop ART further.

**Ante-mortem Collection.**

**Coelomic massage.** Coelomic massage has been used successfully to collect semen from snakes and small lizards. In snakes, semen can be collected by gently massaging the ventral caudal third of the coelomic cavity using light digital pressure in a caudoventral direction toward the cloaca (Watson, 1990; Fitch, 1960; Miller and Watson, 2001; Mengden et al., 1980; Samour, 1986; Fahrig et al., 2007; Zacariotti et al., 2007; Mattson et al., 2007; Tourmente et al., 2007). Similarly, digital ventral coelom massage has been used in small geckos and skinks to collect semen sample (Todd, 2003; Molina et al., 2010).

**Digital phallic massage.** In crocodilians, digital phallic massage has been used to successfully collect semen under mild sedation and manual restraint. Success with this strategy has been reported in American alligators, broad-nosed caiman (*Caiman latirostris*), saltwater crocodiles, and Philippine crocodiles (*Crocodylus mindorensis*). Individuals are placed in ventral recumbency and straddled across two supporting structures with the cloaca being easily accessible. The phallus is then extruded from the proctodeum with a gloved finger by hooking the phallus with downward pressure. The gloved finger is inserted past the phallus base and into the urodem, where it is repeatedly used to stroke the phallus. This allows for semen to flow

Portions of this section was previously published as:
within the sulcus spermaticus (Cardeilhac et al., 1982,1988; Larsen et al., 1982, 1984, 1992; Johnston et al., 2014a). No reports exist of using this method in other species, although investigation may be warranted, especially in chelonians.

**Vibrational method.** One report described the use of a human vibrational device to collect semen from chelonians as an alternative to electroejaculation (EEJ). Male turtles were held upright and an appropriately sized vibrator set to its highest frequency and firmly placed against the carapace and moved along the carapace in linear and circular motions. If no erection was noted after carapacial stimulation, then the vibrator was placed on the plastron for 7-22 minutes. Semen collection success was found to be species-specific, with good success in Blanding’s turtles (*Emydoidea blandingii*) (12/12) and poor success in pond turtles (4/30) and wood turtles (*Glyptemys insculpta*) (2/17) (Lefebvre, 2013).

**Chemical ejaculation.** No study has investigated chemical ejaculation in reptiles. A single report exists in alligators, but this was not documented to improve semen quality (Larsen et al. 1982). Additional studies need to be performed that evaluate chemical emission/ejaculation using pharmacological agents that induce smooth muscle contraction within the epididymis or ductus deferens, such as oxytocin and PGF$_{2\alpha}$. Tricyclic antidepressants, such as imipramine or clomipramine, increase noradrenaline concentration, which triggers an $\alpha$-adrenergic response. Ejaculation is mediated by activating $\alpha$-1 adrenergic receptors. Alpha-2-agonists, such as xylazine, medetomidine, detomidine, and dexmedetomidine, should also be investigated. In horses, imipramine, alpha$_2$ agonists (xylazine and detomidine), and/or oxytocin have been used to successfully collect semen *ex copula* (McDonnel, 2001; Cavalero, 2019).

Portions of this section was previously published as:
Electroejaculation (EEJ). EEJ has been used to successfully and safely collect ejaculates from all reptile taxa other than the tuatara. This is the preferred method to collect semen ante-mortem in larger lizards and chelonians because their anatomy limits the use of some of the other methodologies described here. EEJ semen collection in lizards and chelonians is typically performed under anesthesia or heavy sedation. Different electroejaculation units and protocols have been reported to be successful for collecting semen from reptiles. Most possess a variable voltage/amperage power source with a plastic or metallic probe. Much of the variation in EEJ techniques is due to the different sized animals, unique species anatomy, and variable/limited availability of appropriately sized probes. In lizards, EEJ was first done in green iguanas (Zimmerman et al., 2013). Since then, multiple studies have been performed to evaluate the safety and efficacy of EEJ in lizards, including veiled chameleons (Chameleo calyptatus), spiny lava lizards (Tropidurus spinulosus), spiny lizards (Sceloporus torquatus), and Grand Cayman blue iguana hybrids (Cyclura lewisi x rubila). (Zimmerman et al., 2013, Mitchell et al., 2015, López Juri et al., 2018, Martínez-Torres et al., 2019, Perry et al., 2019). One of these studies performed EEJ under manual restraint. The authors believed that the procedure was not-traumatizing and considered it to be safe because all of the subjects survived a single EEJ. Unfortunately, the authors did not use objective health criteria to conclude that EEJ did not have a negative impact on the lizards (López Juri et al., 2018). Another study evaluated the safety of repeated EEJ in anesthetized veiled chameleons. This study demonstrated that repeated anesthesia and EEJ can be performed safely and successfully at least once weekly in this species. Overall animal health was assessed using physical examination, complete blood counts,

Portions of this section was previously published as:
biochemistry values, cloacal external morphology, and cloacal mucosal inflammation with endoscopy. Transient cloacal inflammation was observed after EEJ, but healed spontaneously. (Perry et al., 2019). These studies demonstrate that semen collection can be done safely using EEJ, and although it is a more invasive method of semen collection, did not appear to impact the long term health of the reptiles. To date, no study has evaluated whether EEJ affects corticosterone (stress) levels in reptiles. In mammals, EEJ has been associated with stress but it did not impact sperm quality (Wildt et al., 1984).

In chelonians, electroejaculation is the most common ante-mortem method used to collect semen. EEJ has been used to collect semen from leopard tortoises (*Stigmochelys pardalis*), green sea turtles, red-eared slider turtles, Galapagos tortoises (*Chelonoidis nigra*), a ploughshare tortoise (*Astrochelys yniphora*), olive Ridley turtles, hawksbill turtles, and the black marsh turtle (*Siebenrockiella crassicollis*) (Platz et al., 1980; Wood et al., 1982; Juvik et al., 1991; Tanasanti et al., 2009; Kimskulvech and Suttiyoti, 2012; Kawazu et al., 2014; Zimmerman et al., 2017, Sirinarumitr et al., 2010). EEJ has been performed with and without anesthesia in chelonians with no associated discomfort or trauma being reported (Platz et al., 1980; Zimmerman et al., 2017). An aversion to performing semen collection in chelonians using EEJ exists because a single report of a death that occurred in a ploughshare tortoise 30 days following semen collection due to suspected renal disease (Platz et al., 1980; Juvik et al., 1991). No reports exist in the literature associating renal failure and EEJ in any species. This has been perpetuated as "dogma" throughout the chelonian community and prevented the further development of ART in
chelonians; although as a group chelonians could benefit the most from ART as a conservation tool.

In snakes, EEJ has been reported to be successful in the checkered garter snake as an alternative to coelomic massage. EEJ in snakes pose a special problem because of the distance from the cloaca to the gonads. For the garter snakes, a special long EEJ probe was used due to the high position of the reproductive tract. In larger snakes, EEJ may be the only method that can be used to collect semen because coelomic massage could be challenging (Platz, 1980; Quinn, 1989).

In crocodilians, EEJ has been used successfully to collect semen from American alligators and saltwater crocodiles (Larsen et al., 1982; Johnston et al., 2014a).

Behavioral collection. Voluntary semen collection has been documented in numerous domestic species, including dogs, cats, and horses. Collection methods focused on understanding and exploiting pre-copulatory and copulatory behaviors, including arousal and the stimuli for successful ejaculation and emission (temperature or pressure). Anecdotal reports, including videos on youtube.com, suggest this may be a viable alternative for reptiles. However, it is necessary to understand species-specific courting behaviors, endocrine profiles, environmental stimuli, arousal stimuli (pheromones, visual, and tactile stimuli), and pre-copulatory and copulatory behaviors to develop this method in the future. Preliminary, unpublished studies using species-specific artificial cloaca in a skink and a turtle have shown there is a need to understand the pre-copulatory behaviors of the individuals since they were not willing to copulate with the

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proposed models despite being in the reproductive season (Hobbs, unpublished; Perry, unpublished).

2.3.1.2. Oocytes

No reports exist describing the ante-mortem or post-mortem collection and maintenance of oocytes from reptiles. Oocyte recovery in live reptiles is complicated by numerous factors, including reproductive modality, ovary type, embryo nutrition, and egg shell. The reptiles that should be initially investigated to develop these methods are those that practice viviparity. As these species are reproductively more similar to mammals, techniques are likely more transferable.

2.3.2. Gamete Storage Methodologies

2.3.2.1. Semen Handling and Extension.

Semen handling and extension methods have been successfully investigated in at least one species from each reptile taxon, except the tuatara. A general trend is that reptile spermatozoa can be successfully stored at low temperatures without cryopreservation for short periods. In the American alligator, spermatozoa were successfully recovered from the deferent ducts post-mortem and the spermatozoa maintained 25-50% motility for 2-5 days at 5°C (Larsen et al., 1984). In the saltwater crocodile, the effect of diluent type on spermatozoa % motility, rate of sperm movement, and % intact plasma membranes was evaluated when samples were incubated for 30 and 120 mins at 30°C. It was determined that motility and rate of spermatozoa movement were significantly lower when extended in TRIS compared to PBS and BEST with or without 20% egg yolk (Johnston et al., 2014a,b). Plasma membrane integrity was not affected by

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diluent type or incubation time. The presence or absence of 20% egg yolk in the BEST diluent also had no difference on sperm motility. PBS without egg yolk was determined to be the best diluent for storing saltwater crocodile at 30°C (Johnston et al., 2014a, b). In lizards, semen handling and extension methods have been evaluated in the McCann's skink (Oligosoma maccanni) and the green iguana. In the skink, motility was >70% over 5 days when incubated at 4°C in a chelonian spermatozoa media (Molinia et al., 2010; Gist et al., 2000). The green iguana samples were extended in Ham’s F-10 media and test yolk buffer with albumin and incubated in a refrigerator and an equitainer for 72 hours. Initial baseline motility was 78%, but then declined to 60% at 24 hours and 33% at 48 hours. (Zimmerman et al., 2013). Short term spermatozoa extension has only been evaluated in one species of snake, the corn snake (Pantherophis guttatus). The corn snake semen was extended in modified Ham's F-10 media and test yolk buffer with albumin and stored at 4°C in a refrigerator and equitainer for 120 hours. Motility was >50% in the equitainer for 48 hours and the refrigerator for 72 hours (Farhig et al., 2007).

In chelonians, semen extension appears to be the most challenging. In musk turtles, red-eared sliders, and painted turtles, initial post-mortem motilities were 28-52%, 3-4%, and 2-5%, respectively. These samples were incubated at 23°C and 2°C in F-10 with bovine serum albumin for 1-3 hrs. prior to analysis. Cooler temperatures appeared to maintain motility better in musk turtles as motility was 52% at 2°C and 28% at 23°C. Despite poor motility preservation, samples from this study were determined to be viable and retained the capacity for motility when stored at 4°C for 40 days if 3-isobutyl-1-methylxanthine was added to stimulate the spermatozoa (Gist

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et al., 2000; Clulow and Clulow, 2015). In sea turtles, contradictory information exists on the viability of spermatozoa. In olive Ridley sea turtles and hawksbill turtles, mean motility following EEJ was 28% and 60%, respectively. Spermatozoa from the olive Ridley turtles remained viable following collection for approximately 90 minutes; however, viability increased to 16-22 hours at 4°C (Tanasanti et al., 2009). The sea turtle semen samples were extended with eight different semen extenders, including refrigeration medium test yolk buffer, Tyrode medium with albumin, lactate, pyruvate, Beltsville poultry semen extender, 3% sodium citrate buffer, phosphate buffered saline, EEL, 1% bovine serum albumin, and Ham F-10. Initial motility for this study was poor following collection, with mean initial motilities being 8.3% for olive Ridleys and 14% for hawksbills. Motility declined to zero at 4°C in all treatments within 6 hours of collection. (Sirinarumitr et al., 2010). Semen collected from leopard tortoises using EEJ demonstrated a higher mean baseline (57.3%, range: 10-80%) motility. However, leopard tortoise spermatozoa stored at 4°C in modified Ham-F10 and test yolk buffer at a 1:1 ratio declined to a median motility of 0% within 24 hours. More work needs to be done to further assess chelonian semen extension methods.

2.3.2.2. Semen Cryopreservation/Vitrification.

In reptiles, only a few reports exist describing experiments evaluating spermatozoa cryopreservation. Species that have been studied to date include, the saltwater crocodile, American alligator, Argentine black and white tegu (Tubinambis meriana), Eastern water skink (Eulamprus quoyii), red diamond rattlesnake (Crotalus ruber), and the Burmese python (Python bivittatus). The most extensive work looking at cryopreservation has been performed in saltwater
crocodiles. Saltwater crocodile spermatozoa were exposed to three cryoprotectants (dimethyl sulfoxide [DMSO] dimethylacetamide [DMA], and glycerol with subsequent cryopreservation. All three cryoprotectants demonstrated a negative impact on motility and rate of progression post-exposure at 4°C for one hour. A concentration-dependent decline in motility was also observed with increasing concentrations (0.68M [5%], 1.35 M [10%], and 2.7 M [20%]) of the cryoprotectants. No changes were observed in plasma membrane integrity during the equilibration phase, despite a significant loss of motility. Cryopreservation was performed in this study using 0.25 mL French straws at a freezing rate of 6°C/min from 4°C to -86°C and then plunged into liquid nitrogen (-196°C) and stored from 6 weeks. Although significant losses of motility and rate of progression were observed post-thawing, plasma membrane integrity was preserved in about 25% of spermatozoa. Higher concentrations of cryoprotectant improved plasma membrane preservation (Johnston et al., 2014b). In a subsequent study, the same group evaluated three non-penetrating cryoprotectants (trehalose, raffinose, or sucrose at 0.3M) against glycerol at concentrations of 0.3M, 0.68M, 1.35M, and 2.7M and determined that glycerol at the highest concentration maintained the plasma membrane the best and limited DNA fragmentation following thawing. From this information, glycerol and sucrose cytotoxicity were evaluated prior to cryopreservation and revealed no loss of survival when spermatozoa were diluted with 0.68 M glycerol or 0.2-0.3M sucrose and the cryoprotectants were washed out with PBS or Briggers, Whitten, and Whittingham medium containing sperm capacitation agents (BWWCAP) (Johnston et al., 2017). This combination was found to result in adequate pre-freeze survivability before cryopreservation by preserving motility, rate of movement, and plasma membrane integrity.

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Additionally, freezing rates were evaluated combining the use of penetrating (0.68 and 1.35 M glycerol) and non-penetrating cryoprotectants (0.2 or 0.3 M sucrose) and exposed to a fast freeze (21°C/min) and slow freeze (6 °C/min) from 5°C to -80°C. Post-thaw survival was highest with a combination of 0.2 M sucrose and 0.68 M glycerol, followed by washing of BWWCAP independent of freezing rate (Johnston et al., 2017). In conjunction with this study, the same group validated the spermatozoa chromatin dispersion test (SCDt) to assess DNA fragmentation in saltwater crocodiles (Johnston et al., 2015). This test can likely be used to assess DNA fragmentation in other reptile species, rather than just evaluating motility, motility progression, and plasma membrane integrity. Further investigation into the saltwater crocodilian spermatozoa has revealed some remarkable evidence, including that capacitation may occur in reptiles and may be required for fertilization. Crocodile spermatozoa experience a rapid and sustained, cyclic-AMP mediated increase in progressive motility following incubation under conditions optimized for the induction of capacitation in mammalian species. Elevations in protein kinase A and tyrosine kinase within the sperm flagellum were observed, suggestive of a process similar to capacitation observed in mammals (Nixon et al., 2016). Additional work using proteomic profiling demonstrated a difference in protein phosphorylation between capacitated and non-capacitated saltwater crocodile spermatozoa; the identified phosphorylated proteins share significant evolutionary overlap with those proteins documented for capacitation in mammalian spermatozoa. Comparatively, serine residues were phosphorylated compared to tyrosine, which is seen in mammals (Nixon et al., 2018). This needs to be investigated further in other reptile species.

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species and will be critical in handling semen for ART. A single report of attempted
cryopreservation in alligators demonstrated low spermatozoa recovery (Larsen et al., 1984).

There have been two attempts at cryopreserving lizard semen. The first attempt was made in Argentine black and white tegus. Post-mortem spermatozoa samples were collected in order to evaluate two cryoprotectants, DMSO and glycerol, at three different concentrations (8%, 12%, and 16%), and three different freezing rates (0.3°C/min, 1°C/min, and 6.3°C/min). Samples were frozen in cryovials in a Test-Yolk-M199 with HEPES combination at a concentration of 50 x 10^6 mL. Overall, the slowest freezing rate had the higher cryosurvival. Additionally, DMSO provided better plasma membrane integrity and acrosome integrity compared to glycerol. To determine the best cryopreservation method, a sperm quality index was developed to weight the three measured indicators, including motility, plasma membrane integrity, and acrosome integrity. Based on this metric, a slower freezing rate of 0.3°C/min with a 12% DMSO concentration had the highest sperm quality index. Additionally, a significant male effect was observed for post-thaw sperm quality parameters. This phenomenon has also been reported in mammals and birds (Young et al., 2017; Young et al., 2017; Johnston et al., 2017).

Spermatozoa were collected from Eastern water skinks using ventral coelomic massage and spermatozoa volume, concentration, progressive and total motility, and membrane integrity were measured. Motility could be maintained up to 70% for 16 hours when the spermatozoa were extended in PBS, TLHepes, or Ham's F-10 at room temperature. Cryotoxicity was examined using PBS and the following cryoprotectants: DMSO, DMA, and glycerol at 4°C for 2 hrs. Spermatozoa diluted in 1.35 and 2.7 M DMSO had higher motilities and proportions of live

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sperm compared with the controls or the DMA group. Cryopreservation was evaluated using 1.35 M of cryoprotectant in Tris-yolk buffer, Beltsville poultry semen extender, and PBS. There was no benefit to the complex extenders compared to PBS for skinks. Post-thaw progressive motility was significantly higher, 5%, with 1.35 M DMSO Tris-yolk buffer than all other treatment groups. The authors concluded that DMSO may hold promise as a cryoprotectant for skinks (Hobbs et al., 2018).

Initial reports of semen cryopreservation in snakes were promising, with Mengden et al. (1980) reporting approximately 30% motility recovery using a pellet freezing method (Clulow and Clulow, 2016). However, since that time, follow-up studies in other species have proven less fruitful. In red-diamond rattlesnakes, spermatozoa stored in Lake's extender with DMSO (2% and 4%) showed no post-thaw survival, no matter the cryopreservation method or cryoprotectant concentration (Zacariotti et al., 2011, Clulow and Clulow, 2016). Semen frozen in Test-Yolk-M199 with HEPES combination exhibited motility post-thaw, 8% glycerol frozen in cryovials in a controlled-rate freezer and cooled at 1°C min⁻¹ to −20°C, then 50°C min⁻¹ to −80°C resulted in the highest post-thaw motility in all treatments. Another study using Burmese pythons as a model species, evaluated cryopreservation methods for spermatozoa extended in Test-yolk buffer with final DMSO or glycerol concentrations of 8, 12, or 16%, or combinations of DMSO and glycerol with final concentrations of 4:4, 6:6, or 8:8% (Young et al., 2017). Samples were frozen in vials at 0.3°C/min to −40°C before exposure to liquid nitrogen. Sperm frozen in the 6%DMSO:6% glycerol and 4%DMSO:4% glycerol had the highest post-thaw motilities. These same protocols also exhibited the highest plasma membrane integrity post-thawing.
In chelonians, one limited report on cryopreservation exists. In the study, spermatozoa could not be recovered after exposing them to pellet freezing. The freezing rate of this method was estimated to be -20-30°C/min. (Platz et al., 1980).

2.3.2.3. **Oocyte Collection and Handling.**

Successful oocyte collection before fertilization remains the major hurdle in the development of *in-vitro/ex-ovo* assisted reproductive technologies for reptiles. Further work needs to be completed to determine the timing of ovulation, develop collection techniques and oocyte handling methods, and develop *in-vitro/ex-ovo* culture development and incubation/techniques. We will briefly review some of the embryology literature that where recently, some of these techniques have been translated from avian species to reptiles for developmental studies.

*In vitro ex ovo* methods have been primarily used reptiles for developmental biology studies. Species that have been studied include garter snakes, Madagascar ground geckos (*Paroedura pictus*), Chinese softshell turtles, veiled chameleons, and Japanese striped snakes (*Elaphe quadrivirgata*) (Holtzman and Halpern, 1989; Diaz et al., 2017; Nagashima et al., 2007; Nomura et al., 2015; Tschopp et al., 2014). Embryos are collected from oviposited eggs, and the fertilized ovum and/or embryo are carefully removed from the egg and placed into culture media. In many of these studies, the embryos are fixed and analyzed at certain timepoints based on the proposed experimental design, so we do not know if these exact methods could be used to culture reptile embryos to maturation. The *in vitro* culture systems used for *ex-ovo* studies are whole embryo culture systems with a sterilized glass bottle system with 95% oxygen and 5%...
carbon dioxide maintained at 30°C. This system can keep early embryos alive for up to two days following removal from the egg. Further investigation into these methodologies is warranted because they could be used to manage animals that have disease in their oviducts, especially if an \textit{ex-ovo} culture system similar to birds can be produced that leads to a fully developed hatched chick.

\textit{In-ovo} embryo manipulation using electroporation has been used for gene transduction in Madagascar ground geckos and Chinese softshell turtles. Gene transduction using electroporation was successful in both species; however, these embryos were sacrificed prior to complete development so it is unknown if the embryos would have completely developed. Lentiviral vectors have also been used to successfully introduce fluorescent genes upon injection into the germinal disk in leopard geckos (\textit{Eublepharis macularius}). This method resulted in a reduced hatch rate compared to non-injected eggs; however, 41 (36.6% hatch rate) viable offspring were produced from 112 embryos (Kaitlyn et al., 2014).

\textbf{2.3.2.4. Oocyte Cryopreservation/Vitrification.}

No attempts have been made to cryopreserve or vitrify oocytes in reptiles. Oocyte size, composition, and structure are limiting factors in method development. Oocyte collapse and re-inflation should be investigated as possible methods for oocyte vitrification. Technologies should be developed to overcome these hurdles.

\textbf{2.3.3. Genome Resource Banking}

A fully functional genome resource bank for reptiles is necessary with the current extinction crisis. A complete genome resource bank should include multiple, essential
components to maximize our ability to store reproductive cells and tissues. The technology and techniques required to develop, maintain, and grow a genome resource bank include our ability to routinely collect spermatozoa, oocytes, embryos, and somatic cells. Once these samples are collected, then indefinite storage is required, and this could be achieved using cryopreservation. Technologies that allow for us to collect these samples from reptiles will lead to the development of methods to perform artificial insemination, in-vitro fertilization, embryo transfer, and nuclear transfer. These methodologies are at the heart of ART and are required to produce live offspring from eternally stored cellular material. The goal of this bank would be to preserve current biodiversity before it declines further and stop extinction, return genetic diversity to populations, and bring back species that may have gone extinct.

For reptiles, a realistic genome resource bank could be developed using technologies that already exist and focus on collecting and storing spermatozoa both in the short term and long term, and performing artificial insemination to produce live offspring from stored material. Methodologies to cryopreserve reptile ovarian and testicular tissues do not exist at this time. Although cryopreservation and cellular culture of somatic cells have been successful for reptiles, successful oocyte collection, culture, cryopreservation, and in-vitro methods such as IVF and ICSI remain a hurdle due to the inherent diverse reproductive anatomic and physiologic variations in reptiles and our limited understanding of oocyte biology. Once technologies and methodologies are developed and applied to female reptiles, the potential application for in-vitro methodologies will increase because many reptiles are oviparous and embryo development occurs outside of the female’s body.

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2.3.4. Artificial Insemination

Artificial insemination with successful fertilization and offspring production has only been reported in a handful of reptiles, including three species of snakes and one crocodilian. At this point in time, artificial insemination has only been successful in reptiles using freshly collected or short-term extended semen. Successful insemination using cryopreserved spermatozoa has not been reported in any reptile.

2.3.4.1. History of Artificial Insemination in Reptiles.

The American alligator was the first reptile to be successfully artificially inseminated with fresh spermatozoa and produce live offspring (Cardeilhac et al., 1982, 1988; Larsen, 1982, 1988). This was initially intended to assist in the recovery of the American alligator and the leather industry; however, other conservation efforts and strategies implemented at the same time proved to be more fruitful. Thus, at this time these methods may be of limited value for American alligators but should be pursued for threatened crocodilians.

Successful artificial insemination has been reported in three species of snakes: corn snakes on two separate occasions a garter snake (Thamnophis marcianus), and an Amazon tree boa (Corallus hortulanus) (Mattson et al., 2007; Quinn, 1989; Oliveri et al., 2010). The first documented case in corn snakes demonstrated that offspring could be produced using fresh and cooled (3 days at 4°C in TL-HEPES media) semen following insemination post-hibernation. No follicular monitoring was performed during this study and they inseminated the animals at the same time. The spermatozoa were deposited into the oviduct of each animal using a metallic ball tip syringe. Insemination was performed in 10 females; only three females produced eggs and...
only two of these clutches produced offspring with a 33% (5/15) and a 38% (5/13) hatch rate, respectively. Genetic testing was performed on the offspring from both clutches to determine paternity with microsatellites. Paternity -by-exclusion confirmed the offspring were from the artificial insemination (Mattson et al., 2007). The other report successful insemination in a corn snake and an Amazon tree boa used monitored follicular progression and performed artificial insemination using endoscopic assisted oviductal catheterization using a trans-cervical insemination catheter. The corn snakes used in this study had successfully produced eggs in prior years from natural breeding, while the Amazon tree boas were wild-caught adults and their reproductive history was unknown. Paternity was never confirmed for either species.

Insemination success was reported to be much higher in the corn snakes, with 14 and 16 egg clutches, respectively, and all of which were fertile and hatched. The authors reported that the Amazon tree boa underwent parturition four months after insemination, and successfully produced 2/7 live young. Unfortunately, the proposed timeline does not fit with the known gestation for this particular species, which ranges from 175-225 days. Thus, the offspring produced by the boa were not a result of artificial insemination and suggest the snake was gravid at the time of acquisition. Additionally, because the female corn snakes had produced offspring in the past, it is possible that they two were gravid as a result of sperm storage and not artificial insemination. Paternity testing for these cases is important to confirm the outcome. Previous studies have described the normal ejaculate and spermatozoa characteristics for this species, and the ejaculates from the males were not reported in this study, so our knowledge of spermatozoa quality used for insemination is unknown (Farhig et al., 2007; Mattson et al., 2007).
Additionally, a spermatozoa insemination dose and volume were not reported, although insemination methodologies were different (Oliveri et al., 2010).

There is a single report of artificial insemination in a lizard. A micropipettor was used to inseminate a McCann’s skink at the lateral side of the cloaca. No offspring were produced; however, environmental issues and a disease outbreak likely contributed to this outcome (Molinia et al., 2010).

In chelonians, artificial insemination has only been attempted in chelonians in the ploughshare tortoise (*Astrochelys yniphora*) and the Yangtze giant softshell turtle (*Rafetus swinhoei*). In October 1982, artificial insemination was attempted in a ploughshare tortoise following sample collection via EEJ from a male. This female laid a clutch of 7 eggs twelve days after the AI procedure. A single dead embryo was reported in this clutch, although it was determined that this was not secondary to the artificial insemination due to the timing between the artificial insemination and oviposition. Additional clutches were laid with dead embryos and one live hatching in December 1982 and February 1983; these events could have been related to the artificial insemination events or sperm storage from natural copulation that occurred in 1982. This was never further investigated in this species because the male conspecific died from suspected renal disease in 1983 (Juvik et al., 1991). In the case of the Yangtze giant softshell turtle (*Rafetus swinhoei*), artificial insemination has been performed repeatedly since 2015 in the last known female, and she successfully laid eggs following insemination but none of them have been found to be fertile. Unfortunately, the lone remaining female died following an insemination attempt secondary to complications from anesthesia in 2019.
2.3.4.2. Assessing Eggs Following Oviposition for Fertilization and Embryo Development.

Gross egg appearance in oviparous squamates, crocodilians, and chelonians can be used to determine egg viability. Most healthy eggs should be an off-white coloration with a symmetrical shell. Eggs with any deformities are likely not viable. In chelonians and crocodilians, egg viability can be assessed by looking for egg banding or chalking. This site is where the vitelline membrane attaches to the inner shell membrane and causes an opaque spot to form; as the embryo develops the spot expands. Note, in some species, especially chelonians, an embryonic diapause occurs naturally. Candling can be successfully performed using a focal high-intensity light source in a darkened room to look for the presence or absence of an embryonic disc, developing vasculature, and embryo visualization/movement. Nonviable eggs often show a uniform yellow to white appearance without a vascular pattern. Care must be taken to not expose the egg to a high-intensity light for an extended period of time, as prolonged heat exposure could affect embryonic development. An egg Doppler can be used to evaluate heart rate in reptile eggs (hard or soft) once the embryo has completed cardiac development. These Dopplers use infrared technology that could also harm the embryo with prolonged use. High frequency ultrasound (8-15 mHz) can be used to evaluate eggs at any stage of development, and color-flow or pulse wave Doppler can be used to evaluate the blood flow and heart rate of the developing embryo. Water soluble acoustic coupling ultrasound gel can be applied to the egg without any detriment to the developing embryo; however, the gel can clog the pores within the shell and limit gas exchange, so minimal gel and a probe with a small footprint should be used to minimize potential complications (Keller et al., 2019).

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Differentiating between true infertility and early embryonic loss, as well as assessing fertilization, has only recently been evaluated in reptiles. Perivitelline membrane-bound sperm detection has been used successfully to assess fertilization in crocodilian and chelonians eggs. Investigation into other reptile species, such as squamates, needs to be conducted. Ideal candidate species will be one with minimal in-utero development. This technique is being applied to reptiles from birds. In birds, sperm-egg interactions using perivitelline membrane stained with Hoechst stain and reviewed under ultraviolet microscopy has allowed for scientific assessment of sperm competition, sperm storage, estimated sperm production, and to egg fertility. This technique could also be used for confirming paternity with DNA isolation from sperm-heads and species identification of eggs. Hopefully, this method will lead to further investigation of these same processes in reptile species. In chelonians, incubation time and microbial infection did not preclude the ability to detect PVM bound sperm. Additionally, eggs can be stored at -20°C or in formalin prior to analysis. This method will prove to be valuable in assessing the egg-sperm interaction in reptiles (Croyle et al., 2016; Augustine et al., 2017).

2.3.4.3. Confirming Paternity is Required for Reptiles.

In reptiles, multiple paternity is ubiquitous. It has been documented in all major groups of reptiles and is suggestive of high levels of female promiscuity. Multiple paternity arises in reptiles via two routes; either mating occurs with more than one male during the same reproductive cycle or that mating with one or more males during each reproductive cycle coupled with sperm storage across reproductive cycles (Uller and Olsson, 2008). In reptiles, direct benefits to multiple paternity often do not play a role, including no paternal care among reptiles.
and no evidence that ejaculates are utilized as resources. Even if there were, ejaculate sizes are unlikely to be large enough to contribute to female resource levels (Uller and Olsson, 2008). Although maternal care has been documented in numerous reptile species, indirect benefits can be found in reptiles with multiple paternity, including cryptic female choice, bet-hedging, promoting sperm competition, trading up, and sexual conflict.

In order to truly determine the value of ART for reptiles, certain tests are necessary. Tests for multiple paternity in animals with sperm storage is one of them. In order to confirm success with artificial insemination, paternity testing is needed for all offspring due to multiple paternity and parthenogenesis. In order to do this, molecular methods such as genotype polymorphic microsatellite DNA loci or single nucleotide polymorphisms (SNP) to assign paternity can be used. Another more recent methodology to evaluate is double digest restriction site associated DNA (RAD) sequencing. This form of analysis simultaneously achieves SNP discovery and genotyping steps, which is optimized to return a statistically powerful set of SNP markers. This has been found in birds to outperform microsatellite loci in assigning paternity and estimating relatedness by improving power to discriminate among potential relatives and provide more precise estimates of relatedness coefficients (Uller and Olsson, 2008; Thrasher et al., 2018).

2.3.5. Pharmacological Control of the Reptile Reproductive Cycle.

Pharmacological control of reproduction is a vital component to any ART program. These compounds can be used to time ovulation and semen collection in order to maximize our success in producing offspring. Clinical application of exogenous hormones for reptiles is

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scarce. Here I will try to summarize the main pharmacologic agents used to control reproduction in veterinary medicine and cover any applications for reptiles.

**GnRH and Gonadotropins.**

Gonadotropin-releasing hormones can be used to induce follicular development, induce ovulation, and manage infertility disorders. Supraphysiologic doses and long-term doses can be used for contraception.

**GnRH.** GnRH is a decapeptide that regulates the release of FSH and LH by the anterior pituitary. In many species, the release of GnRH is controlled by a neural pulse generator in the hypothalamus. Intermittent release is necessary for proper synthesis and release of gonadotropins, which are released in a pulsatile manner. GnRH acts on a G protein-coupled receptor, and continuous administration of GnRH can lead to desensitization and downregulation of GnRH receptors on the pituitary gonadotropins. This is the basis of the administration of GnRH agonists for chemical castration. There are two principal usages for GnRH: management of infertility and suppression of gonadotropin secretion.

**Gonadorelin.** Gonadorelin has been historically used to treat animals with infertility disorders secondary to GnRH deficiency or dysfunction in GnRH secretion. Additionally, this has been used in mammals for cystic ovaries, estrus synchronization, embryo transfer, terminating estrus in induced ovulators, and for diagnostic purposes with pituitary dysfunction. No reports exist of using this medication in reptiles.

**Leuprolide acetate.** Leuprolide acetate, a synthetic Gonadotropin Releasing Hormone (GnRH) agonist, has been found to reduce sex hormone production in vertebrates (Kirchgessner et al.,

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2009; Dlugi et al., 1990; Millam, 1993; de Oliveira et al. 2004). One study exists in reptiles using leuprolide acetate to decrease testosterone concentrations in green iguanas (Iguana iguana). Three groups of green iguanas were administered either a sham treatment or a leuprolide acetate injection of 0.2 mg/kg or 0.4 mg/kg intramuscularly. Blood was collected for testosterone assays on day 0, before treatment, and days 1, 4, 7, 14, 21, 28, and 35 post-treatment (Kirchgessner et al., 2009). A significant difference in testosterone concentrations was observed within subjects over time, although there was not a significant difference in testosterone between treatment groups. The results of this study suggests that mammalian leuprolide acetate, at the doses used, do not significantly reduce testosterone levels in captive male green iguanas (Kirchgessner et al., 2009).

Deslorelin. Deslorelin is a synthetic GnRH agonist that is used to induce ovulation and chemical castration. It is manufactured in multiple forms, including ovuplant, a long-acting implant, SucroMate, an injectable formulation, and Suprelorin, an implant. In the reptile literature, Suprelorin is most commonly used. There is a single study evaluating the effects of a single 4.7-mg deslorelin acetate implant on plasma testosterone concentrations in yellow-bellied sliders (Trachemys scripta). Animals were split into two groups, one control and one with a 4.7 mg deslorelin acetate implant. Plasma testosterone concentrations were sampled at six-time points between April and September. No significant difference was found between the control group and the deslorelin treatment group, except for the mean plasma testosterone concentration at the first timepoint. The treatment group had a significantly higher testosterone, suggesting that the 4.7 mg deslorelin acetate implant has a transient stimulatory effect on the anterior pituitary in

Portions of this section was previously published as:
yellow-bellied sliders but will not inhibit the reproductive tract (Potier et al., 2017). A single case report in a green sea turtle showed that after the 4th deslorelin implant, the seasonal change in testosterone expected with reproduction was abolished (Graham et al., 2016). 4.7 mg deslorelin acetate implants inserted intracoelomically in female leopard geckos did not inhibit reproduction (Korste, 2018). Deslorelin has been reported to suppress reproductive activity in female green iguanas, but did not suppress reproductive activity in female veiled chameleons kept in groups (Kneidiner et al., 2009; Knotek, 2014).

*Follicle Stimulating Hormone.* FSH has been used to develop follicles for ovulation or superovulation and oocyte collection for embryo production/embryo transfer in mammals. Folltropin and Ovagen are the two commercial preparations that are available. FSH has been used in experimental studies to promote follicular recruitment in reptiles; this may be the primary exogenous hormone needed to promote follicular recruitment in reptiles, especially squamates, because it is believed that FSH is the primary gonadotropin in squamates. In metallic skinks (*Niveoscincus metallicus*), exogenous FSH can induce recruitment of new follicles, but only if administered during early vitellogenesis (Jones, 2000). The effects of FSH on inactive ovarian follicles of different sizes has been evaluated in two lizard species, the little brown skink (*Leiolopisma laterale*) and green anole. In the little brown skink, 1 µg of FSH daily for 21 days did not stimulate the growth of follicles. In the larger ovary, dosages of 10 and 50 µg of FSH did not stimulate growth of follicles less than 0.35 mm in diameter, but did stimulate growth of follicles greater than 0.43 mm (only 50µmg FSH) or 0.48 mm (10 and 50 µg FSH) (Jones et al., 1973). Similar FSH effects occurred in the smaller ovary, except tiny 0.25 mm follicles

Portions of this section was previously published as:
responded to 10 and 50 µg FSH, and biased growth of FSH-treated ovaries occurred in different initial follicular sizes in the larger and smaller germ lines (0.79 mm and 0.47 mm, respectively) (Jones et al., 1973). In green anoles, the effects of FSH treatment were similar to those seen in the skinks except that all follicles measured responded to 10 and 50 µg FSH. 1 µg of FSH stimulated the growth of larger follicles, and biased growth occurred whenever the higher FSH dosages stimulated growth (Jones et al., 1973). These results demonstrate that some follicular selection does occur (Jones et al., 1973). FSH causes the recruitment of follicles into the size hierarchy by starting new follicle development in the GB and movement of one follicle at a time into the stroma. Gonadotropin also changes the number of some cell types in the granulosa and stimulates yolk deposition in larger follicles (Jones et al., 1975).

*Human Chorionic Gonadotropin.* hCG is a gonadal-stimulating hormone obtained from the urine of pregnant women. It is synthesized by syncytiotrophoblast cells of the placenta. hCG is a glycoprotein and non-pituitary gonadotropin with long-lasting effects. It primarily acts with LH-activity and serves as a substitute for LH to promote follicle maturation, ovulation, and formation of the corpus luteum. hCG is used to induce ovulation and to assist in animals with hypogonadism due to pituitary hypofunction. Additionally, it can be used to treat cystic ovaries, induce the estrous cycle, manage impotence, and inducing pseudopregnancy in felids. hCG has been used to increase plasma testosterone concentrations in reptiles and evaluate the effect(s) of this hormone on the gonad (Arslan et al., 1977; Edwards et al., 2004; Eyeson, 1971; Haider et al., 1987; Jadhav and Padgaonkar, 2010; Jalali et al., 1976; Jones, 1973; Lance et al., 1985; Licht 1969, 1971; Prasad and Sanyak, 1969; Reddy and Prasad, 1970a, 1970b; Sonar and Patil, 1994;
Of all the exogenous medications used to control reproduction, hCG has been evaluated the most in reptiles.

*Equine Chorionic Gonadotropin/Pregnant Mare Serum Gonadotropin (eCG/PSMG)*. eCG is a glycoprotein and non-pituitary gonadotropin with long-lasting biological effects. eCG is a hormone that is typically secreted from the endometrial cups of pregnant mares in early pregnancy to induce secondary corpus lutea by developing accessory follicles and maintaining the primary corpus lutea, and thus continuing progesterone secretion in the mare. It has significant gonadotropic activity and is primarily FSH-like and increases ovarian follicular growth; however, it also has sufficient LH-like activity to induce ovulation and luteinization. eCG/PSMG has been utilized in experimental studies to increase plasma testosterone concentrations in Indian spiny-tailed lizards (*Uromastix hardwicki*) and follicular growth in desert iguanas (Arslan, 1977).

**Ecbolics.**

*Oxytocin*. Oxytocin (C$_{43}$H$_{66}$N$_{12}$O$_{12}$S$_{2}$ or H-Cys-Tyr-Ile-Glu (NH$_2$)-Asp (NH$_2$)-Cys-Pro-Leu-Gly-NH$_2$) is an exogenously administered chemically synthesized hormone widely used by medical doctors and veterinarians. In veterinary medicine, Oxytocin (Pitocin®, Syntocinon®, or generic Oxoject, Vetus Pharmaceuticals) is primarily used to induce labor, promote milk letdown in domestic species, and used in bovine species to treat mastitis caused by *Escherichia coli*. In it's currently available formulations, oxytocin can be administered intravenously or intramuscularly.

Portions of this section was previously published as:
Endogenous oxytocin is a non-peptide hormone that is produced by the hypothalamus and released via the posterior pituitary. It’s synthesized as a large precursor molecule in the cell bodies of the paraventricular nucleus (PVN). Formation occurs in the nerves as an oxytocin-neurophysin complex. Secretion occurs from nerve endings that terminate at the posterior pituitary that are stimulated by sensory input from the cervix, vagina or suckling of the mammary gland. Oxytocin is tonically inhibited by GABAergic (γ-aminobutyric acid) magnocellular neurons in the hypothalamus. Rapid depolarization and release of oxytocin containing neurons is caused by disinhibition of the tonic inhibition from neurons in the hypothalamus. Oxytocin acts at a specific G protein-coupled membrane receptor, which, when activated, leads to the formation of inositol triphosphate (IP3) from phosphoinositide hydrolysis. The formation of IP3 causes an increase in intracellular Ca\(^{2+}\) and leads to the contraction of smooth muscle tissue containing oxytocin receptors. Expression of the G protein-coupled receptors has been documented to increase during the later stages of pregnancy. Oxytocin also alters transmembrane ionic currents, increasing the sodium permeability of the uterine myofibrils causing the myometrium to produce sustained uterine contractions (Reddy, 2009; Perry, 2013). If oxytocin is administered around luteolysis it stimulates the secretion of prostaglandin F2-alpha (PGF2-alpha) and disrupts luteolysis. If oxytocin is administered prior to luteolysis, it does not induce PGF2-alpha, and disrupts luteolysis and prolongs corpus luteum function (Papich, 2010).

Oxytocin distributes throughout the extracellular fluid and appears to have a short half-life of approximately 1-6 minutes in mammals. Intravenous administration demonstrates a uterine response almost immediately with a one-hour duration of action. Intramuscular

Portions of this section was previously published as:
administration demonstrates a uterine response occurring within 3-5 minutes and duration of action lasting approximately 2-3 hours. Metabolism and excretion of oxytocin from plasma occurs by the liver and kidney. A small amount of oxytocin is degraded in circulation because of an enzyme oxytocinase. A small amount is excreted in the urine unchanged (Perry, 2013). No withdrawal times have been reported for oxytocin; however, a 24-hour withdrawal time is suggested because of rapid clearance after administration and the low risk of residues (Papich, 2010).

Oxytocin is primary use is to induce oviposition or maintain normal labor and delivery in pregnant mammals by stimulating uterine contractions. After cesarean section, oxytocin may be administered postoperatively to facilitate involution and resistance to the large inflow of blood. Oxytocin helps stimulate lactation, when oxytocin is released smooth muscle cells of the mammary gland contract for milk letdown in mammals if the udder is physiologically capable. Oxytocin does not increase milk production but stimulates contraction leading to milk ejection. It has also been used to help in the expulsion of placenta after delivery, although its efficacy in treating retained placentas is questionable. If oxytocin is administered before luteolysis it can prolong corpus luteum function and suppress estrus behavior (Perry, 2013).

Numerous reports of oxytocin use in reptiles do exist throughout several families. In reptiles, AVT is the endogenous equivalent oxytocin-like hormone that is responsible for oviposition in oviparous or parturition in viviparous species. An exogenous form of this hormone does exist; however, it is only available as a research drug at this time. Because of the

Portions of this section was previously published as:
limited availability of AVT oxytocin is often used to treat dystocia's in reptilians (DeNardo, 2006).

Cree et al. reported using oxytocin in forty-one adult female tuatara (*Sphenodon guntheri*) at 1 U/100 g intracoelomically to induce oviposition for artificial incubation. Eight females laid eggs in response to the administration of oxytocin, a 19.5% response rate. This may not be an accurate response rate due to female tuatara's laying eggs once every four years, although oviposition has been documented up to every two years in captivity (Cree et al., 1991)

In true non-obstructive dystocia’s in reptiles, oxytocin is best initiated within 48-72 hours of nesting behavior or straining. Oxytocin’s effectiveness at treating non-obstructive dystocia is variable between reptilian clades. In snakes, effectiveness is reported to be about 50%, with lizards being only slightly higher, which is drastic when compared to chelonians that have an approximately 90% response rate (DeNardo, 2006; Sykes, 2010; Stahl, 2002). Oxytocin can be used in lizards without mechanical obstruction as a medical therapy for dystocia at 5-30 U/kg intramuscularly or intracoelomically. In snakes, a dose of 5-20 U/kg intramuscularly may be effective, especially if it is administered 2 to 3 days from the onset of dystocia (DeNardo, 2006; Sykes, 2010). Often a second dose (50%-100% of the initial dose) can be given 20-60 minutes after the first. All reptiles administered oxytocin should be maintained or near their optimal body temperature because temperature likely influences the effect of oxytocin on the oviduct. If two to three dosages are given without any response it is unlikely that medical therapy will not be effective.

Portions of this section was previously published as:
In chelonians, oxytocin appears to have the best response rate of all reptiles. Various treatment protocols exist. McArther suggests using 1-3 U/kg IM, IV, IO. The intraosseous infusion is suggested to be more effective than bolus injections. If egg expulsion has not occurred following a single oxytocin injection, 50%-100% of the initial does can be repeated intramuscularly 1 to 12 hours later. For best results, oxytocin should be used in conjunction with a therapy protocol of rehydration, cloaca lubrication, environmental changes (ie. nesting area, heat, and humidity), calcium supplementation (hypocalcemic individuals), and beta-blockers (atenolol 7 mg/kg orally or propranolol 1 mg/kg intracoelomically). Oxytocin and beta-blockers should be administered the morning after the initial change in environment, rehydration, lubrication, and calcium supplementation. This therapeutic protocol can be continued with fluid therapy and beta-blockers, while eggs are deposited at a rate greater than one every 24 hours. This protocol should be discontinued once oviposition stops (Skyes, 2010; McArthur, 2004).

Prostaglandins (PGF2-alpha) used at 1.5 mg/kg subcutaneously has been reported to aid in oviposition in red-eared sliders (Trachemys scripta elegans) when used in conjunction with oxytocin at 7.5 mg/kg subcutaneously (Sykes, 2010).

In Crocodillians, one case report exists using oxytocin to induce oviposition in a saltwater crocodile (Crocodylus porosus). A dose of 25 U was administered intramuscularly via dart gun in the tail base of an approximately 25 kg individual (1 U/kg). Weak abdominal contractions were observed 35 minutes after injection, indicating signs of uterine contractions. At 70 minutes after injection, strong contractions were observed. At 90 minutes after injection, the first egg was observed and over the following 30 minutes, 26 eggs were observed (Carmel, 1991).

Portions of this section was previously published as:
Prostaglandins. PGF$_{2\alpha}$ mediates a decrease in circulating progesterone via luteolysis by corpus leuta regression and a decrease in placental progesterone production. PGF$_{2\alpha}$ acts via activation of G protein-coupled receptors linked to the IP$_3$-Ca$^{2+}$ protein kinase C pathway, which leads to a decrease in steroidogenesis and luteolysis. PGF$_{2\alpha}$ is a luteolytic agent that is typically produced by the endometrium in mammals to help signal the induction of parturition. PGF$_{2\alpha}$ is primarily used to regulate estrous cycles, induce abortion, and to induce parturition in animals. For estrus synchronization, PGF$_{2\alpha}$ decreases the estrus cycle length and can hasten the onset of estrus, PGF$_{2\alpha}$ alone, or in conjunction of progestins/progesterone treatment withdrawal, can be used. Additionally, it can be used with GnRH for timed AI. Induction of abortion can be completed with PGF$_{2\alpha}$, as it obliterates the corpus leuta in pregnancies that are leutal dependent. Other usages of PGF$_{2\alpha}$ include induction of uterine contractions, mating induced persistent endometritis, managing uterine infections, and to induce parturition. PGF$_{2\alpha}$ increases smooth muscle tone and can lead to significant discomfort following administration. In reptiles, some studies have been performed evaluating PGF$_{2\alpha}$. Oviposition was not induced in gravid female lizards treated with PGF$_{2\alpha}$ in vivo; however, when oviducts were isolated in vitro, then PGF$_{2\alpha}$ did induce oviposition (Guillette et al., 1991c). In late-term viviparous lizards, PGF$_{2\alpha}$ administration stimulated parturition in vivo within 2 hrs (Guillette et al., 1992). Animals midway through the pregnancy did not respond to PGF$_{2\alpha}$ in vivo (Guillette et al., 1991a). In New Zealand common geckos, pre-treatment with a β-adrenoreceptor antagonist before administration of PGF$_{2\alpha}$ stimulated parturition (Cree and Guillette, 1991).

Portions of this section was previously published as:
**Prolactin.** Prolactin is synthesized in the lactotrophs within the anterior pituitary and is considered a somatomammotrophic hormone. Prolactin stimulates lactation in the post-partum period in mammals and promotes mammary gland development in mammals in concert with other hormones such as progesterone and estrogen. Additionally, prolactin inhibits ovarian function by suppressing the hypothalamic-pituitary-gonadal axis. Discussion of this hormone in detail is noted previously. Drugs that influence prolactin release include Metoclopramide and Domperidone. The difference between the two drugs is that domperidone does not cross the blood-brain barrier and is primarily experimental for animals in the United States. Metoclopramide acts via dopamine antagonism and increases prolactin secretion. Studies using both of these medications in reptiles are lacking.

**Progestins.**

Progesterone is the major progestin in most species. Progesterone is rapidly metabolized in the liver and often has a low bio-availability with a short half-life. Synthetic progestins such as altrenogest and melengesterol acetate have been used to suppress ovarian activity and synchronize the estrous cycle following progestin withdrawal. Progestins act on progesterone receptors in the hypothalamus/pituitary, increasing the negative feedback and decreasing FSH and LH output; once the progestin is removed, then a new estrous cycle and ovulation occurs. *Altrenogest.* Altrenogest is a synthetically active progestin indicated for controlling cyclicity. PGF$_{2\alpha}$ following the withdrawal of altrenogest can induce estrus; additionally, it can help maintain pregnancy in animals and prevent abortion due to luteal insufficiency. To date, no studies have been performed in reptiles using altrenogest.

Portions of this section was previously published as:
2.3.6. Advanced ART-Future Technology Development and Application in Reptiles

2.3.6.1. In-vitro/ex-ovo Fertilization/Intracytoplasmic Sperm Injection (ICSI).

*In-vitro/ex-ovo* fertilization and ICSI methods or successes have not been reported in any reptile to date. This is due to our limited knowledge of ovulation timing in these species and our limited ability to harvest the ovum following ovulation but before fertilization. Successful ovum collection with our current technologies would require invasive surgical or terminal procedures following ovulation due to their large size, as is performed in avian species to successfully collect ovum (Mizushima et al., 2017). Reptiles, like birds, undergo polyspermic fertilization, thus *in-vitro/ex-ovo* fertilization would likely be the preferred technique to allow for successful fertilization and developmental progression. In birds, ICSI is not successful when only one sperm is injected into the oocyte. Higher success has been reported using a co-injection of sperm extract; this has enhanced the full-term development of ICSI-generated zygotes to hatching (Mizushima et al., 2017). For *in-vitro/ ex-ovo* methods to be successful in reptiles, we should look at birds as a model to develop our techniques and methods.

2.3.6.2. Cloning-Somatic Cell Nuclear Transfer (SCNT).

No reports exist within the literature of successful cloning or somatic cell nuclear transfer in reptiles. Further, oocyte collection methods and culture need to be developed prior to these technologies being applied to reptiles.

2.3.6.3. Genetic Editing in Reptiles.

Reports of genetic editing in reptiles are scarce. Manipulation of gene function in reptiles is limited to manipulation of whole embryos in culture using viral- or electroporation methods to
alter gene expression (Nagashima et al., 2007; Nomura et al., 2015; Tschopp et al., 2014). Transgenic leopard geckos and ball pythons (*Python regius*) have been successfully developed using lentiviral vectors to insert genes, including fluorescent proteins (Kaitlyn et al., 2014; Mozdziak and Petitte, 2010). Recently, the first successful report of genetic editing using the CRISPR/Cas9 system was reported in the brown anole (*Anolis sagrei*). Ovarian microinjection was used to administer a Cas9 protein coupled to a mixture of three different synthetic tyr guide RNAs into immature oocytes to terminate the tyrosinase gene, which resulted in phenotypic albino brown anoles (Rasys et al., 2019).
CHAPTER 3. DETERMINING THE SAFETY OF REPEATED ELECTROEJACULATION IN VEILED CHAMELEONS CHAMAELEO CALYPTRATUS

3.1. INTRODUCTION

Reptile biodiversity loss is an accelerating anthropogenic created crisis. Current estimates are that one in five reptile species are threatened with extinction (Alroy, 2015; Anderson et al., 2013; Böhm et al., 2013, 2016; Ceballos et al., 2015; Gumbs et al., 2018). As anthropogenic and climatic pressures continue to mount leading to extinction, ectotherms, such as reptiles, are increasingly being negatively impacted. To prevent extinction, it is important for scientists to develop comprehensive conservation programs to protect reptiles. Reptiles are particularly sensitive to habitat degradation because of their comparatively low dispersal, morphological specialization on substrate type, relatively small home ranges, and thermoregulatory constraints. While the ultimate goal of herpetologic conservation is to maintain wild reptile populations within their native ranges, it may not be possible in every case and captive populations within zoos and aquariums may be needed to serve as a proverbial “ark” for threatened and endangered species.

Based on recently published data, 23% of 867 lizard species surveyed were categorized as critically endangered, endangered, vulnerable, or near threatened (Alroy, 2015; Anderson et al., 2013; Böhm et al., 2013, 2016; Ceballos et al., 2015; Gumbs et al., 2018). Further, based on the IUCN data, more than half (51%, 107/207) of all known chameleon species are categorized as threatened or near-threatened. Of the 193 species of chameleons included on the IUCN Red List, 11 species are classified as critically endangered, 39 species as endangered, 22 species as
vulnerable, and 35 species as near threatened. These data suggest that chameleons are at a greater extinction risk with over half of their familial diversity at risk, and that special efforts should be made to provide further assistance towards conservation for this group. Many wild and captive chameleon populations are not self-sustaining because of geographic isolation from potential mates and environmental differences from their native habitats, respectively, resulting in abnormalities in physiologic and behavioral cues necessary to stimulate reproduction (Cote et al., 2010; Mattson et al., 2008, 2007). To counter these negative impacts, assisted reproductive technologies can serve as an important component of a species conservation plan and should be pursued to develop methods to assist reproduction and increase genetic diversity, in combination with other methods such as habitat restoration and protection.

Semen collection is a commonly used assisted reproductive technology for fish, amphibians, birds, and mammals; however, its use in reptiles has been limited (Clulow and Clulow, 2016; Johnston et al., 2014, 2014a; Juri et al., 2018; Larsen et al., 1988; Larsen et al., 1984; Larsen et al., 1992; Lierz et al., 2013; Mattson et al., 2008, 2007; Mitchell et al., 2015; Molina et al., 2010; Quinn et al., 1989; Schulte-Hostedde et al., 2006; Tanasanti et al., 2009; Tourmente et al., 2007; Zacariotti et al., 2007; Zimmerman and Mitchell, 2017; Zimmerman et al., 2013). In reptiles, both post-mortem and ante-mortem semen collection methods have been described. Post-mortem semen collection can be collected directly from the ductus deferens or epididymis, and commonly yields higher numbers of uncontaminated spermatozoa (Ceballos et al., 2015). While post-mortem semen collection allows for higher quality samples, relying on these types of samples for a conservation program is not sustainable. To preserve population numbers, ante-mortem techniques have been developed to collect semen in reptiles, including
massage, a method utilizing a vibrational device, and electroejaculation. However, much of the current literature on semen collection in reptiles is focused on snakes, chelonians and crocodilians, with only four reports on lizards in the peer-reviewed literature, including the green iguana (*Iguana iguana*), the common house gecko (*Hemidactylus frenatus*), Spiny lava lizard (*Tropidurus spinulosus*) and the McCann’s skink (*Oligosoma maccanni*) (Fahrig et al., 2007; Johnston et al., 2014, 2014a; Juri et al., 2018; Larsen et al., 1988; Larsen et al., 1984; Larsen et al., 1992; Mattson et al., 2008, 2007; Mitchell et al., 2015; Molina et al., 2010; Quinn et al., 1989; Schulte-Hostedde et al., 2006; Tourmente et al., 2007; Zacariotti et al., 2007; Zimmerman and Mitchell, 2017; Zimmerman et al., 2013). The green iguana report used electroejaculation for semen collection at a single point in time, while the other two reports used massage. A limited understanding of semen collection in lizards, along with the high percentage of animals currently under threat, reinforces a need to expand the current knowledge in this field and develop assisted reproductive techniques that can be used to protect species before it is too late.

This study’s goal was to evaluate whether repeated electroejaculation under anesthesia could be done safely in a species of chameleon. The objectives for this study were to perform four weekly electroejaculations under alfaxalone anesthesia in veiled chameleons (*Chamaeleo calyptratus*). The specific hypotheses being tested in this study were that: 1) electroejaculation could be used to collect semen from veiled chameleons, and 2) that four weekly repeated electroejaculations under anesthesia would have no effect on survival, the physical examination findings, or hematological results of the chameleons.
3.2. MATERIALS AND METHODS

This longitudinal, prospective study was performed in accordance with the rules and regulations established by Louisiana State University’s institutional animal care and use committee (protocol # 16-095). Twelve wild caught, invasive veiled chameleons collected from southern Florida were used for this study. The sample size for this study was determined using the following a priori information: an alpha=0.05, a power=0.80, and a likelihood of being able to collect semen in at least 60% of the chameleons during the study.

Animals were individually housed at Louisiana State University in 18in x 18in x 36in (45.27 cm x 45.27 cm x 91.44) screen cages (Zoo Med, Paso Robles, California 93446, USA). A MistKing misting system (Jungle Hobbies Ltd, Ontario N9E4R3, Canada) was incorporated into the caging and used to mist the animals for three minutes, three times a day. Animals were provided a 12hr photoperiod. Daytime ambient room temperature and a basking spot were maintained at 27.8-28.8°C (82-84°F) and 35-37.8°C (95-100°F) utilizing central heating and incandescent lighting (Fluker Farms, Port Allen, Louisiana 70767, USA), respectively. Nighttime room temperature was 23.3-25.5°C (74-78°F). Animals were exposed to ultraviolet lighting 12 hours per day (Reptisun 10.0 Fluorescent bulbs, Zoo Med). A combination of Repta-Vines (Fluker Farms), treated sand blasted grape vines (Fluker Farms) were used to provide cover and climbing surfaces within the cages. Chameleons were fed a diet consisting of gut-loaded house crickets (Acheta domestica) and superworms (Zophobas morio). Insects were gut loaded 24 hours prior to feeding with a combination of vegetables.

A physical examination was performed on each unsedated chameleon upon intake to confirm that the chameleons were healthy. Direct saline smears and fecal flotations using zinc...
sulfate were performed. Animals were found to harbor undescribed flagellates, *Strongyloides* sp., and trematodes. All chameleons were all treated with fenbendazole (Panacur, Merck Animal Health, Madison, New Jersey 07940, USA; 25 mg/kg p.o. s.i.d for 3 days), praziquantel (Praziquantel, Bimeda, Inc., Le Suer, Minnesota 05058, USA; 6 mg/kg i.m. every 7 days for 2 treatments), and metronidazole (compounded metronidazole 50 mg/mL, Louisiana State University, Baton Rouge, Louisiana 70803, USA; 20 mg/kg p.o s.i.d. for 3 days) prior to initiating the study with a negative fecal. Animals were acclimated to captivity for one month prior to initiating the study. Fenbendazole and praziquantel were administered at the beginning of the acclimatization period. Metronidazole therapy was initiated at the start of the study to reduce the flagellate load observed at the time of initial semen collection likely secondary to colonic overflow with cloacal flushing. Blood collection was performed via manual restraint at the beginning of the study, prior to the first electroejaculation event, and after the last electroejaculation event to measure complete blood counts and plasma biochemistries. Blood was collected from the ventral coccygeal vein using a 25 gauge needle fastened to a 1-mL syringe. Manual complete blood counts were performed by boarded clinical pathologist utilizing previously described methods (Mayer et al., 2011). Biochemistries were performed using the VetScan Classic analyzer and Vetscan Avian-Reptile Profile- Plus rotors (Abaxis Inc., Union City, California 94587, USA) (Mayer et al., 2011).

Electroejaculation under alfaxalone anesthesia was performed once weekly over a 4-week period to determine whether serial electroejaculation could be used to collect semen from veiled chameleons and whether electroejaculation or serial anesthesia affected the health of the chameleons. Animals were sedated for each electroejaculation procedure with alfaxalone 15
mg/kg intravenously in the ventral tail vein. Heart rate and respiratory rates were monitored throughout the procedure, and the chameleons spontaneously ventilated throughout the procedure. Animals were maintained on a warm water re-circulating blanket Gaymar TP 500 (Gaymar Industries, Orchard Park, New York 14127, USA) set at 35°C (95°F). Chameleons were hand recovered under a 100 W ceramic heat emitter (Fluker Farms). The chameleons were returned to their enclosure once they had resumed their righting and escape reflexes.

Vent photographs (Figure 3.1.) were collected once the chameleon was anesthetized and prior to and after each electroejaculation event using a Nikon D5100 digital single lens reflex camera (Nikon USA, Melvill, New York 11747, USA) with a Sigma 105 mm f/2.8 DX lens (Sigma, Ronkonkoma New York 1179, USA) and a Nikon SB- 700 speedlight (Nikon USA). Following photographic documentation of the external vent appearance and prior to electroejaculation, the vent was lavaged with 20 mL plasmalyte (Baxter International, Deerfield Illinois 60015, USA) to clean the vent and improve endoscopic visualization. Cloacoscopy was performed using a Storz Hopkins II 2.7 mm 30° telescope (Karl-Storz Endoscopy America, El Segundo, California 90245, USA) with a MDS-Vet portable LED light source (MDS incorporated, Valrico Florida 33596, USA). Visualization of the internal cloacal structures and images were captured using the Envisioner EndogoHD Portable Endoscopy System (Envisioner Medical Technologies, Rockville, Maryland 20859, USA) before and after each electroejaculation procedure.

The electroejaculator used for this study has a variable amperage power source and a 360° circumferential metallic probe as previously described. This bipolar probe consists of a metallic portion measuring and a non-conducting plastic where two electrical contacts are placed.
Figure 3.1. External vent photos used for evaluation. A. Image taken before electroejaculation event. This image was graded as a score of 1 based on the vent scoring system. B. Image taken after electroejaculation event. This image was scored as a 2 based on the vent scoring system. Note the mild erythema and moderate eversion of the mucosa due to repeated penetration.

at the sides (Lierz et al., 2013). The metallic portion of the probe measures 20 mm in length and 3 mm in diameter. A procedural description of the electroejaculation can be found in Figure 3.2. Animals were electroejaculated by performing 15 cloacal intromissions at 0.1 mAmps for up to 3 events, with a rest period of 3 minutes in between each series of intromissions. Electroejaculation was discontinued once a semen sample was collected. Ejaculates were collected using a 1 mL Monoject™ syringe (Coviden, Mansfield, Massachusetts, 02048 USA) and confirmed to have spermatozoa under microscopy by placing a drop of the ejaculate on a microscope slide with a coverslip and reviewed by SMP or MAM using a Nikon Eclipse E 400 (Nikon USA) at 400 x. Presence or absence of spermatozoa was recorded for each chameleon at the conclusion of each weekly trial.

Vent and cloacal rank scoring systems were developed to assist with the interpretation of the photographic and endoscopic images collected pre- and post-electroejaculation, respectively (Tables 3.1. and 3.2., Figure 3.1., 3.3., 3.4.). A single blinded reviewer interpreted and scored the

Figure 3.2. Photos of the electroejaculation procedure in sequence. A and B. Forward insertion of the electroejaculation probe into the cloaca while the C. The electroejaculation probe is fully inserted into the cloaca. Light dorsal pressure is applied to ensure the nerves of the pelvic plexus are engaged. The variable amperage power source is discharged for a single intromission at this point. D. The metallic portion of the probe is pulled out of the cloaca slowly when the power source is discharging. This cycle is considered one intromission.

image. The images were forwarded to the reviewer as randomly assigned files with no identification as to the animal, whether it was a pre- or post-electroejaculation event, or regarding the week of collection.

3.2.1. STATISTICAL METHODS

The distributions of the continuous data (weight, complete blood counts, biochemistry results) were evaluated using the Shapiro-Wilk test, skewness, kurtosis, and q-q plots. Normally distributed data are reported by the mean, standard deviation (SD), and minimum-maximum (min-max) values, while non-normally distributed data are reported as the median, 25-75 quartiles (%), and min-max values. Normally distributed weights were analyzed using a repeated measures ANOVA. Normally distributed complete blood count and biochemistry results were analyzed using paired samples t-tests. Non-normally distributed data were analyzed using Wilcoxon paired ranked sums tests. Vent and cloacoscopy image data were reported as ordinal data.
### Table 3.1. Vent scoring system

<table>
<thead>
<tr>
<th>Vent Scoring System</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
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<tr>
<td>No color change to the external vent structures, Mild eversion of mucosa, no inflammation or swelling of visible mucosa observed</td>
<td>Normal appearing vent</td>
<td>Mild inflammation</td>
<td>Severe inflammation</td>
</tr>
<tr>
<td>No color change or swelling to the external vent structures, moderate eversion of mucosa, mild inflammation and swelling observed to visible mucosa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Color change and swelling to the external structures of the vent, severe eversion of mucosa, severe inflammation and swelling to visible mucosa</td>
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### Table 3.2. Cloacoscopy scoring system

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<thead>
<tr>
<th>Cloacoscopy Scoring System</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
<th>Grade 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>No erythema, swelling, petechiation present</td>
<td>Normal mucosa</td>
<td>Mild inflammation</td>
<td>Moderate inflammation</td>
<td>Severe inflammation</td>
<td>Chronic/Life threatening changes</td>
</tr>
<tr>
<td>Mild erythema, no swelling, some petechiation present, no hemorrhage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cloacal perforation or Cloacal stricture</td>
</tr>
<tr>
<td>Moderate /segmental erythema, numerous petechiation or ecchymosis, mild hemorrhage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse erythema, swelling of mucosa, moderate hemorrhage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


148
Figure 3.3. Cloacoscopy scoring images. A. Grade, note no erythema present within the mucosa; B. Grade 2; C. Grade 3; and D. Grade 4. No grade 5 images were observed at any point

Figure 3.4. Cloacoscopy images used for grading based on the cloacoscopy grading system. A. Image taken before electroejaculation event. This was scored as a 1. B. Image taken after the electroejaculation. This was scored as a 2; note the increased erythema in the cloacal mucosa.

data. The frequencies of ordinal data are reported, and Friedman’s repeated measures ANOVA tests were used to compare the results over time. When a difference was found, a post-hoc Wilcoxon paired ranked sums test was used to determine the point(s) in time (pre-post or week) that the difference occurred. Statistical analyses were performed using SPSS 23.0 (IBM Statistics, Armonk, NY, 10504 USA). A p≤0.05 was used to determine statistical significance.

3.3. RESULTS

All 12 chameleons were successfully anesthetized with alfaxalone and electroejaculated once weekly for 4 weeks. None of the chameleons showed any negative side effects during the study or for the four months. There was a significant increase in bodyweight over the duration of the study (F=41.14, df=3, P<0.001), with the weights of the chameleons increasing, on average, 11% over the course of the study (baseline: 151.6±34.41 g, Min-Max 90.0-214.0 g; 4 weeks: 169.57±31.8 g, Min-Max 113.2-215 g). Post hoc pairwise comparison showed a significant difference between weights at week one and week four (P<0.001). The mean duration of anesthesia was 17.93±4.3 minutes, Min-Max 10-28 minutes.

In total, 48 electroejaculation events were performed in the 12 chameleons over the 4 weeks and semen was successfully collected in 50% (24/48) of the procedures. Successful collection was defined as spermatozoa that were too numerous to count with in a field without further dilution at 10x. Semen was successfully collected from all individuals at least once during this study. In five individuals, semen was collected only once during the study period. Semen was successfully collected in four individuals in three of four electroejaculation attempts, all of these collections sequential by week. In two individuals, semen was collected 50% of the time. Only one subject produced semen at each collection attempt. The majority (n=20) of the This chapter was previously published as Perry SM, Konsker I, Lierz M, Mitchell MA. 2019. Determining the safety of repeated electroejaculations in veiled chameleons (Chamaeleo calyptratus). Journal of Zoo and Wildlife Medicine, 50(3): 557-569. Reprinted by permission of Allen Press
semen samples were collected after the first set of probe intromissions, with semen also being collected in 3 electroejaculation events after 2 intromissions, and in 1 event after 3 intromissions; no semen samples were collected in 24 electroejaculation events after 3 sets of intromissions.

The median volumes for the samples collected after the first, second, and third sets of intromissions were 100 µL (25-75 %:100-250 µL, min/max: 0.0-0.53 µL), 39 µL (25-75 %: 0-20 µL, min/max: 0-350 µL), and 0 µL (25-75 %: 0-8 µL, min/max: 0-100 µL), respectively.

Ejaculate volume was significantly different between the first set of intromissions compared to the second (p=0.005) and third (p<0.001) set of intromissions. There was no significant difference in ejaculate volume between the second and third set of intromissions(p=0.07), but the comparison approached significance.

There was a significant increase in the white blood cell counts (t=2.88, p=0.015), absolute heterophil counts (t= 4.19, p=0.02), absolute monocyte counts (t=3.772, p=0.003), and albumin (t=2.35, p=0.038) between the pre- and post-ejaculation sampling periods (Tables 3.3a and 3.3b). AST (t=-4.26, p=0.001) and potassium (t=-2.38, p=0.036) were found to significantly decrease between the pre- and post-sampling periods (Table 3.3a). There were no significant differences in any of the other complete blood count or biochemistry parameters between the pre- and post-electroejaculation sampling periods (Tables 3.3a and 3.3b).

Vent and cloacoscopy image scores for the pre- and post-ejaculation sampling periods can be found in Tables 3.5 and 3.6, respectively. Vent images scored significantly higher (Z=-2.0, p=0.046) between the pre- and post-electroejaculation images collected during week 1; however, no significant difference were noted between pre- and post-electroejaculation images for weeks 2 (Z=-1.857,p=0.063), 3 (Z=-1.782,p=0.083), or 4 (Z=-1.732,p=0.083). Cloacoscopy
Table 3.3: Complete blood count and biochemical values for normally distributed data between sampling times in Veiled chameleons (n=12). *Indicates significant difference change between the start and end of the study period (P<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Standard deviation</th>
<th>95% Confidence interval</th>
<th>Minimum-Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Packed Cell Volume (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start</td>
<td>28.5</td>
<td>2.74</td>
<td>26.75-30.24</td>
<td>25.0-35.0</td>
</tr>
<tr>
<td>End</td>
<td>25.27</td>
<td>3.2</td>
<td>23.06-27.48</td>
<td>21.0-32.0</td>
</tr>
<tr>
<td><strong>White blood cell count (K/μL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start</td>
<td>8.86</td>
<td>4.89</td>
<td>5.75-11.97</td>
<td>1.5-18.30</td>
</tr>
<tr>
<td>End</td>
<td>13.8</td>
<td>4.03</td>
<td>11.23-16.36</td>
<td>6.5-20.0</td>
</tr>
<tr>
<td><strong>Heterophils (K/μL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start</td>
<td>2.75</td>
<td>1.71</td>
<td>1.67-3.84</td>
<td>0.7-6.10</td>
</tr>
<tr>
<td>End</td>
<td>6.38</td>
<td>3.5</td>
<td>4.14-8.6</td>
<td>0.30-11.9</td>
</tr>
<tr>
<td><strong>Bands (K/μL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>End</td>
<td>0.29</td>
<td>0.75</td>
<td>-0.18-0.77</td>
<td>0.0-2.6</td>
</tr>
<tr>
<td><strong>Lymphocytes (K/μL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start</td>
<td>4.48</td>
<td>3.43</td>
<td>2.29-6.66</td>
<td>0.4-12.0</td>
</tr>
<tr>
<td>End</td>
<td>4.68</td>
<td>2.16</td>
<td>3.31-6.05</td>
<td>1.3-8.2</td>
</tr>
<tr>
<td><strong>Monocytes (K/μL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start</td>
<td>0.86</td>
<td>0.44</td>
<td>0.58-1.14</td>
<td>0.3-1.6</td>
</tr>
<tr>
<td>End</td>
<td>1.65</td>
<td>0.87</td>
<td>1.09-2.21</td>
<td>0.3-3.30</td>
</tr>
<tr>
<td><strong>Basophils (K/μL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start</td>
<td>0.75</td>
<td>0.70</td>
<td>0.31-1.20</td>
<td>0.10-2.3</td>
</tr>
<tr>
<td>End</td>
<td>0.79</td>
<td>0.58</td>
<td>0.42-1.16</td>
<td>0.0-1.8</td>
</tr>
<tr>
<td><strong>Eosinophils (K/μL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>End</td>
<td>0.03</td>
<td>0.62</td>
<td>-0.0145-0.064</td>
<td>0.0-0.20</td>
</tr>
<tr>
<td><strong>AST (U/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start</td>
<td>748.3</td>
<td>271.7</td>
<td>575.65-921.01</td>
<td>452.0-1353.0</td>
</tr>
<tr>
<td>End</td>
<td>402.0</td>
<td>92.0</td>
<td>343.53-460.46</td>
<td>272.0-601.0</td>
</tr>
<tr>
<td><strong>CK (K/μL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Start</td>
<td>3186.16</td>
<td>1540.61</td>
<td>2207.30-4165.02</td>
<td>810.0-5782.0</td>
</tr>
</tbody>
</table>

(table cont’d)

images scored significantly higher between the pre- and post-electroejaculation events for each week: week 1 ($Z=-2.486, p=0.013$), week 2 ($Z=-2.251, p=0.024$), week 3 ($Z=-2.165, p=0.030$), and week 4 ($Z=-2.588, p=0.010$). No significant difference was observed between study week 1

Table 3.4: Complete blood counts and biochemical values for non-normally distributed data between sampling times for Veiled chameleons (n=12).

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>25-75% Quartiles</th>
<th>Minimum-Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK (K/uL) End</td>
<td>2111.5</td>
<td>1525.7-3162.0</td>
<td>272.00-601.00</td>
</tr>
<tr>
<td>Total Protein (g/dL) End</td>
<td>6.0</td>
<td>5.47-6.17</td>
<td>4.50-6.40</td>
</tr>
<tr>
<td>Globulin (g/dL) End</td>
<td>2.90</td>
<td>2.57-2.97</td>
<td>2.10-3.0</td>
</tr>
</tbody>
</table>

prior to electroejaculation and study week 4 after electroejaculation in vent external morphology, grade 1-3 (Z=-1.414, p=0.157), and internal cloacal scoring, grade 1-5 (Z=-1.897, p=0.058); however, the cloacal scoring did approach significance.

Table 3.5. Frequency of vent morphology scoring evaluation before and after electroejaculation (n=12). *Indicates a significant difference was observed in vent morphology before and after electroejaculation each week (P<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Before EEJ</th>
<th>After EEJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Grade 2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Grade 3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Week 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Grade 2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Grade 3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Week 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Grade 2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Grade 3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

(table cont’d)

Table 3.6. Frequency of cloacoscopy scoring before and after electroejaculation (n=12). *Indicates a significant difference change before and after electroejaculation each week (P<0.05).

<table>
<thead>
<tr>
<th>Week 1</th>
<th>Before EEJ*</th>
<th>After EEJ*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Grade 2</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Grade 3</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Grade 4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Grade 5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Week 2</th>
<th>Before EEJ*</th>
<th>After EEJ*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Grade 2</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Grade 3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Grade 4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Grade 5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Week 3</th>
<th>Before EEJ*</th>
<th>After EEJ*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Grade 2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Grade 3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Grade 4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Grade 5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

3.4. DISCUSSION

This study represents the first attempt at developing a method for collecting semen from chameleons. The results of this study also represent only the third attempt at electroejaculation in a species of lizard, and the second to perform serial electroejaculations in any reptile species (Mitchell et al., 2015). No deaths or serious adverse effects were observed during this study. Suggesting, serial electroejaculation and anesthesia can be performed safely to collect semen samples from veiled chameleons. The results of this study also suggest that the dogma that chameleons are fragile and unable to handle stress associated with serial handling or anesthetic events is not true.

Electroejaculation has been used successfully to collect semen from a number of reptiles; however, we still do not have a complete understanding of how it works in these animals (Juri et al., 2018; Juvik et al., 1991; Mitchell et al., 2015; Quinn et al., 1989; Tanasanti et al., 2009; Zimmerman and Mitchell, 2017; Zimmerman et al., 2013). In mammals, electroejaculation is thought to work by stimulating the prostate, although no experimental studies exist proving this theory in mammals. A recent experimental study utilizing a porcine model, lesioned the nervous

<table>
<thead>
<tr>
<th>(table cont’d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before EEJ</td>
</tr>
<tr>
<td>Week 4</td>
</tr>
<tr>
<td>Grade 1</td>
</tr>
<tr>
<td>Grade 2</td>
</tr>
<tr>
<td>Grade 3</td>
</tr>
<tr>
<td>Grade 4</td>
</tr>
<tr>
<td>Grade 5</td>
</tr>
</tbody>
</table>
system both pharmacologically and manually to evaluate the nervous systems influence on electroejaculation, this study suggests that stimulation of the pelvic musculature contractions may be the primary stimulus for ejaculation, utilizing electroejaculation (Groh et al., 2018). Reptiles do not have traditional accessory sex glands. This impacts ejaculate volume, with reptiles producing very small volumes without any seminal plasma. Testicular positioning is also different between mammals and reptiles, with reptile testicles located intracoelomic. More specifically, chameleon testicles lie cranial and ventral to the kidneys. The epididymides arise along the cranial pole of the testicles and course caudal to the ductus deferens, terminating in the ampulla leading into the urodeum. Semen travels through within the urodeum and into the sulcus spermaticus when intromission is about to occur. We suspect the nervous innervation to the male chameleon reproductive tract is similar to other reptiles and mammals, and that nervous stimulation or smooth muscles stimulation in the male reproductive tract, produce the ejaculate, however more studies need to be performed to determine the underlying mechanisms of electroejaculation in reptiles.

Changes observed with the complete blood count, including an increase in the total white blood cell count, absolute heterophil count, and absolute monocyte count, suggest that mild inflammation may be associated with electroejaculation in chameleons. The blood findings correlate with the mild changes noted on cloacoscopy over the course of the study. While significant, the changes noted in the complete blood count remain within the reference standards for this species (Romano et al., 2013). Additional blood samples collected in between the serial electroejaculations, or at some point after the study (i.e., 2-4 weeks after the last collection), would have provided additional insight into the degree of inflammation associated with the
procedure. A significant reduction in the AST activity was seen in these animals, which was not expected. In reptiles, AST activity has been associated with both skeletal and smooth muscle cell leakage, and it would be expected to increase in this study after the electroejaculation procedures (Bakst et al., 2006; Bogan et al., 2014; Wagner and Wetzel, 1999). An increase in creatinine kinase (CK) would have also been expected because of its association with muscle too (Bakst et al., 2006; Bogan et al., 2014; Wagner and Wetzel, 1999). The absence of these changes suggest that electroejaculation causes limited cellular damage in veiled chameleons. A mild elevation in albumin was also observed in the chameleons between the start and conclusion of the study. The increase in albumin was attributed to the overall improved condition of the animals noted over the course of the study because of the provision of standardized diet and husbandry. If the observed mild inflammatory response in the complete blood count was more severe, an albumin may have decreased and globulins may have increased.

External vent appearance did not change between the beginning and end of the study period, other than the initial change noted after the first electroejaculation procedure. In contrast, gross changes to the cloacal mucosa were observed before and after each electroejaculation procedure. The changes observed with the cloacal mucosa after each electroejaculation event could be attributed to either the repeated electric stimulus associated with the electroejaculation, the repeated penetration into the cloaca with the electroejaculation probe, or a combination of both. There was no significant difference in the cloacal scores between weeks 1 and 4; although the alpha did approach significance. Regardless, the changes do not appear to have caused any chronic or permanent disease and these chameleons were transferred to other research protocols with no ill effects.
Electroejaculation has been associated with side effects in mammals. Elevations in cortisol concentrations, rectal temperature, and heart, pulse and respiratory rates have been demonstrated in ruminants. Additionally, changes in hematological and biochemical parameters indicative of a stress response have been reported (Damián and Ungerfeld, 2011). However, this is not true in all cases, as ejaculation has been shown to not induce a stress response in sheep and it is the electrical impulses that cause the associated physiological changes (Abril-Sánchez et al., 2017). In rams (Ovis aries), electroejaculation was found to significantly increase heart rate and cortisol concentrations once the electricity was applied, while probe insertion was not associated with any significant change (Orihuela et al., 2009). In pampas deer (Ozotoceros bezoarticus), heart, pulse, and respiratory rates, as well as CK, were found to increase after electroejaculation. Unlike the ram study, the pampas deer study used general anesthesia, similar to this chameleon study. The authors suggested that the physiological changes observed in their study could be attributed to the capture and immobilization. In O. bezoarticus, the authors suggested that the procedure with anesthesia was stressful but it can be repeated at regular intervals without negative effects (Fumagalli et al., 2015; Fumagalli et al., 2012). Electroejaculation induces muscular contractions, and these electrical pulses alone, have been demonstrated to increase serum creatine kinase (CK) activity (Damián and Ungerfeld, 2011; Fumagalli et al., 2015; Fumagalli et al., 2012). In this chameleon study, no significant changes in CK was observed and AST activity decreased over the study period. However, this study only evaluated these enzymes at the beginning and end of the study, not after each procedure, and therefore only suggest there is no cumulative change. Further investigation is needed to evaluate this objectively in reptiles. Additionally, measuring corticosterone could be useful for determining whether a stress response

occurs with the procedure. The absence of any obvious clinical side effects, as well as the long-term survival of the chameleons, suggests that any stress developing from the procedure did not cause significant morbidity. In reptiles, only one report exists of complications associated with electroejaculation in the ploughshare tortoise. This animal died secondary to suspected renal failure months after the electroejaculation event (Burke et al., 1990; Juvik et al., 1991). The findings in the mammalian studies, and this study, suggest that electroejaculation is not an innocuous procedure, and that the animals should be monitored during the procedures for side effects. Ultimately, the risk of such a procedure needs to be weighed against the benefit for a particular species.

The authors elected to anesthetize the chameleons for the electroejaculation procedure because of the potential for pain and discomfort associated with the procedure. In conscious rams and bulls, vocalizations are associated with the electrical pulses from the electroejaculator probe, suggesting that the procedure is painful or uncomfortable (Damián and Ungerfeld, 2011; Falk et al., 2001; Mosure et al., 1998; Whitlock et al., 2012). Alfaxalone was selected as an anesthetic for the chameleons because of its availability, short duration of action, multiple routes of administration, previously documented safety in reptiles, and mechanism of action (Knotek et al., 2011). Additionally, alfaxalone was selected as a single agent and did not include any opioids because of potential side effects this class of drugs can have on spermatozoa (Agirregoitia et al., 2006; Xu et al., 2013). Propofol and isoflurane have also been used to anesthetize reptiles for electroejaculation procedures with excellent results (Mitchell et al., 2015; Zimmerman and Mitchell, 2017; Zimmerman et al., 2013). In reptiles, it is not known whether anesthesia and/or stress may affect semen quantity or quality. While the presence of GABA\textsubscript{A} receptors have not

been documented on reptile spermatozoa, it has been reported in humans and other species (Calogero et al., 1996; László and Wekerle, 1990). Thus anesthetics used for reptiles to perform electroejaculation could alter spermatozoa motility. Further work is needed to determine if anesthetics influence semen and ejaculate in reptiles.

There were several limitations to this study. First, electroejaculation was not performed without anesthesia. Based on the authors experience with these lizards, and other reptiles, the authors do not feel comfortable performing it without anesthesia. Utilization of lower electrical discharge or massage could be used to collect semen from reptiles. Second, further research needs to be performed to determine if these events are sufficiently stressful to consider their value. Logistically (financially) the study was restricted to evaluating complete blood counts and biochemistry parameters at the beginning and end of the study. Collecting and evaluating these parameters weekly would have been ideal. However, none of the animals experienced any ill effects from the procedures. Third, semen parameters are not reported from the semen samples collected in these animals, which was not the goal of this study. Finally, since the chameleons were wild caught and had parasite burdens, it was necessary to treat them for parasites. Anti-parasitics, such as fenbendazole, have been shown to decrease semen motility in the turkeys (Bakst et al., 2006). This effect could have biased results. To the authors’ knowledge, there are no studies evaluating the effect on semen characteristics of reptile species.

Semen collection success observed in this study utilizing electroejaculation was similar to previously published reports and experience by the authors (Mitchell et al., 2015; Zimmerman and Mitchell, 2017; Zimmerman et al., 2013). In 30 different species of reptiles, 22 lizards and 8 chelonians, semen was collected from the lizard species 45% (10/22) of the time while in
chelonians collection was observed in 62.5% (6/8) of collection attempts (Mitchell et al., 2015). Increased success was observed in green iguanas (*Iguana iguana*) during reproductive season where successful collection with electroejaculation was performed in 80% of the animals sampled (Zimmerman et al., 2013). In leopard tortoises (*Stigmochelys pardalis*), semen was collected in 81.2% (13/16) individuals during breeding season (Zimmerman and Mitchell, 2017). Successful semen collection is likely associated with the reproductive season which in captive or acclimated reptiles may be mediated by several environmental factors, including husbandry, environment, photoperiod, thermal gradient, social structure, stress, and diet. Further work needs to be completed evaluating the reproductive cycle of male reptiles like the veiled chameleons to fully understand and improve scientists and veterinarians ability to collect semen to further develop assisted reproductive technologies in reptiles.
CHAPTER 4. CHARACTERIZING THE ANNUAL REPRODUCTIVE CYCLES OF CAPTIVE MALE VEILED CHAMELEONS CHAMAЕLΕΟ CALΥPTRATUS AND PANTHER CHAMELEON FURCIFER PARDALIS.

4.1. INTRODUCTION

Biodiversity loss is the most critical environmental problem threatening ecosystem health and human well-being today (Ceballos et al., 2015). Humans are now inciting the sixth mass extinction through co-opting resources, fragmenting habitats, introducing non-native species, spreading pathogens, killing species directly, and changing global climate (Barnosky et al., 2011). Some say recovery of biodiversity will not occur on any timeframe meaningful to humans. Global climate change affects organisms in all ecosystems and may cause two natural compensatory responses, species adaptation or extinction. Climate change is thought to be a major contributing factor behind this increased extinction rate that has been demonstrated within most taxa in the animal kingdom (Thomas et al., 2004). These anthropogenic activities have led to a significant increase in extinction rates across all taxa and a reduction in reptile populations worldwide. Approximately 20% of reptile species are threatened, with some believing the rate at which reptiles are declining is similar to amphibians. Reptiles are particularly sensitive to habitat degradation because of their comparatively low dispersal rates, morphological specialization on substrate type, relatively small home ranges, and thermoregulatory constraints (Kearny et al., 2009).

As of January 2020, 202 species of chameleons are included on the IUCN Red List; 10 species are classified as critically endangered, 42 species as endangered, 22 species as vulnerable, and 39 species as near threatened. The remaining animals are either classified as least concern or there is insufficient data for them to be classified. Based on the IUCN data, more than half (55%, 113/202) of all known chameleon species are categorized as threatened (i.e.,

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critically endangered, endangered, or vulnerable) or near-threatened (Jenkins et al., 2014, IUCN, 2019). For comparison, the global average for lizard species that are similarly categorized is 23% (n= 867 species surveyed) (Böhm et al., 2013). These figures suggest that chameleons are at a much greater risk of extinction than other lizard species and that special attention should be provided to further assist in their conservation efforts. Many wild and captive populations of chameleons are not self-sustaining because of geographic isolation from potential mates or environments different from their native environment, resulting in abnormalities in physiologic and behavioral cues necessary to stimulate reproductive activity (Mattson et al., 2008; Mattson et al., 2007; Romero et al., 2005; Cote et al., 2010). Habitat fragmentation, habitat loss, and even the pet trade are considerable threats to sustaining in-situ chameleon populations. Since 1977, more than 1.37 million chameleons have been exported from their native habitats in Africa. Of all the chameleon species in the reptile trade, few are reliably produced in captivity in any significant quantity and there is a high mortality rate among wild caught imports (Carpenter, 2004). Over the past several decades, research focusing on improving habitats has been initiated to identify methods to assess reptile populations and protect these animals from being extirpated from their native range; however, in many cases the socio-economics of a region may limit the success of these programs. While it is ultimately desired to maintain wild populations of reptiles within their native ranges, it may not be possible in every case. When these limitations arise, it is imperative for veterinarians and herpetologists to develop programs that can be used in the long term to preserve the genetics of a species until improvements in its natural habitat can be made or to prevent a species extinction.

Fortunately, assisted reproductive technologies can be used to counter these threats and increase genetic diversity. The creation of a functional and sustainable reproductive assistance
program for chameleons could be an excellent pre-emptive method for strengthening conservation efforts for these unique evolutionary gems, alongside other methods such as habitat restoration and protection. Gamete collection and preservation, hormone analysis, and artificial insemination have all become integral components of in situ and ex situ conservation programs for threatened and endangered species of mammals, birds, amphibians, and fish; however, few studies have been conducted to investigate and characterize such technologies for reptiles. (Mattson et al., 2008; Mattson et al., 2007; Fahrig et al., 2007; Zacariotti et al., 2007; Zimmerman et al., 2013; Zimmerman et al., 2017; Browne et al., 2011; Martínez-Torres et al., 2019, Martínez-Torres et al., 2019; Perry et al., 2019; Johnston et al., 2014; Johnston et al., 2014).

In order to further develop and advance assisted reproductive technologies for chameleons, an understanding of their basic reproductive biology is necessary. Initial study of the female veiled chameleon (Chamaeleo calyptratus) reproductive cycle of a has been done (Kummerrow et al., 2010a, b, c); however, there has been no attempt to characterize the reproductive cycle of male chameleons. The purpose of this study was to characterize the annual reproductive cycles of two male chameleon species, the veiled chameleon and the panther chameleon (Furcifer pardalis), under captive conditions. The objective of this study was to measure plasma testosterone concentrations, testicular size, and semen parameters bi-monthly over the course of one year to determine if these parameters could be used to characterize the annual reproductive cycle of these animals under captive conditions. The hypotheses tested in this study were that an increase in plasma testosterone would either precede or would be associated with increased testicular size (e.g., length, width, height and volume) and semen
output/quality (e.g., electroejaculation success, ejaculate volume, ejaculate concentration, and spermatozoa morphology).

4.2. MATERIALS AND METHODS

This longitudinal, prospective study was performed in accordance with the rules and regulations established by Louisiana State University’s institutional animal care and use committee (protocol # 16-096). Sixteen panther chameleons and sixteen veiled chameleons were used for this study. The panther chameleons were born in captivity and acquired from a breeder (Panther-Ranch, Matiland, FL, USA), while the veiled chameleons were acquired from a reptile distributor (The Turtle Source, Ft. Meyers, FL, USA) that captured them in southern Florida. The sample size for this study was determined using the following a priori information: an alpha=0.05, a power=0.80, and a likelihood of being able to collect semen in at least 60% of the chameleons during the study. Animals were sampled bi-monthly (2 samples/month) over one year to characterize the annual plasma testosterone concentrations, testicular dimensions, and semen parameters under captive conditions.

Husbandry

Animals were individually housed at Louisiana State University (Baton Rouge, LA, 70802, USA) in 18 in x 18 in x 36 in (45.27 cm x 45.27 cm x 91.44 cm) screen cages (Zoo Med, Paso Robles, CA, 93446, USA). A MistKing misting system (Jungle Hobbies Ltd, Ontario N9E4R3, Canada) was incorporated into the caging and used to mist the animals for three minutes, three times a day. Animals were provided a 12-hour photoperiod. For veiled chameleons, the daytime ambient room temperature and a basking spot were maintained at 27.8-28.8°C (82-84°F) and 35-37.8°C (95-100°F), respectively, using central heating and incandescent lighting (Fluker Farms, Port Allen, LA, 70767, USA). Panther chameleons were
maintained at a daytime ambient room temperature of 27.8- 28.8°C (82- 84°F) and a basking spot of 32.2-35°C (90- 95°F) using central heat and incandescent lighting. Nighttime room temperature was 23.3-25.5°C (74- 78°F) for both species. Animals were exposed to ultraviolet lighting 12 hours per day (Reptisun 10.0 Fluorescent bulbs, Zoo Med). A combination of Repta-Vines (Fluker Farms) and treated sand blasted grape vines (Fluker Farms) were used to provide cover and climbing surfaces within the cages. Chameleons were fed a diet consisting of gut-loaded house crickets (*Acheta domestica*) and superworms (*Zophobas morio*). Insects were gut loaded 24 hours prior to being offered to the chameleons (SuperLoad, Repashy Ventures, Oceanside, CA, 92056, USA).

A physical examination was performed on each unsedated chameleon upon intake to confirm that they were healthy. Direct saline smears and fecal flotations using zinc sulfate were performed on both species. Veiled chameleons were found to harbor a significant number of a single undescribed species of flagellate, *Strongyloides* sp., and trematodes. Thus, all veiled chameleons were treated with fenbendazole (Panacur, Merck Animal Health, Madison, NJ, 07940, USA; 25 mg/kg p.o., q 24 h for 3 days), praziquantel (Praziquantel, Bimeda, Inc., Le Suer, MN, 05058, USA; 6 mg/kg i.m. every 7 days for 2 treatments), and metronidazole (compounded metronidazole 50 mg/ml, Louisiana State University, Baton Rouge, LA, 70803, USA; 20 mg/kg p.o., q 24 h for 3 days). Panther chameleons were found to have *Strongyloides* sp. and were treated with fenbendazole (25 mg/kg p.o., q 24 h for 3 days). Two consecutive negative fecals were needed prior to initiating the study. Both species were acclimated to captivity for one month prior to initiating the study.
Blood collection

Blood was collected bimonthly for one year to quantify plasma testosterone concentrations. The blood samples were collected under manual restraint from either the ventral coccygeal vein or jugular vein using a 25-gauge needle fastened to a 1 mL syringe. Blood samples were immediately placed into a lithium heparin microtainer (B-D Vacutainer Systems New Jersey 07471) after collection and centrifuged at $894 \text{ G}$ for 10 minutes. Plasma was aliquoted into cryovials and frozen at $-80^\circ \text{C}$ until being analyzed.

Semen collection/analyses and testicular measurements analysis

Semen collection and testicular measurements were performed bimonthly for one year. Chameleons were anesthetized for these procedures with alfaxalone (Alfaxan, Jurox Animal Health, North Kansas City, MO, 64116, USA) 10 mg/kg intravenously in the ventral tail vein (Perry et al., 2019). Heart and respiratory rates were measured throughout the procedure; the chameleons were found to spontaneously ventilate under this protocol, so no positive pressure ventilation was required. Animals were maintained on a warm water re-circulating blanket Gaymar TP 500 (Gaymar Industries, Orchard Park, NY, 14127, USA) set at $35^\circ \text{C}$ ($95^\circ \text{F}$) during the procedure.

Once anesthetized, the cloaca was cleaned with a kimwipe (Kimberly-Clark Professional, Corinth, MO, 63134, USA) to remove debris. An intermediate ColorpHast pH strip pH 5-10 (Millipore Sigma, St Louis, MO, 63103, USA) was inserted into the cloaca for one minute and read by a single individual (SP) to measure cloacal pH. Interpretation of the test strip was based on the chromogenic scale provided by the manufacturer. Next, each animal was electroejaculated using a variable amperage power source and a $360^\circ$ circumferential metallic
probe as previously described (Lierz et al., 2013; Perry et al., 2019). Each animal was
electroejaculated up to three times by performing 15 cloacal intromissions at 0.1 mAmps, 0.2
mAmps, and 0.3mAmps. A rest period of three minutes was provided in between each series of
intromissions. Electroejaculation was discontinued once a semen sample was collected.

The semen sample was collected from the cloaca using a single channel pipettor (2-20
μL) and placed into a 1.5 mL microcentrifuge tube. The volume was then quantified using either
a 0.5-10 μL or 2-20 μL single channel pipettor depending on the estimated sample volume.
Samples were evaluated for the presence or absence of semen by taking 2 μL of the collected
sample, placing it on a microscope slide with a coverslip, and reviewing it under light
microscopy (100-400x). Motility was evaluated by observing an average of five high powered
fields at 400x magnification. The sample on the microscope slide was then submerged in 0.1 mL
formal saline and added to the remaining neat sample of the ejaculate within the microcentrifuge
tube. A dilution was then subsequently calculated based on the volume of ejaculate and total
volume of formal saline it was suspended in. The spermatozoa concentration was measured using
a Makler sperm counting chamber (Sukcharoen et al.,1994; Wells et al., 2012). Once a
concentration was determined for the formal saline sample, the total number of spermatozoa was
determined by multiplying by the dilution factor. Additional data calculated with this final
spermatozoa number included total spermatozoa/ejaculate. Spermatozoa morphology was
determined by screening 100 spermatozoa (1,000X) stained with Eosin-Nigrosin stain (Björndahl
et al., 2003). The percentage of each spermatozoa morphological type was determined for each
semen sample as described by Barth et al., 1989. Live/Dead assessment using Eosin-Nigrosin
was not performed because the spermatozoa used for the motility assessment were suspended in
formal saline and killed.
Testicular size was measured following semen collection. A Sonoscape S8 Ultrasound (Sonoscape, Centennial, CO, 80112, USA) with a 10-15 mHz linear array hockey stick probe was used to measure the length, width, and height (mm) of the testicles. Length measured the distance between the cranial and caudal poles of the testicle. Width was collected in the same view, and measured the dorsal to ventral borders at the center of the testicle. The probe was then rotated 90° to obtain an orthogonal view and measure the height of the testicle. If the height of the testicle was difficult to appreciate, the width was used as an estimate (Figure 4.1). Testicular volume was estimated using the following equation \( V (\text{mm}^3) = 0.52 \times \text{LW}^2 \) (Watson-Whitmyre and Stetson 1985). Once completed, the chameleons were recovered in a Brinsea TLC 50 intensive care unit at a temperature of 31.6°C (89°F) (Brinsea, Titusville, FL, 32796, USA). The chameleons were returned to their enclosure once they had resumed their righting and escape reflexes.

Figure 4.1. A. Chameleons were placed in lateral recumbency for the ultrasound examination. B. Ultrasound image of a panther chameleon left testicle (see digital caliper marks outlining, 1. Testicular length: 10.30 mm, 2. Testicular width: 6.11 mm testicle).
Testosterone methods

Plasma testosterone concentrations were measured using a fluorescence enzyme immunoassay (FEIA; AIA 360 TOSOH BioScience Inc., South San Francisco, CA, 94080, USA). The FEIA was validated in-house for both species by comparing it with an enzyme immunoassay (EIA, Arbor assays, K032-H5, Ann Arbor, Michigan, 48108, USA), which was previously validated in veiled chameleons (Chapter 5). Bland-Altman plots and Passing-Bablok regression were used for the agreement analyses (unpublished data). The FEIA assay range was from 7-2200 ng/dL and assay sensitivity was 10 ng/dL. FEIA machine detection limit is 5 pg/mL. If a sample fell outside the region of linearity on the standard curve, it was diluted to a 1:4 or 1:8 dilution using reagents provided by the manufacturer until the samples read within linearity. Final testosterone concentration was based on a dilution conversion factor. The machine was calibrated based on the manufacturer’s standards to ensure accuracy and precision. Coefficient of variation upon calibration was determined to be <10%. Inter- and intra-assay variation was <10%.

4.2.1. STATISTICAL METHODS

SPSS 24.0 (IBM Statistics, Armonk, NY, 10504, USA) was used to analyze the data. A p<0.05 was used to determine significance for all statistical comparisons. The distributions of the continuous data (body weight, testicular measurements, plasma testosterone concentrations, ejaculate volume, spermatozoa concentration, and spermatozoa morphology) were evaluated using the Shapiro-Wilk test, skewness, kurtosis, and q-q plots. Normally distributed data are reported as the mean, standard deviation (SD), and minimum-maximum (min-max) values, while non-normally distributed data are reported as the median, 25-75 quartiles (%), and min-max values. The exception was for testosterone, which used standard error (SE) to define variability.
Non-normally distributed continuous data were log-transformed for parametric analysis. Dichotomous data, such as ejaculation success, are reported as frequencies. Chi-square tests were used to compare ejaculate collection success by month and semen collection success with number of intromissions performed. Mixed linear models were used to determine if the outcome variables for semen characteristics and testicle volume differed over time and by species. Study subject was used as the random variable in the model, while month and species of chameleon were included as the fixed variables. Akaike’s information criterion (AIC) was used to assess model fit. Bonferroni’s test was used to further characterize differences between predictors for each independent variable. Linear regression models were used to determine if there was a relationship between body weight, testosterone concentration, and ejaculate characteristics (ejaculate volume, spermatozoa concentration). Linear regression was also used to determine if there was a relationship between cloacal pH with spermatozoa motility and spermatozoa morphology. To evaluate the annual cycle of plasma testosterone concentrations, baseline concentrations of testosterone were determined through an iterative process described by Moreira et al 2001. Values in excess of two SD of baseline were removed from the dataset until no values exceeded 2 SD of the baseline mean. Mean baseline hormone concentrations are expressed as the mean ± standard error (SE) (Table 4.1).

Table 4.1. Baseline plasma testosterone concentrations for veiled chameleons (*Chamaeleo calyptratus*) and panther chameleons (*Furcifer pardalis*).

<table>
<thead>
<tr>
<th></th>
<th>Veiled chameleon</th>
<th>Panther chameleon</th>
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<tbody>
<tr>
<td>Baseline plasma testosterone concentrations</td>
<td>12.93 ng/mL</td>
<td>11.64 ng/mL</td>
</tr>
<tr>
<td>Standard error</td>
<td>1.24 ng/mL</td>
<td>1.54 ng/mL</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>5.28 ng/mL</td>
<td>6.57 ng/mL</td>
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</tbody>
</table>
Elevations in plasma testosterone were determined to be physiologically significant when a cluster of two or more values were found to be 1.5 SD above baseline; this was considered a hormonal peak and the highest value within the cluster was considered the maximum value of the peak. The authors elected to use +1.5 SD for characterizing an elevation in plasma testosterone rather than +2 SD because this data appeared to be more biologically relevant. A linear mixed model was used to determine if plasma testosterone concentrations differed over time or for species. Study subject served as the random variable, while month and species were the fixed variables. AIC and Bonferroni test were again used to assess model fit and further characterize differences by month, respectively.

4.3. RESULTS

Electroejaculation results

The results of the electroejaculation procedures with confirmed semen collection can be found for veiled and panther chameleons in Figures 4.2 and 4.3, respectively. A significant difference in semen collection was found by month for veiled chameleons ($X^2 = 47.38, \ df: 11, P<0.001$), with success being highest during May (94%; $p=0.007$). Semen collection was also high in April (61%; $p=0.294$) and June (57%; $p=0.52$), but these times were not significant. A significant difference in semen collection by month was also found for panther chameleons ($X^2 = 63.902, \ df: 11, P<0.001$), with success being highest in March (75%), April (85%), May (82%), and June (78%) (Figures 4.2 and 4.3).
Semen collection was found to differ significantly in veiled chameleons based on the number of electroejaculation series that had to be done ($X^2=76.793$, df:2, $P<0.001$), with semen samples being significantly ($p<0.001$) more likely to be collected after the third set of intromissions (56.9%, 45/79) than the first (29%, 23/79; $X^2=26.04$, df:1, $P<0.001$) or second (13.9%, 11/79; $X^2=9.65$, df:1, $P=0.002$) set of intromissions. There was also a significant difference in the number of electroejaculation series required to collect semen samples from panther chameleons ($X^2=53.680$, df:2, $P<0.001$); however, for this species, semen was significantly ($p<0.001$) more likely to be collected after the first set of intromissions (64.6%, 102/158) compared with the second (21.5 %, 34/158; $X^2=23.78$, df:1, $P<0.001$) or third series (13.9%, 22/158; $X^2=24.03$, df:1, $P<0.001$).
Figure 4.3. Plasma testosterone concentrations (Mean +/- S.E.M.) for the panther chameleon (*F. pardalis*) during the year (solid black line). Percentage electroejaculation success for each month (green bars). Baseline testosterone concentration (red dotted line).

**Cloacal pH**

The results for cloacal pH for can be found in Table 4.2 for both species. Cloacal pH was significantly different by species (F:10.733, df:1, P=0.03) and time (F:2.494, df:11, P=0.08), but not the interaction between species and time (F:1.032, df:10, P=0.423). Cloacal pH was significantly different by month for both veiled chameleons (F:2.951, df:11, P=0.02) and panther chameleons (F:3.867, df:11, P<0.001), with cloacal pH being lowest in April, May, August and September for veiled chameleons and May-September for panther chameleons (Table 4.2).
Table 4.2. Cloacal pH measurements of the veiled chameleons and panther chameleons over one year.

<table>
<thead>
<tr>
<th></th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
<th>Jun</th>
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<th>Sept</th>
<th>Oct</th>
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<tr>
<td><strong>Veiled Chameleon</strong></td>
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<tr>
<td>Median:</td>
<td>6.8</td>
<td>6.8</td>
<td>6.5</td>
<td>6.5</td>
<td>6.0</td>
<td>6.5</td>
<td>7.1</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
<td>6.8</td>
<td>6.5</td>
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<tr>
<td>25-75%:</td>
<td>6.5-7.47</td>
<td>6.5-6.9</td>
<td>6.5-6.8</td>
<td>6.5-6.57</td>
<td>6-6.66</td>
<td>6.5-7.4</td>
<td>6.5-7.5</td>
<td>6.5-6.8</td>
<td>6.5-6.87</td>
<td>6.5-6.95</td>
<td>6.5-7.1</td>
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<tr>
<td>Min-Max:</td>
<td>6.5-8.5</td>
<td>6.5-8.1</td>
<td>6.5-7.9</td>
<td>6.5-8.10</td>
<td>5-7</td>
<td>5-8.5</td>
<td>6-8.5</td>
<td>5-7.9</td>
<td>5.5-8.3</td>
<td>5-8.5</td>
<td>6.5-7.9</td>
<td>6.5-8.3</td>
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<td><strong>Panther Chameleon</strong></td>
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<tr>
<td>Median:</td>
<td>7.9</td>
<td>7.1</td>
<td>7.8</td>
<td>7.7</td>
<td>6.8</td>
<td>7.4</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
<td>7.25</td>
<td>7.9</td>
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<tr>
<td>25-75%:</td>
<td>7.1-8.3</td>
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<td>7.32-8.3</td>
<td>6.72-8.15</td>
<td>6.5-7.1</td>
<td>6-8.1</td>
<td>6.5-7.7</td>
<td>6.5-8</td>
<td>6.5-7.5</td>
<td>6.8-7.9</td>
<td>6.5-8.3</td>
<td>7.4-8.5</td>
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<td>6.5-8.7</td>
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<td>5-8.3</td>
<td>5.5-8.3</td>
<td>6-8.2</td>
<td>6-8.5</td>
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<td>6.5-8.7</td>
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</table>
Characterization of spermatozoa

Spermatozoa parameters, including ejaculate volume, spermatozoa concentration, motility, clumping frequency, and morphological characteristics, can be found for veiled and panther chameleons in Tables 4.3 and 4.4, respectively. Specific images of spermatozoa morphological changes can be found in Figures 4.4-4.9. Total ejaculate volume was significant over time (p=0.006, Df: 11, F:2.488) and with the interaction of species and time (p=0.027, Df: 11, F:2.083). Spermatozoa concentration was found to be significant over time (p=0.037, Df: 11, F:1.971), species (p=0.026, Df: 1, F:5.481), and with the interaction of species and time (p≤0.001, Df: 10, F:3.832). For both chameleon species, a significant difference was observed in motility over time (p=0.012, Df:11, F:2.278), but no significant difference was observed based on species (p=0.779 Df:1, F:0.080) or the interaction between species and time (p=0.717, Df:10, F:0.707) (Table 4.5). Normal morphology was found to be not different by time (p=0.420, Df:11, F:1.036), species (p=0.513, Df:1, F:0.438), or the interaction of species and time (p=0.542, Df:1, F:0.894). The appearance of proximal droplets was not different by time (p=0.454, Df:11, F:1.007), species (p=0.059, Df:1, F:3.860), or the interaction of species and time (p=0.705, Df:10, F:0.720). Distal droplets were found to be significantly different based on time (p=0.002, Df:11, F:2.838), but not species (p=0.265, Df:1, F1.291) or the interaction of species and time (p=0.760, Df:10, F:0.659). Bent tails were found to be significant based on time (p≤0.001, Df:11, F:3.671), but not species (p=0.392, Df:1, F:0.761) or the interaction of species and time (p=0.454, Df:10, F:1.003). Detached heads were not different by time (p=0.880, Df:11, F:0.532), species (p=0.862, Df:1, F:0.031), or the interaction of species and time (p=0.511, Df:10, F:0.926). Macrocephalic heads were not different by time (p=0.940, Df:11, F:0.433), species (p=0.840, Df:1, F:0.041), or the interaction of species and time (p=0.817, Df:10, F:0.595).
Table 4.3. Results for semen samples collected from captive veiled chameleons and panther chameleons over one year. Significance set at (P<0.05); *significant predictor: time, **significant predictor: species, *** significant predictor: species and time

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Veiled chameleons</th>
<th>Panther chameleon</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ejaculate volume (µL)</strong></td>
<td>Median: 2 µL</td>
<td>Median: 2.9µL</td>
</tr>
<tr>
<td>*</td>
<td>25-75&lt;sup&gt;th&lt;/sup&gt;: 1 - 6.25 µL</td>
<td>25-75&lt;sup&gt;th&lt;/sup&gt;: 2 - 8 µL</td>
</tr>
<tr>
<td>***</td>
<td>Min-Max: 1-81 µL</td>
<td>Min-Max: 0.5-350 µL</td>
</tr>
<tr>
<td><strong>Spermatozoa concentration</strong></td>
<td>Median: 6.66 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Median: 7.52 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>(spermatozoa/ml)</td>
<td>25-75&lt;sup&gt;th&lt;/sup&gt;: 2.31 x 10&lt;sup&gt;7&lt;/sup&gt; - 1.33 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>25-75&lt;sup&gt;th&lt;/sup&gt;: 3.33 x 10&lt;sup&gt;7&lt;/sup&gt; - 1.66 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>*</td>
<td>Min-Max: 1.12 x 10&lt;sup&gt;5&lt;/sup&gt; - 5.33 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>Min-Max: 1.75 x 10&lt;sup&gt;5&lt;/sup&gt; - 2.39 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
</tr>
<tr>
<td>**</td>
<td></td>
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</tr>
<tr>
<td><strong>Motility (%)</strong></td>
<td>Median: 23</td>
<td>Median: 25</td>
</tr>
<tr>
<td></td>
<td>25-75&lt;sup&gt;th&lt;/sup&gt;: 0-50</td>
<td>25-75&lt;sup&gt;th&lt;/sup&gt;: 0-50</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 0-100</td>
<td>Min-Max: 0-100</td>
</tr>
<tr>
<td><strong>Clumping (%)</strong></td>
<td>83.7% (77/91)</td>
<td>78.0 (128/164)</td>
</tr>
</tbody>
</table>

Testicular volume

In veiled chameleons, right testicular volume was significantly different by month (F:2.880, Df:11, p=0.002), with testicular volume being greatest in August (p=0.014), September (p=0.026), November (p=0.018), and December (p=0.033) compared with other months. There was no significant difference in left testicular volume by month (F:1.791, Df: 11, p=0.061). In the panther chameleon, left testicular volume was significantly different by month (F:3.900, Df:11, P<0.001), with left testicular size being greatest in April (p=0.001), May (p=0.003), June
Table 4.4. Spermatozoa morphology characterized for both the veiled chameleon (*C. calyptratus*) and panther chameleon (*F. pardalis*). Significance set at (P<0.05); *significant predictor: time, **significant predictor: species, *** significant predictor: species and time

<table>
<thead>
<tr>
<th>Spermatozoa morphology:</th>
<th>C. calyptratus</th>
<th>F. pardalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (%)</td>
<td>Mean: 56.5</td>
<td>Mean: 55</td>
</tr>
<tr>
<td></td>
<td>SD: 17.79</td>
<td>SD: 21.05</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 13-95</td>
<td>Min-Max: 1-99</td>
</tr>
<tr>
<td>Proximal droplet (%)</td>
<td>Median: 0</td>
<td>Median: 0</td>
</tr>
<tr>
<td></td>
<td>25-75\textsuperscript{th}: 0-0</td>
<td>25-75\textsuperscript{th}: 0-0</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 0-2</td>
<td>Min-Max: 0-40</td>
</tr>
<tr>
<td>Distal droplet (%)</td>
<td>Median: 30</td>
<td>Median: 32</td>
</tr>
<tr>
<td>*</td>
<td>25-75\textsuperscript{th}: 0-44.5</td>
<td>25-75\textsuperscript{th}: 15-44</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 0-76</td>
<td>Min-Max: 0-83</td>
</tr>
<tr>
<td>Bent tails (%)</td>
<td>Median: 11</td>
<td>Median: 6</td>
</tr>
<tr>
<td>*</td>
<td>25-75\textsuperscript{th}: 1.5-22</td>
<td>25-75\textsuperscript{th}: 0-16</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 0-64</td>
<td>Min-Max: 0-98</td>
</tr>
<tr>
<td>Macrocephalic (%)</td>
<td>Median: 0</td>
<td>Median: 0</td>
</tr>
<tr>
<td></td>
<td>25-75\textsuperscript{th}: 0-0</td>
<td>25-75\textsuperscript{th}: 0-0</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 0-48</td>
<td>Min-Max: 0-81</td>
</tr>
<tr>
<td>Detached head (%)</td>
<td>Median: 0</td>
<td>Median: 0</td>
</tr>
<tr>
<td></td>
<td>25-75\textsuperscript{th}: 0-0</td>
<td>25-75\textsuperscript{th}: 0-0</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 0-9</td>
<td>Min-Max: 0-15</td>
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</tbody>
</table>
Table 4.5. Median spermatozoa motility per month for each chameleon species.

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<tr>
<th></th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
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<th>July</th>
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<th>Sept</th>
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<th>Dec</th>
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<tbody>
<tr>
<td><strong>Veiled Chameleon</strong></td>
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<tr>
<td>Median motility %:</td>
<td>15</td>
<td>30</td>
<td>10</td>
<td>25</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>10</td>
<td>0</td>
<td>40</td>
<td>50</td>
<td>42.5</td>
</tr>
<tr>
<td>25-75%:</td>
<td>0-65</td>
<td>0-60</td>
<td>0-45</td>
<td>0-72.5</td>
<td>0-40</td>
<td>0-30</td>
<td>0-20</td>
<td>0-27.5</td>
<td>0-13.75</td>
<td>5-80</td>
<td>21.25-82.5</td>
<td>5-85</td>
</tr>
<tr>
<td>Min-Max:</td>
<td>0-85</td>
<td>0-80</td>
<td>0-70</td>
<td>0-100</td>
<td>0-90</td>
<td>0-95</td>
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<td>0-95</td>
<td>0-95</td>
<td>15-90</td>
<td>0-90</td>
</tr>
<tr>
<td><strong>Panther Chameleon</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Median motility %</td>
<td>40</td>
<td>30</td>
<td>10</td>
<td>20</td>
<td>10</td>
<td>15</td>
<td>5</td>
<td>7.5</td>
<td>0</td>
<td>27.5</td>
<td>50</td>
<td>55</td>
</tr>
<tr>
<td>25-75%:</td>
<td>5-78.75</td>
<td>5-58.75</td>
<td>2.5-32.5</td>
<td>0-70</td>
<td>0-30</td>
<td>3.75-33.75</td>
<td>0-20</td>
<td>3.75-36.25</td>
<td>0-17.5</td>
<td>3.75-60</td>
<td>21.25-82.5</td>
<td>15-85</td>
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<tr>
<td>Min-Max:</td>
<td>0-85</td>
<td>0-70</td>
<td>0-60</td>
<td>0-100</td>
<td>0-80</td>
<td>0-95</td>
<td>0-40</td>
<td>0-95</td>
<td>0-95</td>
<td>0-90</td>
<td>15-90</td>
<td>5-90</td>
</tr>
</tbody>
</table>
Figure 4.4. Two panther chameleon (*F. pardalis*) spermatozoa at 200x with phase contrast, one is normal (N) in morphology and the other has a distal droplet present (DD).

Figure 4.5. Panther chameleon (*F. pardalis*) spermatozoa with scanning electron microscopy with a 2µm filter at 6,500x magnification. A: Acrosome, H: Head, M: Midpiece.

Figure 4.6. Panther chameleon (*F. pardalis*) spermatozoa morphologic abnormalities observed with eosin-nigrosin stained samples at 1,000x. Macrocephalic head (M), Kinked tails (KT), Distal Droplet (DD), and spermatozoa clump (C).
**Clumping**

Images of semen clumping can be found in Figures 4.7 and 4.8.

---

Figure 4.7. Spermatozoa clump under light microscopy (40x) observed following electroejaculation in both chameleon species *C. calyptratus* and *F. pardalis*.

---

Figure 4.8. Panther chameleon (*F. pardalis*) spermatozoa clump evaluated with scanning electron microscopy A. Clump image with 10 µm filter at 1,000x magnification B. Clump image with 5 µm filter at 3,000x magnification C. Clump image with 5 µm filter at 5,000x magnification.

(p<0.044), and August (p=0.015). Right testicular volume was also found to be significantly higher by month (F:3.218, Df:11, p<0.001), with right testicle volume being greatest in February (p=0.015), March (p=0.013), April (p=0.015), May(p=0.026), June (p=0.014), and July(p=0.026) (Figures 4.9 and 4.10; Table 4.6 and 4.7).
**Plasma testosterone concentrations**

Plasma testosterone concentrations were analyzed on an individual, bi-weekly, and monthly basis, and based on the trends it was decided to use the monthly data for reporting. A significant difference was observed between species (F:10.163, dF:1, p=0.02) and the interaction of species and month (F:2.571, dF:11, p=0.04), indicating two separate reproductive cycles between species. In veiled chameleons, plasma testosterone concentrations were significantly different by month (F=2.318, dF: 11, p=0.010), and plasma testosterone concentrations were closest to baseline during the months of July (p=.891) and August (p=0.123). Plasma testosterone was significantly elevated during the months of January (p=0.017), February (p=0.020), March (p=0.017), April (p=0.050), October (p=0.002), November (p=0.028), and December (p=0.008) (Figure 4.3). May is when this decline was first appreciated, and September is when the rise in testosterone begins for the next cycle (Figure 4.3). In panther chameleons, plasma testosterone concentrations were also significantly different by month (F=1.939, dF: 11, p=0.039). Plasma testosterone concentrations were closest to baseline during the months of January (p=0.518), February (p=0.509), March (p=0.580), April (p=0.933), and May (p=0.475) with concentrations being the highest in July (p=0.029), August (p=0.05), September (p=0.007), and December (p=0.046) compared with the other months (Table 4.8). June is when an increase is first appreciated, and October is when the decline in testosterone begins; a subsequent increase in December is also noted before decreasing again in January.
Table 4.6. Testicular volume calculated based on the equation $V = 0.52 \times LW^2$ for the veiled chameleon (*C. calyptratus*) over one year.

<table>
<thead>
<tr>
<th></th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
<th>Jun</th>
<th>July</th>
<th>Aug</th>
<th>Sept</th>
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<th>Dec</th>
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<tbody>
<tr>
<td><strong>Left</strong></td>
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</tr>
<tr>
<td>Median: (mm$^3$)</td>
<td>165.08</td>
<td>140.75</td>
<td>164.93</td>
<td>164.62</td>
<td>161.62</td>
<td>181.29</td>
<td>141.26</td>
<td>129.19</td>
<td>158.12</td>
<td>150.39</td>
<td>103.12</td>
<td>142.16</td>
</tr>
<tr>
<td>25-75%:</td>
<td>130.0-191.15</td>
<td>116.43-211.44</td>
<td>120.38-201.72</td>
<td>124.29-216.46</td>
<td>133.01-292.22</td>
<td>91.15-192.74</td>
<td>77.98-193.07</td>
<td>88.96-196.89</td>
<td>101.82-189.84</td>
<td>60.34-180.73</td>
<td>100.26-160.94</td>
<td></td>
</tr>
<tr>
<td>Min-Max:</td>
<td>101.22-368.09</td>
<td>85.94-342.89</td>
<td>89.27-312.31</td>
<td>54.84-483.94</td>
<td>51.40-472.46</td>
<td>85.31-510.01</td>
<td>66.82-631.72</td>
<td>36.07-271.16</td>
<td>39.70-259.98</td>
<td>34.53-272.32</td>
<td>26.21-258.05</td>
<td>75.86-410.05</td>
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<tr>
<td><strong>Right</strong></td>
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<td></td>
</tr>
<tr>
<td>Median: (mm$^3$)</td>
<td>183.11</td>
<td>151.71</td>
<td>175.14</td>
<td>135.51</td>
<td>207.48</td>
<td>158.38</td>
<td>146.46</td>
<td>95.31</td>
<td>107.21</td>
<td>130.49</td>
<td>113.06</td>
<td>143.54</td>
</tr>
<tr>
<td>25-75%:</td>
<td>154.87-221.30</td>
<td>113.46-240.24</td>
<td>119.70-194.10</td>
<td>156.56-277.91</td>
<td>127.01-219.17</td>
<td>96.15-189.32</td>
<td>70.93-165.73</td>
<td>81.30-161.64</td>
<td>107.23-211.07</td>
<td>63.30-158.86</td>
<td>97.47-177.19</td>
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</tr>
<tr>
<td>Min-Max:</td>
<td>46.17-323.57</td>
<td>78.02-542.80</td>
<td>65.12-464.20</td>
<td>39.0-404.20</td>
<td>65.56-631.14</td>
<td>66.30-440.38</td>
<td>49.67-425.84</td>
<td>36.63-263.82</td>
<td>38.33-307.04</td>
<td>28.17-326.62</td>
<td>40.08-231.74</td>
<td>56.73-241.48</td>
</tr>
</tbody>
</table>
Table 4.7: Testicular volume calculated based on the equation \( V = 0.52 \, LW^2 \) for the panther chameleon (*F. pardalis*) over one year.

<table>
<thead>
<tr>
<th><em>F. pardalis</em></th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
<th>Jun</th>
<th>July</th>
<th>Aug</th>
<th>Sept</th>
<th>Oct</th>
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</tr>
<tr>
<td>Median:</td>
<td>137.40</td>
<td>141.26</td>
<td>180.38</td>
<td>183.25</td>
<td>156.06</td>
<td>158.38</td>
<td>134.51</td>
<td>169.05</td>
<td>149.34</td>
<td>104.73</td>
<td>113.87</td>
<td>100.79</td>
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<td>(mm³)</td>
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</tr>
<tr>
<td>Min-Max:</td>
<td>52.26-265.12</td>
<td>47.74-318.63</td>
<td>29.91-343.70</td>
<td>39.00-404.20</td>
<td>44.71-499.65</td>
<td>49.10-273.87</td>
<td>58.40-304.46</td>
<td>71.69-336.73</td>
<td>70.85-255.29</td>
<td>16.14-205.14</td>
<td>9.24-224.62</td>
<td>26.85-341.27</td>
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<tr>
<td><strong>Right</strong></td>
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<tr>
<td>Median:</td>
<td>106.26</td>
<td>168.03</td>
<td>151.31</td>
<td>163.03</td>
<td>142.99</td>
<td>168.37</td>
<td>169.12</td>
<td>142.26</td>
<td>141.57</td>
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<tr>
<td>25-75%:</td>
<td>89.59-154.09</td>
<td>99.55-212.87</td>
<td>106.07-252.00</td>
<td>113.07-233.61</td>
<td>104.81-216.43</td>
<td>130.86-252.54</td>
<td>112.88-234.80</td>
<td>114.29-213.37</td>
<td>96.18-191.09</td>
<td>78.57-191.09</td>
<td>49.14-126.97</td>
<td>57.13-147.52</td>
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<tr>
<td>Min-Max:</td>
<td>42.32-206.36</td>
<td>60.25-468.97</td>
<td>67.55-322.93</td>
<td>42.32-311.77</td>
<td>79.09-341.66</td>
<td>40.81-332.93</td>
<td>53.43-334.89</td>
<td>55.63-384.12</td>
<td>70.70-309.19</td>
<td>18.89-330.59</td>
<td>60.69-233.41</td>
<td>24.97-318.03</td>
</tr>
</tbody>
</table>
Figure 4.9. Graphs demonstrating veiled chameleon (*F. pardalis*) estimated left and right testicular volume (mean +/- S.D.) based on the equation for an ellipsoid \( V=0.52LW^2 \). Volume is reported as \( \text{mm}^3 \).
Figure 4.10. Graphs demonstrating panther chameleon (*C. calyptratus*) estimated left and right testicular volume (mean +/- S.D.) based on the equation for an ellipsoid (*V*=0.52*L*W²). Volume is reported as mm³.
4.4. DISCUSSION

This study was the first to characterize the annual reproductive cycles of captive male veiled and panther chameleons. The findings of this study confirm the complexity of these animals and reinforce that reptiles, like mammals, require a multi-step approach to characterizing their reproductive cycle. In this study, changes in plasma testosterone, ultrasound-measured testicle volume, and the success in collecting and the quality of semen samples were used to characterize the annual reproductive cycles in these animals.

Plasma testosterone concentrations for both species were found to increase during defined months of the year, suggesting that both species are seasonal in nature, even under static captive environmental cues. In the veiled chameleon, plasma testosterone concentrations increased 1.5 times from baseline during the months of January through June and October through December. In Yemen, the wet season is typically between April and September, while the dry season occurs between October through March (Buhairi et al., 2010). In the panther chameleons, plasma testosterone concentrations were elevated 1.5 times above baseline during the months of February, June thru September, and December. In Madagascar, the wet season is typically from November through March, while the dry season occurs from April through October. The elevations in testosterone noted for both species coincide with their natural respective dry seasons. While the reproductive cycles of wild chameleons are not known, James and Shine (1985) reported that semi-arid species of Australian lizards were more likely to reproduce in the dry season vs. the wet season, while mesic-adapted species reproduced during the wet season. From the authors’ experience, there can be a great deal of variation with this, as even some tropical mesic-adapted species of lizards, such as the green iguana (*Iguana iguana*), reproduce in the dry season vs. the wet season. Ultimately, objective, evidence-based data should
Table 4.8. Mean plasma testosterone concentrations based on month. Bolded values are statistically significant.

<table>
<thead>
<tr>
<th></th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
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<tr>
<td><strong>Veiled Chameleon</strong></td>
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<tr>
<td>S.E.:</td>
<td>5.13</td>
<td>6.02</td>
<td>4.71</td>
<td>3.79</td>
<td>2.35</td>
<td>3.35</td>
<td>2.34</td>
<td>1.28</td>
<td>2.38</td>
<td>4.61</td>
<td>3.33</td>
<td>6.13</td>
</tr>
<tr>
<td>Min-Max:</td>
<td>5.18-84.43</td>
<td>3.15-108.87</td>
<td>2.73-80.54</td>
<td>7.05-64.88</td>
<td>1.14-44.52</td>
<td>0.85-51.46</td>
<td>1.21-52.20</td>
<td>1.19-28.98</td>
<td>4.39-53.03</td>
<td>6.15-70.25</td>
<td>7.03-55.06</td>
<td>5.57-117.89</td>
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<td><strong>Panther Chameleon</strong></td>
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<tr>
<td>Mean plasma testosterone (ng/ml):</td>
<td>14.82</td>
<td>19.58</td>
<td>17.06</td>
<td>14.27</td>
<td>17.10</td>
<td>21.56</td>
<td>30.98</td>
<td>18.83</td>
<td>42.89</td>
<td>11.57</td>
<td>13.44</td>
<td>28.38</td>
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<tr>
<td>S.E.:</td>
<td>2.29</td>
<td>3.81</td>
<td>2.78</td>
<td>2.58</td>
<td>3.68</td>
<td>5.43</td>
<td>5.85</td>
<td>2.97</td>
<td>10.26</td>
<td>2.23</td>
<td>1.64</td>
<td>8.51</td>
</tr>
<tr>
<td>Min-Max:</td>
<td>0.33-34.13</td>
<td>0.90-73.35</td>
<td>0.29-45.63</td>
<td>0.47-35.62</td>
<td>1.27-54.96</td>
<td>1.26-79.08</td>
<td>2.07-74.44</td>
<td>3.82-73.23</td>
<td>8.52-160.66</td>
<td>0.73-37.35</td>
<td>0.61-25.27</td>
<td>1.86-146.53</td>
</tr>
</tbody>
</table>
be used to determine whether a species reproduces seasonally or not. The plasma testosterone results of this study confirm that both of these species of chameleons are seasonal breeders, and that their seasons coincide with their native dry seasons. These findings should be used when developing assisted reproduction programs for these animals. It is important to note that reptiles are ectotherms and that many of their reproductive behaviors and cyclicity are tied to environmental cues (Krohmer and Luitterschmidt, 2011; Vivien-Roels et al., 1988; Rawding Hutchison, 1992; Tilden and Hutchison 1993; Moyer et al., 1995; Garcia-Allegue et al., 2001).

This study demonstrated that electroejaculation success is seasonal in seasonal reptile species, and that electroejaculation success varied based on month. In veiled chameleons, semen collection was highest in April, May, and June, while in panther chameleons semen collection success was most successful March, April, May, and June. Based on these findings and the testosterone concentrations, veiled chameleons appear to follow a prenuptial strategy. The testosterone concentrations were elevated over a prolonged period, prior to peak semen production. In this species, spermatogenesis likely peaks during the months of February and March when testosterone is highest. One of the challenges with confirming this is the variability in sample collection using electroejaculation. Because we don’t fully understand the physiologic methods behind electroejaculation in reptiles, we may be underestimating sample success. Panther chameleons appear to follow a post-nuptial reproductive strategy. Semen collection was highest well-after the apparent peak in testosterone. The elevated testosterone concentration noted in December does appear out of place, but this may be associated with the lack of seasonal change inherent to our “captive” study design.

This study was the first to confirm that electroejaculation can be used to collect serial semen samples over the course of a year in lizards. In previous studies, electroejaculation was
only performed at a single time point (Zimmerman et al., 2013, Mitchell et al., 2015, López Juri et al., 2018, Martínez-Torres et al., 2019), except for a single-study evaluating serial weekly sampling over a month in veiled chameleons (Perry et al., 2019). Previous authors have suggested that electroejaculation might be harmful to reptiles, citing the death of a ploughshare tortoise (Astrochelys yniphora) (Juvik et al, 1991; Zimmerman et al., 2017). There have been no mortalities reported in any of the other publications to date (Zimmerman et al., 2013, 2017; Perry et al., 2019, Mitchell et al., 2015, López Juri et al., 2018, Martínez-Torres et al., 2019), but there was a single mortality associated with this procedure in the current study. A panther chameleon did not recover from the anesthetic event after its tenth month of sample collection. A necropsy was performed on this individual and it was determined to have concurrent renal disease. The previous concern with the ploughshare tortoise case was that the electroejaculation procedure may have induced renal disease. It is not possible to confirm whether these mortalities were ultimately associated with the electroejaculation procedure, but it is important to monitor for potential complications.

While electroejaculation is routinely performed on some species (e.g., cattle) without anesthesia, the authors believe that the discomfort associated with the procedure should be controlled using appropriate sedation or anesthesia. Previous studies have used various anesthetics to collect semen from reptiles, including propofol, isoflurane, alfaxalone, ketamine/dexmedetomidine/midazolam combination, and sodium pentobarbital (Zimmerman et al., 2013, 2017; Perry et al., 2019; Martínez Torres et al., 2019). The authors elected to reduce the alfaxalone dose used in the original pilot study (Perry et al., 2019) from 15 mg/kg to 10 mg/kg intravenously to decrease the duration of anesthesia. When compiling the results of this study with that previous study (Perry et al., 2019), a total of 536 anesthetic events were
completed. With the single death noted, the authors experienced a 0.19% mortality rate with these two species of chameleons. Compared to other elective anesthetic mortality rates for dogs (0.05%), cats (0.11%), and humans (0.17%), our findings appear appropriate (Matthews et al., 2017; Heeney et al., 2014). López-Juri et al. (2018) recently published a study where they performed electroejaculation without anesthesia. While those authors did not report any side effects with their study subjects, the discomfort that the authors of the current study have seen in this and other studies were categorized as “Type D-Pain or distress will be relieved by appropriate therapy” at our institution. Therefore, the authors suggest that sedation or anesthesia be used when performing electroejaculation on a species of reptile.

Semen sample collection was found to vary month-to-month, with samples collected every month in both species. When comparing the findings of this study to the only other study in veiled chameleons, there was a difference noted in sample collection success during the same month (January). In the previous study (Perry et al., 2019), semen collection success was 50%, while in the current study it was 30%. This difference was most likely associated with the duration the animals were in a captive setting. Animals in Perry et al. 2019 were only housed for one month prior to initiating the study, while animals in the current study were used from that study then serially introduced into the study based on animal availability. The highest success rates for semen collection using electroejaculation in the veiled and panther chameleons were 88% and 82%, respectively. These findings are similar to those reported for other species that were electroejaculated during their respective breeding seasons, including green iguanas (89%, 16/18), Chaco spiny lizards (*Tropidurus spinulosus*) (80%, 12/15), and Texas rock lizards (*Sceleporus torquatus*) (94%, 16/17) (Zimmerman et al., 2013; López-Juri et al., 2018; Martínez-Torres et al., 2019).
Spermatozoa concentration for both chameleon species appears to be similar to other small lizard species, but much smaller than a larger lizard. In green iguanas, spermatozoa concentrations were reported to be 258.0 x 10^6 spermatozoa/mL to 1,030.2 x 10^6 spermatozoa/mL, which is much more concentrated. Chaco spiny lizards were found to produce an average of 2.1 x10^6 spermatozoa/mL and the Texas rock lizards 17-340 x 10^6 spermatozoa/mL, which is similar to what is reported in our study (Zimmerman et al., 2013; López- Juri et al., 2018; Martínez-Torres et al., 2019). As described previously, spermatozoa clumping was common in these samples and likely confounded the data by underestimating the spermatozoa concentration and spermatozoa/ejaculate. Additionally, these samples had to be screened first on a microscope slide to confirm they were an ejaculate, and then be aspirated from the slide to increase recovery and complete the counts. This procedural step likely led to a decreased sample volume available for analysis using the Makler counting chamber. Again, this would be expected to further underestimate sample counts. Ejaculate volumes in our study were lower than what is reported in the above-mentioned studies, despite the fact that both the Chaco spiny lizards and Texas Rock lizards are smaller than both of the chameleons. We suspect this finding could be due to methodology, as these studies used capillary tubes and one of the studies flushed the cloaca to recover spermatozoa following electroejaculation (Zimmerman et al., 2013; López- Juri et al., 2018; Martínez-Torres et al., 2019).

While there has been success with collecting semen using electroejaculation, there remains questions whether it is a best practice, or whether other techniques (e.g., manual stripping) can produce more consistent samples. Ultimately, to develop consistent assisted reproduction methods for semen collection, it is important to identify those predictors that can help with decision making. The results of this study suggest that season alone may not be enough
of a predictor; however, semen collection success is likely linked to the appearance of the epididymis rather than testicular size and volume. When using electroejaculation for semen collection, it is important to consider the number of times the procedure will be applied for a specific sampling period. The methods used here mimic the techniques originally described for green iguanas (Zimmerman et al., 2013). There appears to be some degree of variance between species. In this study, veiled chameleons were primarily collected during the third series of intromissions (56%), while panther chameleons were primarily collected after the first series of intromissions (65%).

In Chaco spiny lizards, 2.9 stimulation cycles were needed, on average, to collect semen, while in green iguanas samples were primarily collected during the second set (62.5%) of electroejaculations (Martínez-Torres et al., 2019; Zimmerman et al., 2013). Based on the results of these studies, the authors suggest that programs should be developed that include performing three series of electroejaculations to maximize semen collection success, unless adverse effects are seen at the level of the cloaca. The only study evaluating potential adverse effects noted that mild changes may occur, but that the changes were transient (Perry et al., 2019).

The male reptile cloaca is a three-chambered organ that receives fecal material from the colon, urine from the ureters, and semen from the ductus deferens. As one might expect, the microbiome and pH of this chamber may vary based on what is being excreted and mixed within it, and how these factors affect spermatozoa is not well understood. The results of this study suggest that cloacal pH is associated with the increased likelihood of distal droplet formation in the spermatozoa of both veiled and panther chameleons. The pH in the cloaca of both species varied over the course of the year, with veiled chameleons typically having an acidic cloacal pH and panther chameleons a neutral cloacal pH. The authors are unsure of the reason for this
difference, as both species were fed the same diet. pH test strips were used to measure the pH, so the sensitivity of the strips may account for this difference. These distal droplets are often referred to as physiologic in bulls and are found in spermatozoa as they mature in the epididymis, ultimately being lost when the spermatozoa are ejaculated (Cooper, 2011). The distal droplets were the most common non-normal spermatozoa finding in the chameleons and may be associated with the spermatozoa being discharged from the epididymides with electroejaculation. This finding may also be preliminary evidence that the microenvironment within the cloaca is important for the final maturation of spermatozoa and that pH may influence the shedding of the distal droplet. Intracellular and environmental pH has been demonstrated to influence spermatozoa motility in invertebrates, fish, birds, amphibians, and multiple mammalian species (e.g., bovine, swine, equine, and humans) (Carr and Acott, 1989; Babcock et al., 1983; Lee et al., 1983, Alavi and Cosson, 2005; Zhou et al., 2015, Holm and Wisehart, 1998; Browne et al., 2015). A pH shift has also been suggested as possibly being important for inducing spermatozoa motility in reptiles (Sirinarumitr et al., 2010). Additional study into the potential impacts of cloacal pH and the cloacal microbiome on reptile spermatozoa are needed.

This is the first study to characterize spermatozoa clumping in electroejaculated semen samples from reptiles. Clumping was observed in a majority of the ejaculates collected from veiled (77%) and panther chameleons (78%). In these cases, the sperm form a mesh-like aggregation (Figures 4.7 and 4.8). Microscopic analysis, including scanning electron microscopy, demonstrated that some material is connecting the spermatozoa. Other studies evaluating spermatozoa in reptiles immediately placed the semen samples in an extender (e.g., F-10 or F-10 with human serum albumin) prior to evaluation, rather than evaluating the sample raw samples (Zimmerman et al., 2013, 2017; López Juri et al., 2018; Martinez-Torres et al., 2019),
and didn’t report such a finding. The authors have now observed this phenomenon in samples collected from both electroejaculation and epididymal collection in several species. Clumping likely confounds the spermatozoa concentration data, and thus the data presented here is likely an underestimation of true counts because we were unable to dissolve the clumps. At this time, we have not found a way to dissolve the clump to release the spermatozoa. To date, we have used pipetting, vortexing, F-10, bovine serum albumin, and acetylcysteine to dissolve the clumps, all without success. Pre-copulatory ejaculation has been documented in the marine iguana (*Amblyrhynchus cristatus*) as a reproductive strategy to reduce copulation time in lekking subordinate males (Wikelski and Baurle, 1996). Spermatozoa clumping may be a reproductive strategy to enhance a male’s ability to copulate because, in conjunction with pre-copulatory ejaculation, may allow for reduced copulation time and increased success. These strategies may be in response to alternative mating strategies in reptiles such as lekking and “rock, paper, scissor” systems (Wikelski and Baurle, 1996; Sinervo and Lively, 1996). Further research is needed to characterize the material in the clump and determine if it is necessary or an artifact.

Semen morphology of chameleons is similar to other lizard species, including green iguanas, Chaco spiny lizards, Texas rock lizards, and *Cyclura lewisi x nubila* hybrids, all of which maintain a filiform like structure (Zimmerman et al., 2013; López-Juri et al., 2018; Martínez-Torres et al., 2019). In both veiled and panther chameleons, a true primary spermatozoa defect, a macrocephalic head, was detected; the remaining spermatozoa defects appeared to be secondary in nature. The most common defect observed was distal droplets. Distal droplet shedding is one of the final stages of spermatozoa maturation and indicates that the proportion of the sample originated from the distal epididymis.
Spermatozoa motility was highly varied in both species. There was a significant
difference in spermatozoa motility in the panther chameleons over time, with the highest
motilities observed in November (median: 51%) and December (median 55%), and the lowest
motilities observed in July (median: 5%), August (median 7.5%) and September (median 0%). In
veiled chameleons spermatozoa motility was highest in October (median: 40%), November
(median: 50%) and December (median 42.5%), and the lowest motilities were observed in June
(median: 10%), July (median: 5%), August (median 10%) and September (median 0%). It is
generally thought that motility must be over 50% for successful insemination, although previous
work in corn snakes (*Pantherophis guttatus*) and checkered garter snakes (*Thamnophis
carcianus*) found these species could be successfully inseminated with 30% and 50%,
respectively (Mattson et al. 2007; Quinn et al.,1989). The poor median motility noted in this
study was thought to be confounded by the clumping observed in many of the samples.
Additionally, in the other reptile studies that reported higher motilities, extenders were used for
sample preparation and could have influenced the initial motilities because of the available
substrates (e.g., glucose, calcium, bicarbonate). Some authors believe that reptile spermatozoa in
an ejaculate are not motile until they are exposed to some induction agent (Sirinarumitr et al.,
2010). This could also be why we observed low motilities in these chameleons and why higher
average motilities have been observed in other studies. Chameleons live in isolation, except for
breeding. Thus, it is possible that they have developed specific adaptions to their spermatozoa to
offset the potential length of time associated with spermatozoa development and female
encounters, similar to the alternative mating strategy observed in marine iguana. Depending on
species, spermatozoa motility may also differ based on the semen collection method used. In
human males with a spinal cord injury, sample quality was better when semen was collected with
a vibrational method rather than electroejaculation (Bracket et al., 1997); whereas in bulls, semen collected via electroejaculation was found to be comparable to samples collected with an artificial vagina (Austin et al., 1961). More research is needed to elucidate the importance of collection method on sample quality in reptiles.

This was the first study to measure testicular size in a species of reptile over time using a non-invasive method. Changes in testicular size have been reported in other species based on season, including green anoles, Nerodia spp., and cottonmouths (Agkistrodon piscivorus) (Gribbins and Gist 2003, Gribbins et al., 2003, 2005, 2006, 2008, 2009; Rhuebert et al., 2009, 201; Gribbins and Rheubert, 2011); however, these studies were terminal and only one measurement was collected from each individual. The hypothesis for this part of the study was that changes in testicular size would occur over time because of the suspected seasonal spermatogenesis. We elected to use an equation that estimates testicular volume based on the volume of an ellipsoid (Watson-Whitmyre and Stetson, 1985) because it was found to be an accurate method for three different species of hamsters that are similar in weight to the chameleons (Watson-Whitmyre and Stetson, 1985). In addition, this equation only required two measurements to estimate volume. Other equations may be better suited for post-mortem sampling, where the testicle can be removed and measured; however, when performing non-invasive ultrasound, the two-dimensional image provided in a single frame only allows two forms of measurement. Another image is required for a third dimensional measurement. The authors were concerned that collecting a second image could result in too much variability between the measurements. Further study validating different equations to determine if there is a better estimator for testicular size, volume, and weight would be ideal; however, surgical
castration, terminal studies, or computed tomography (3-dimensional imaging) would be needed to validate the equations.

There were significant differences in testicular volume in the panther chameleons over time in both testicles. In the veiled chameleon, no significant difference in testicular volume over time was observed for the left testicle, however, a difference was observed for the right testicle. Small differences in testicular height could highly influence volume due to the proportion of height that contributes to the equation since the value is squared \((V=0.52 \text{ LH}^2)\). Although we did not consistently see the same pattern in the left or right testicle in the veiled chameleon, we were able to see changes in testicular volume and thus non-invasive estimations may still be useful. Measurement error could have occurred with the ultrasound unit and caliper placement based on the small size (mm) of the measurements; however, to limit potential bias all measurements were collected by the same individual (SMP).

There were several limitations to this study. Both these species are ectotherms and originate from areas that experience wet and dry seasons. In this study, the husbandry conditions did not fluctuate over the course of the study. Because temperature and photoperiod did not fluctuate, it is possible that certain cues required to stimulate the synthesis of testosterone or spermatogenesis were not available, or mixed signals were sent. Induction of testicular recrudescence may not have been appropriately stimulated secondary to no fluctuation in photoperiod; testosterone production may have not been appropriately signaled or the signal attenuated. Melatonin production is directly influenced by environmental cues such as photoperiod and temperature. Plasma melatonin concentrations are modulated by a number of extrinsic cues, including photoperiod phase and duration, light intensity, and temperature.
In cold diamondback water snakes (*Nerodia rhombifer*), cold and warm temperature extremes decreased the amplitude of the melatonin cycle (Krohmer and Luitterschmidt, 2011; Tilden and Hutchison, 1993). This pattern has been observed in multiple reptile species, including three toed box turtles (*Terrapene carolina*), marbled geckos (*Christinus marmoratus*), and green anoles (Krohmer and Luitterschmidt, 2011; Vivien-Roels et al., 1988; Rawding Hutchison, 1992; Tilden and Hutchison 1993; Moyer et al., 1995; Garcia-Allegue et al., 2001). In both green anoles and Western fence lizards, removal of the pineal gland blocked reproductive responsiveness to photoperiod (Underwood, 1981a; Underwood, 1985 a,b), which allowed the animals to become reproductively active in the non-breeding season when exposed to inhibitory photoperiods (Underwood 1985a; Lutterschmidt et al. 2004). Clear evidence exists in reptiles that the environment influences endogenous neuro-endocrine response. Due to the numerous environmental factors important to reptiles in captivity (e.g., photoperiod, temperature, humidity), it is important to consider these variables when designing a study. The fixed environmental factors in this study may have influenced some of the results and explain some of the variability noted. While the static husbandry may have impacted the results of this study, it is common for many captive reptiles to be held under these types of fixed conditions in captivity. The purpose of this study was to determine how these static conditions impacted the reproductive functions of these animal, with the intent that follow-up studies could then evaluate specific changes (e.g., photoperiod, humidity, temperature) to the environment to assess their impact on their reproductive cycle. Ultimately, we need to develop “best practices” for each species so that we can develop species focused assisted reproductive programs.
CHAPTER 5. EFFECTS OF EXOGENOUS hCG ADMINISTRATION ON PLASMA TESTOSTERONE AND SEMEN PRODUCTION IN THE VEILED CHAMELEON CHAMAeleO CalypTratus

5.1. INTRODUCTION

As of January 2020, 202 species of chameleons are included on the IUCN Red List; 10 species are classified as critically endangered, 42 species as endangered, 22 species as vulnerable, and 39 species as near threatened. The remaining animals are either classified as least concern or there is insufficient data for them to be classified. Based on the IUCN data, more than half (55%, 113/202) of all known chameleon species are categorized as threatened (i.e., critically endangered, endangered, or vulnerable) or near-threatened (Jenkins et al., 2014, IUCN, 2019). For comparison, the global average for lizard species that are similarly categorized is 23% (n= 867 species surveyed) (Böhm et al., 2013). These figures suggest that chameleons are at a much greater risk of extinction compared to other lizard species and that it is essential that we develop conservation plans to preserve these evolutionary gems.

Many wild and captive populations of chameleons are not self-sustaining because of geographic isolation from potential mates or inadequate environmental conditions, resulting in abnormalities in physiologic and behavioral cues necessary to stimulate reproductive activity (Jenkins et al., 2014; Romero and Reed, 2005; Cote et al., 2010). Habitat fragmentation, habitat loss, and the pet trade represent considerable threats to sustaining in-situ chameleon populations. Since 1977, more than 1.37 million chameleons have been exported from their native habitats in Africa. Of all the chameleon species in the reptile trade, few are reliably produced in captivity in any significant quantity, and there is a high mortality rate among wild caught imports. (Jenkins et al., 2014; Carpenter, 2004).
Fortunately, assisted reproductive technologies can be developed to counter these threats and increase genetic diversity. The creation of a functional and sustainable reproductive assistance program for chameleons would be an excellent pre-emptive method for strengthening conservation efforts for these unique animals, alongside other methods such as habitat restoration and protection. Gamete collection and preservation, hormone analysis, and artificial insemination have all become integral components of *in situ* and *ex situ* conservation programs for threatened and endangered species of mammals, birds, amphibians, and fish; however, few studies have been conducted to investigate and characterize such technologies for reptiles (Clulow and Clulow, 2016). For chameleons, this work is limited to semen (male gamete) collection. The authors have found that electroejaculation can be used to safely collect serial semen samples from veiled chameleons (*Chamaeleo calyptratus*) and panther chameleons (*Furcifer pardalis*); however, the success with sample collection can vary (Perry et al., 2019; Chapter 4). In fish, amphibians, birds, and mammals, semen collection can be improved by collecting the samples during the breeding season. In lower vertebrates (fish and amphibians), hormonal stimulation can also aid in semen collection success irrespective of season (Viveiros et al., 2002; Saad and Billard, 1987; Kouba et al., 2012). Unfortunately, breeding seasons for chameleons are not well-documented, and captive conditions can further alter the reproductive cycle of chameleons because of variable temperature and photoperiod. Thus, the use of exogenous hormones to control the reproductive cycle of a male chameleon should be evaluated to further develop an assisted reproductive program for these animals.

Exogenous hormonal stimulation has been evaluated in male lizards from the following species: little brown skink (*Leiolopisma laterale*), common agama (*Agama agama*), green anole (*Anolis carolinensis*), common wall lizard (*Lacerta muralis*), broadhead skink (*Eumeces laticeps*),
Indian spiny-tailed lizard (*Uromastix harkwickii*), Indian wall lizards (*Hemidactylus flaviviridis*), and oriental garden lizard (*Calotes versicolor*). The exogenous hormones used in these studies were ovine luteinizing hormone (LH), human chorionic gonadotropin (hCG), ovine follicle stimulating hormone (FSH), FSH and LH, porcine FSH, ovine prolactin, hCG, and equine LH and pregnant mare serum gonadotropin (PMSG) (Arslan et al., 1977; Edwards et al., 2004; Eyeson, 1971; Haider et al., 1987; Jadhav and Padgaonkar, 2010; Jalali et al., 1976; Jones 1973; Lance et al., 1985; Licht 1969,1971; Prasad and Sanyakl,1969; Reddy and Prasad, 1970a, 1970b; Sonar and Patil 1994; Vijaykumar et al., 2002). In squamates, a one gonadotroph, two-cell theory is accepted with the primary gonadotroph being FSH-like. Although hCG demonstrates LH activity at the level of the testicle in mammals, conflicting studies exist in reptiles on the gonadal and endocrine responses to hCG. In the common agama, hCG (100 IU/animal) did not have an effect on testicular size, but PMSG did (Eyeson, 1971). Unfortunately, testosterone was not measured in the study to assess the effect of hCG and PMSG on testosterone concentrations. In the New Guinea bockadam (*Cerberus rynchops*), hCG (50 IU/day) over 14 and 28 days did not impact the on seminiferous tubules; however, histologic changes with the epididymis were observed, despite these animals being out of season (Jadhav and Padgaonkar, 2010). In the little brown skink, changes were observed in the testicle following the administration of hCG, including an increased interstitial cell number, stimulated interstitial cell hypertrophy, cytoplasmic granulation, and increased epididymidal and sexual segment epithelial height. In the oriental garden lizard, hCG administration during the non-breeding season resulted in an increased weight and diameter of the testis. The seminiferous tubular diameter was also increased and the tubules were filled with spermatogenic elements like spermatogonia, primary and secondary spermatocytes, and spermatids. These results suggest that mammalian
nonpituitary gonadotrophins like hCG also possess the ability to stimulate spermatogenesis in the reptilian testis (Sonar and Patil, 1994). The results of these studies suggest that the delivery of exogenous hormones could be used to control male chameleon reproduction.

The purpose of this study was to determine if an exogenous hormone, hCG, could be used to impact testosterone concentrations and semen collection in a model species of chameleon. Our first objective was to determine an effective dose of hCG that could be used to increase plasma testosterone concentrations in the veiled chameleon. Our second objective was to determine if serial injections of hCG over a single month could be used to increase the success of collecting a semen sample. The hypotheses being tested in this study were that: 1) the administration of exogenous hCG would increase circulating testosterone concentrations within 24 hours of administration, 2) repeated injections of hCG would maintain elevated testosterone concentrations, 3) repeated injections of hCG would increase testicular size, and 4) that repeated injections of hCG would increase the likelihood of collecting a semen sample.

5.2. MATERIALS AND METHODS

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 # 17-061).

Study species

Twenty-four wild caught veiled chameleons were used for this study. The chameleons were collected from an invasive population reproducing in southern Florida. To determine an appropriate hCG dose, eleven individuals were enrolled for the first experiment. The sample size for this study was determined using the following a priori information: an alpha=0.05, a
power=0.80, and an estimated 50% increase in testosterone concentrations following administration of hCG. To evaluate semen production following serial administration of hCG in the second experiment, we used thirteen individuals. The sample size for this experiment was based on the following a priori analysis: an alpha=0.05, a power=0.80, and a 90% likelihood of collecting semen following serial hCG administration compared to a 10% likelihood without hCG (baseline).

**Husbandry**

Animals were individually housed at Louisiana State University in 18 in x 18 in x 36 in (45.27 cm x 45.27 cm x 91.44 cm) screen cages (Zoo Med, Paso Robles, CA, USA). A MistKing misting system (Jungle Hobbies Ltd, Ontario, Canada) was incorporated into the caging and used to mist the animals for three minutes, three times a day. Daytime ambient room temperature and a basking spot were maintained at 27.8-28.8°C (82-84°F) and 35-37.8°C (95-100°F) using central heating and incandescent lighting (Fluker Farms, Port Allen, LA, USA), respectively. Animals were exposed to ultraviolet lighting 12 hours per day (Reptisun 10.0 Fluorescent bulbs, Zoo Med). Nighttime temperature was based on the ambient room temperature (27.8-28.8°C, 82-84°F). A combination of Repta-Vines (Fluker Farms) and treated sand blasted grape vines (Fluker Farms) were used to provide cover and climbing surfaces within the cages. Chameleons were fed a diet consisting of gut-loaded house crickets (*Acheta domestica*) and superworms (*Zophobas morio*). Insects were gut loaded 24 hours prior to being offered to the chameleons with a gutload diet (SuperLoad, Repashy Ventures, Oceanside, CA, USA).

A physical examination was performed on each chameleon under manual restraint upon intake to confirm that they were healthy. Fecal samples were collected and screened for parasites using direct saline smears and fecal flotations using zinc sulfate. *Strongyloides* sp. were
identified on the fecals; thus, all chameleons were treated with fenbendazole (Panacur, Merck Animal Health, Madison, NJ, USA; 25 mg/kg per os, once daily for 3 days) prior to initiating the study. The chameleons were acclimated to their captive setting for one month prior to initiating the study.

*Dose determination study* (Experiment 1):

A prospective complete crossover study was performed to determine an effective hCG dose for increasing plasma testosterone concentrations in veiled chameleons. The study was conducted during the months of February and March, 2018, because it represented the transition period from non-breeding to breeding season for this species based on previous work by the authors (Chapter 4), where the gonads are more likely to be responsive to exogenous hormone administration based on seasonal influences. Eleven veiled chameleons were randomly divided into three treatment groups using a random number generator (random.org). The doses of hCG (Human chorionic gonadotropin, lyophilized powder, 2,500 IU, Sigma Aldrich, C1063, St Louis, MO, USA) selected for testing were 100IU, 200IU, and 300IU; these hCG doses were based on previous work done with amphibians and reptiles (Eyeson, 1971, Kouba, 2012). The hCG was reconstituted with 5 mL of 0.9% sodium chloride to 500 IU/ml and each treatment animal administered the appropriate dose subcutaneously in the right cranial epaxial region. The order of dosing was as follows: Group 1 (n=3): 100 IU, 200 IU and 300 IU; Group 2 (n=4): 200 IU, 300 IU, and 100 IU; and Group 3 (n=3): 300 IU, 100 IU and 200 IU. A two-week washout period was provided between treatments and was done because of concerns for a priming effect to the testicles that could impact the response of subsequent injections. Whole blood was collected from each individual prior to the hCG injection (time 0, baseline) and then 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 12 hours and 24 hours after the injection.
Blood was collected from either the ventral coccygeal vein or jugular vein using a 25-gauge needle fastened to a 1 mL syringe. A total of 0.2 mL was sampled at each timepoint, which ensured that the total blood volume collected was <1.0% body weight (all chameleons >160 grams). Blood samples were placed into lithium heparin microtainers (B-D Vacutainer Systems NJ, USA) and separated into components using centrifugation at 894 G for 10 minutes. Components were aliquoted into cryovials and frozen at -80°C until being analyzed for plasma testosterone.

Repeated hCG injections influence on semen parameters (Experiment 2):

A prospective complete crossover design was performed to determine if serial hCG injections could be used to increase plasma testosterone concentrations, testicular volume, and semen production (electroejaculation success, ejaculate volume, spermatozoa motility, and morphology). This study was completed during the months of May, June, July, and August, 2019. These dates were selected because they too represented the transition period to the non-breeding season for this species based on previous work by the authors (Chapter 4). Thirteen chameleons were randomly assigned to a treatment group and a control group using a random number generator (random.org). Animals were administered 0.2 mL of 100 IU hCG
subcutaneously weekly using the methods described previously; this dose was based on the findings of the first experiment. Control animals were administered 0.2 mL 0.9% sodium chloride (Baxter Healthcare Corporation, Deerfield, IL, USA). Following an initial 4-week treatment period, animals had a one-month washout period prior to switching the treatment and control groups. Whole blood was collected as described previously from each individual prior to any injection at day 0, day 15, and day 30 of the experimental trial.

Figure 5.2. Experimental design for experiment 2 to determine the influence of weekly administration of hCG 100 IU/animal on plasma testosterone concentration and ejaculate parameters.

*Electroejaculation/Semen collection/Semen analysis:*

Semen was collected using electroejaculation prior to (time 0) and following the series of four injections of saline or hCG (day 30). Animals were sedated for each electroejaculation procedure with alfaxalone 10 mg/kg intravenously in the ventral tail vein. Heart and respiratory rates were monitored throughout the procedure, and the chameleons spontaneously ventilated throughout the procedure. Once anesthetized, the cloaca was cleaned with a kimwipe (Kimberly-Clark Professional, Corinth, MO, 63134, USA) to remove debris. While anesthetized, each
animal was electroejaculated using a variable amperage power source and a 360° circumferential metallic probe (Perry et al., 2019). This bipolar probe consists of a metallic portion measuring 20 mm in length and 3 mm in diameter and a non-conducting plastic with two electrical contacts at the sides. The electroejaculation probe was fully inserted into the cloaca and light dorsal pressure applied to ensure the nerves of the pelvic plexus were engaged. The variable amperage power source was discharged for a single intromission at this point. The metallic portion of the probe was then slowly retracted from the cloaca, while the power source was discharged. This cycle was considered one intromission. Each animal was electroejaculated by performing 15 cloacal intromissions at 0.1 mAmperes for a single series. If semen was not collected, the amperage was increased to 0.15 mAmperes for an additional series. If no semen was observed after a second series, then the amperage was increased to 0.2 mAmperes for a third and final series. A rest period of 3 minutes was provided in between each series of intromissions. Electroejaculation was discontinued once a semen sample was collected. Semen samples were collected from the cloaca utilizing a single channel pipettor (2-20 µL) and placed into a 1.5 mL microcentrifuge tube. The volume was then quantified using either a 0.5-10 µL or 2-20 µL single channel pipettor, depending on the estimated sample size.

Samples were evaluated for the presence or absence of semen by placing 2 µL of the sample on a microscope slide with a coverslip and reviewing it under light microscopy (100x and 400x). Motility was measured by counting an average of 5 fields at 400x magnification. The sample remaining on the microscope slide and coverslip were then aspirated with 0.1 mL formal saline into a 1 mL syringe to recover the remaining spermatozoa for evaluation. This 0.1 mL of formal saline was added to the remaining neat ejaculate sample. Sperm concentration sample collected in formal saline was determined with a Makler chamber (Sukcharoen et al., 1994; Walls
et al., 2012). Once a concentration was measured, the total number of spermatozoa was determined by multiplying by the dilution factor. Additional data calculated with this final spermatozoa number included total spermatozoa/ejaculate.

**Testicular measurement:**

Testicular measurements were made using the Sonoscape S8 (Sonoscape, Centennial, CO, 80112, USA) with the 10-15 mHz linear array hockey stick probe as previously described (Chapter 4). Testicular measurements, including length, width, and height, were collected on Days 0 and 30 at the time of each electroejaculation event. The distance from the cranial to the caudal pole of the testicle represented length. Width was measured in the same view and included the distance between the medial and lateral borders of the testicle at its midpoint. The probe was then rotated 90° in order to obtain an orthogonal view to measure testicular at its dorsal and ventral borders. If the height of the testicle was difficult to appreciate, the width was used as an estimate. Following the testicular measurements, testicular volume was estimated using the following equation $V (\text{mm}^3) = 0.52 \times LW^2$ (Watson-Whitmyre and Stetson, 1985).

**Testosterone assay**

An enzyme immunoassay kit (EIA) (Arbor Assay DetectX Testosterone K032-H5, Ann Arbor, MI, USA) was used to measure circulating testosterone concentrations in the plasma. First, the EIA kit was optimized for this species. The published sensitivity for this assay was reported to be 9.92 pg/mL with a limit of detection at 30.6 pg/mL. Plasma testosterone was extracted using a liquid extraction method based on the manufacturer’s protocol. Plasma samples (100µL) were aliquoted into microcentrifuge tubes, 400µL of ethyl-acetate (Sigma Aldrich, St. Louis, MO, USA) was 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 pipetted 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. Samples were diluted 1:20 with the assay buffer based on the parallelism 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 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 and then allowed to dry. Next, substrate was added to the wells and allowed to incubate for 30 minutes 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. Based on the spiked sample, extraction efficiency from the neat samples was calculated to be 75%.

*Parallelism*

Parallel displacement between the standard curve and serial dilutions of plasma extracts were used to determine immunological similarities between the standard and sample hormones. A pooled sample of plasma extracts was serially diluted from 1:2 to 1:2048 in assay buffer and run with the standard curve. Parallel displacement of the curve was observed with the serial dilution. Sample pool displacement of the label hormone paralleled that of the assay standard based on nonlinear regression ($r^2 = 0.99; P < 0.0001$). The ideal dilution with approximately 50% binding was 1:16; based on this finding, dilutions were done at 1:20.
**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. Values with CV >10% were re-analyzed, while samples with CV <10% were considered data. Inter-assay CV were measured by re-analyzing samples, in duplicate, with 30, 50, and 70% binding. To evaluate interference of the components within the extract with antibody binding, recovery of a known amount of hormone was measured for the assay. Pooled samples of plasma extracts were diluted as previously described. Diluted pooled samples were assayed alone to determine endogenous hormone concentrations. The percent recovery was calculated using the following formula: (amount observed/amount expected) x 100%, where the amount observed is the value obtained in the spiked sample and the amount expected is the calculated amount of standard hormone added plus the amount of endogenous hormone in the unspiked sample. A linear regression showed a significant direct relationship (P<0.001, $R^2=0.9899$; $y=1.0163x+47.916$).

![Plasma testosterone recovery](image)

**Figure 5.3. Plasma testosterone recovery.**
5.2.1. STATISTICAL METHODS

Continuous data (testosterone concentrations, testicular measurements, testicular volume, spermatozoa concentration, ejaculate concentration, and motility) were evaluated for normality using the Shapiro-Wilk test, skewness, kurtosis, and q-q plots. Normally distributed data are reported by the mean, standard deviation (SD), and minimum-maximum (min-max) values, while non-normally distributed data are reported as the median, 25-75 quartiles (%), and min-max values. Non-normally distributed data were log transformed prior to analysis. A mixed linear model was used to determine whether the three different hCG doses had an impact on testosterone concentrations. Chameleon was used as the random variable in the model, while dose, collection time, and order were the fixed factors. Akaike’s information criterion (AIC) was used to assess model fit. Least significant differences (LSD) test was used to further characterize differences between predictors for each independent variable. A related samples Cochran’s Q test was used to determine if semen collection differed over time. Linear mixed models were also used to determine if serial injections of hCG or saline had an impact on plasma testosterone, semen parameters (ejaculate volume, spermatozoa concentration, ejaculate concentration, motility), and testicular volume. Chameleon was used as the random variable in the model, while treatment, order, and time were the fixed factors for plasma testosterone. Chameleon was used as the random variable in the model, while treatment and time, were the fixed factors for semen parameters, cloacal pH, and testicular volume. Akaike’s information criterion (AIC) was used to assess model fit. SPSS 24.0 (IBM Statistics, Armonk, NY, USA) was used to analyze the data. A P<0.05 was used to determine statistical significance.
5.3. RESULTS

*Dose determination study*

Plasma testosterone concentrations based on dose and time can be found in Table 5.1 and Figure 5.4.

Table 5.1. Median plasma testosterone concentrations over 24 hours following injection of hCG at 100 IU, 200 IU, and 300 IU.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Prior to injection (ng/ml)</th>
<th>30 mins (ng/ml)</th>
<th>1 hrs (ng/ml)</th>
<th>2 hrs (ng/ml)</th>
<th>4 hrs (ng/ml)</th>
<th>8 hrs (ng/ml)</th>
<th>12 hrs (ng/ml)</th>
<th>24 hrs (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 IU</td>
<td>p=0.690</td>
<td>102.02</td>
<td>105.43</td>
<td>143.53</td>
<td>130.52</td>
<td>184.29</td>
<td>175.99</td>
<td>159.70</td>
</tr>
<tr>
<td></td>
<td>25-75%: 41.77-194.85</td>
<td>54.64-227.12</td>
<td>69.49-180.06</td>
<td>81.99-252.02</td>
<td>125.49-212.52</td>
<td>105.42-210.82</td>
<td>111.31-245.84</td>
<td></td>
</tr>
<tr>
<td>200 IU</td>
<td>p=0.033</td>
<td>109.31</td>
<td>116.36</td>
<td>160.59</td>
<td>163.12</td>
<td>143.81</td>
<td>161.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25-75%: 34.70-140.88</td>
<td>100.6-153.46</td>
<td>95.46-129.39</td>
<td>110.91-202.85</td>
<td>118.90-177.31</td>
<td>123.16-241.60</td>
<td>130.71-255.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Min-Max: 8.05-323.00</td>
<td>25.38-354.72</td>
<td>45.99-354.13</td>
<td>64.67-244.41</td>
<td>92.58-333.99</td>
<td>65.89-298.37</td>
<td>28.91-421.70</td>
<td></td>
</tr>
<tr>
<td>300 IU</td>
<td>p=0.002</td>
<td>128.87</td>
<td>146.69</td>
<td>138.38</td>
<td>186.57</td>
<td>173.52</td>
<td>186.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25-75%: 50.53-162.20</td>
<td>117.71-194.23</td>
<td>104.18-231.23</td>
<td>121.60-244.26</td>
<td>134.48-230.76</td>
<td>136.67-244.90</td>
<td>123.78-230.76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Min-Max: 28.23-366.46</td>
<td>44.02-335.32</td>
<td>55.08-465.72</td>
<td>66.37-439.78</td>
<td>87.09-264.90</td>
<td>96.06-434.64</td>
<td>91.07-296.31</td>
<td></td>
</tr>
</tbody>
</table>
There was no significant difference in testosterone concentrations based on hCG dose (AIC: 2,568, degrees of freedom (F:0.08, P=0.927). However, there was a significant difference (F: 9.21, P=0.001) in testosterone concentrations observed over time. Pairwise comparisons showed that testosterone concentrations increased significantly one hour after hCG administration (P=0.033) and remained elevated for 24 hours compared to baseline concentrations (P<0.001) (Table 1).

No interaction between treatment and time was observed (F=0.642, P=0.842). There was also a significant difference (F:4.44, P=0.021) in testosterone concentrations observed based on the order of the hCG injections. Pairwise comparisons showed no difference in testosterone concentrations between the first and second injections (P=0.098) or the second and third injections (P=0.235); however, a difference was noted between the first and third injections (P=0.006).
Repeated hCG injections influence on semen parameters

Plasma testosterone concentrations for chameleons receiving serial hCG or saline injections are presented in Table 5.2 and Figure 5.3.

Table 5.2. Plasma testosterone concentrations at days 0, day 15 and day 30 following a series of injections

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 15</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline injections (ng/ml)</td>
<td>Mean: 17.78</td>
<td>Median: 8.87</td>
<td>Mean: 16.61</td>
</tr>
<tr>
<td></td>
<td>SD: 20.37</td>
<td>25th-75th: 5.27-31.95</td>
<td>SD: 10.6</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 0.78-68.11</td>
<td>Min-Max: 0.35-57.6</td>
<td>Min-Max: 5.37-38.49</td>
</tr>
<tr>
<td>hCG injections  (ng/ml)</td>
<td>Mean: 15.77</td>
<td>Mean: 127.27</td>
<td>Mean: 115.76</td>
</tr>
<tr>
<td></td>
<td>SD: 12.74</td>
<td>SD: 80.23</td>
<td>SD: 73.10</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 1.40-48.92</td>
<td>Min-Max: 15.68-259.03</td>
<td>Min-Max: 38.74-274.80</td>
</tr>
</tbody>
</table>

Figure 5.3. Graphical representation of plasma testosterone concentrations in chameleons treated with weekly injections of 100 IU hCG and saline controls over 30 days.
There was no significant difference in semen collection over time (P = 0.536). The number of semen samples collected from the chameleons before hCG administration was 52.2% (12/23), whereas the number of ejaculates collected from animals following hCG administration was 47.8% (11/20). The number of semen samples collected from the chameleons treated with saline was 45% (9/20), while the number of ejaculates collected from animals treated with hCG was 60% (14/23). There were significant differences in plasma testosterone concentrations by time (F: 14.06, P < 0.001), treatment (F: 15.22 P = 0.001), and the interaction of treatment and time (F: 19.82, P < 0.001). Testosterone concentrations were significantly (F: 31.27, P ≤ 0.001) higher in the chameleons receiving the hCG than saline at day 15 (p ≤ 0.001) and day 30 (P ≤ 0.001) following 100 IU/animal every 7 days (Table 5.2). Saline showed no effect on increasing plasma testosterone concentrations. There were no significant differences in total spermatozoa, ejaculate volume, spermatozoa concentration, or spermatozoa motility by treatment, time, or the interaction of these terms (Table 5.3, 5.4).

Table 5.3. Descriptive statistics for spermatozoa characteristics from ejaculates.

<table>
<thead>
<tr>
<th>Spermatozoa parameter</th>
<th>Saline injections</th>
<th>hCG injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total ejaculate volume (µL)</td>
<td>Median: 1.0</td>
<td>Median: 2.5</td>
</tr>
<tr>
<td></td>
<td>25-75th: 1-3.5</td>
<td>25-75th: 1-2.5</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 1-8</td>
<td>Min-Max: 0.5-10</td>
</tr>
<tr>
<td>Spermatozoa concentration (spermatozoa/ml)</td>
<td>Median: 1.01 x10^8</td>
<td>Median: 1.23 x10^8</td>
</tr>
<tr>
<td></td>
<td>25-75th: 3.53 x 10^7 - 1.20 x 10^8</td>
<td>25-75th: 6.40 x 10^7 - 5.29 x 10^9</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 3.33 x 10^7 - 2.02 x 10^8</td>
<td>Min-Max: 3.32 x 10^7 - 6.52 x 10^8</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>Median: 0</td>
<td>Median: 0</td>
</tr>
<tr>
<td></td>
<td>25-75th: 0-20</td>
<td>25-75th: 0-15</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 0-30</td>
<td>Min-Max: 0-60</td>
</tr>
</tbody>
</table>
Table 5.4. Mixed model results for ejaculate and semen parameters following serial hCG injections in veiled chameleons (* is indicative of significance).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Variable</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total spermatozoa</td>
<td>Treatment</td>
<td>2.22</td>
<td>0.171</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>2.17</td>
<td>0.205</td>
</tr>
<tr>
<td></td>
<td>Treatment x Time</td>
<td>0.35</td>
<td>0.574</td>
</tr>
<tr>
<td>Ejaculate volume</td>
<td>Treatment</td>
<td>0.33</td>
<td>0.596</td>
</tr>
<tr>
<td></td>
<td>Order</td>
<td>0.01</td>
<td>0.907</td>
</tr>
<tr>
<td></td>
<td>Treatment x Time</td>
<td>2.84</td>
<td>0.161</td>
</tr>
<tr>
<td>Spermatozoa concentration</td>
<td>Treatment</td>
<td>4.91</td>
<td>0.069</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>1.13</td>
<td>0.310</td>
</tr>
<tr>
<td></td>
<td>Treatment x Time</td>
<td>0.267</td>
<td>0.624</td>
</tr>
<tr>
<td>Spermatozoa motility</td>
<td>Treatment</td>
<td>0.10</td>
<td>0.757</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>0.869</td>
<td>0.368</td>
</tr>
<tr>
<td></td>
<td>Treatment x Time</td>
<td>3.73</td>
<td>0.085</td>
</tr>
</tbody>
</table>

Significant differences for testicular volume were found for interaction terms (Table 5.7). An interaction was observed between testicular volume and time. When comparing based on treatment alone it appears, left testicular volume increased from a mean of 189.07 mm$^3$ (SD:51.09, min-max: 114.22-286.71) to 208.27 mm$^3$ (SD: 54.84, min-max:117.08-354.01) while right testicular volume increased from a mean of 177.71 mm$^3$ (SD: 46.49, min-max: 115.68-307.32) to 212.37mm$^3$ (SD: 71.09, min-max: 113.03-406.97) although these changes were not significant. However, when the data is evaluated further based on treatment and time the results
show on average the testicular volume was larger on the control testicles compared to the treated testicles (Table 5.5 and 5.6)

Table 5.5. Descriptive statistics for testicular volume estimated from the equation \(\text{Volume}=0.52\ LW^2\): mm\(^3\) reported as Mean, (SD) Min- Max during the first month.

<table>
<thead>
<tr>
<th>T0</th>
<th>Control</th>
<th>HCG</th>
<th>T30</th>
<th>Control</th>
<th>HCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left</td>
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<td>Max:</td>
<td>Min:</td>
<td>Max:</td>
<td>Min:</td>
<td>Max:</td>
</tr>
<tr>
<td>101.04-</td>
<td>262.97</td>
<td>144.80</td>
<td>(55.14)</td>
<td>130.90</td>
<td>(46.84)</td>
</tr>
<tr>
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<td>Max:</td>
<td>Min:</td>
<td>Max:</td>
</tr>
<tr>
<td>77.76-</td>
<td>200.97</td>
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<td>(76.06)</td>
<td>260.40</td>
<td>(113.63)</td>
</tr>
<tr>
<td>148.03-</td>
<td>366.83</td>
<td>119.70-</td>
<td>465.95</td>
<td>113.36-</td>
<td>267.34-</td>
</tr>
<tr>
<td>100.04-</td>
<td>262.97</td>
<td>102.04</td>
<td>(32.74)</td>
<td>114.94</td>
<td>(30.29)</td>
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<td>66.09-</td>
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<td>165.13</td>
<td>(35.12)</td>
<td>179.59</td>
<td>(83.85)</td>
</tr>
<tr>
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<td>217.69</td>
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<td>(83.85)</td>
<td>179.59</td>
<td>(83.85)</td>
</tr>
<tr>
<td>74.35-</td>
<td>166.54</td>
<td>150.92</td>
<td>(62.69)</td>
<td>176.42</td>
<td>(103.77)</td>
</tr>
<tr>
<td>74.35-</td>
<td>166.54</td>
<td>150.92</td>
<td>(62.69)</td>
<td>176.42</td>
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<tr>
<td>95.45-</td>
<td>272.45</td>
<td>112.45</td>
<td>(27.09)</td>
<td>147.13</td>
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<td>Max:</td>
</tr>
<tr>
<td>73.04-</td>
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<td>147.13</td>
<td>(71.75)</td>
<td>147.13</td>
<td>(71.75)</td>
</tr>
<tr>
<td>73.04-</td>
<td>148.80</td>
<td>147.13</td>
<td>(71.75)</td>
<td>147.13</td>
<td>(71.75)</td>
</tr>
</tbody>
</table>

Table 5.6. Descriptive statistics for testicular volume estimated from the equation \(\text{Volume}=0.52\ LW^2\): mm\(^3\) reported as Mean, (SD) Min- Max following 1 month washout period and complete cross over of individuals.

<table>
<thead>
<tr>
<th>T0</th>
<th>Control</th>
<th>HCG</th>
<th>T30</th>
<th>Control</th>
<th>HCG</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Max:</td>
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<tr>
<td>102.04-</td>
<td>145.58</td>
<td>114.94</td>
<td>(30.29)</td>
<td>165.13</td>
<td>(35.12)</td>
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<tr>
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<td>145.58</td>
<td>165.13</td>
<td>(35.12)</td>
<td>179.59</td>
<td>(83.85)</td>
</tr>
<tr>
<td>128.36-</td>
<td>217.69</td>
<td>179.59</td>
<td>(83.85)</td>
<td>179.59</td>
<td>(83.85)</td>
</tr>
<tr>
<td>74.35-</td>
<td>166.54</td>
<td>150.92</td>
<td>(62.69)</td>
<td>176.42</td>
<td>(103.77)</td>
</tr>
<tr>
<td>74.35-</td>
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<td>(71.75)</td>
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<tr>
<td>73.04-</td>
<td>148.80</td>
<td>147.13</td>
<td>(71.75)</td>
<td>147.13</td>
<td>(71.75)</td>
</tr>
</tbody>
</table>
Table 5.7. Mixed model results for testicular volume following serial hCG injections in veiled chameleons (* is indicative of significance).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Variable</th>
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</thead>
<tbody>
<tr>
<td>Right Testicular Volume</td>
<td>Treatment</td>
<td>2.38</td>
<td>0.139</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>2.38</td>
<td>0.137</td>
</tr>
<tr>
<td></td>
<td>Treatment x Time</td>
<td>8.85</td>
<td>0.008*</td>
</tr>
<tr>
<td>Left Testicular Volume</td>
<td>Treatment</td>
<td>0.98</td>
<td>0.332</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>0.65</td>
<td>0.801</td>
</tr>
<tr>
<td></td>
<td>Treatment x Time</td>
<td>5.52</td>
<td>0.029*</td>
</tr>
</tbody>
</table>

5.4. DISCUSSION

The results of this study confirmed the authors’ first and second hypotheses, that hCG could be used to increase plasma testosterone concentrations in a species of chameleon after 24 hours and over time. This is the first time this has been done in a species of chameleon and suggests that this method could be used to help further develop assisted reproductive programs for this group of threatened reptiles. Obtaining pharmacological control over the reproductive system would allow scientists to manipulate the reproductive cycles of these animals and reduce the dependency on natural breeding seasons, which can be limited.

The results of this study further support previous research that hCG has a gonadal effect and that it can be used in reptiles elevate testosterone concentrations (Eyeson 1971; Jadhav and Padgaonkar, 2010; Sonar and Patil, 1994). Alternative exogenous hormones, such as LH, LHRH, or PMSG may also be considered; however, they may not be as useful in reptiles. In mammals, the biological effects and immunological cross-reactivity of hCG and LH have been found to be similar (Bell et al., 1969); however, in lizards it has been documented that interstitial cells appear
to be more sensitive to hCG than LH. Follicle stimulating hormone (FSH), hCG, and LH were all found to stimulate the interstitial cells of the common agama (*Agama agama*), with LH having the greatest potency; however, based on the reported methods, there was likely some contamination by FSH (Eyeson, 1971). In Hardwicks’ spiny-tailed uromastyx (*Uromastix harkwickii*) (Arslan et al., 1975;), varying doses of ovine FSH, LH, and a lizard hypophysial extract were found to increase testicular concentrations (Arslan et al., 1981; Jalali et al., 1976). These results suggest more work is needed to further elucidate the role of these hormones in male reptiles, but that hCG should be strongly considered when evaluating the role of an exogenous hormone in a new reptile species, even in squamates.

The doses selected for this study, 100 IU, 200 IU, and 300 IU/animal, 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 and there is a ceiling effect. Based on the weights of the chameleons, the IU/kg dose range in this study varied from 444-1000 IU/kg. In the other species studied, doses varied greatly from 50 IU/animal/ day for 28 days to 1 mg/animal. There has not been any attempt to standardize dosing, but this will be necessary to develop functional reproductive programs. The authors suggest performing a dose-response study for any new species, as we outlined in this study with the three doses and a crossover study design. Ultimately, we did not observe a difference between the three different doses of hCG and selected the 100 IU/animal dose because of the previously noted ceiling that occurs when LH receptors are bound by hCG. It is possible that the minimum effective dose of hCG required to achieve this is lower than 100 IU, and possibly lower than 50 IU/animal based on a study in New Guinea bockadam and little brown skinks (*Scincella lateralis*) (Jadhav and Padgaonkar, 2010; Jones, 1973). In the bockadam, a dose of 50 IU was used, while a 1 IU dose was used in the
skinks. In both studies, testosterone was not measured but gonadal changes were measured and reported. In the current study, the authors chose 100 IU because of limitations in the available sample size and the number of groups that could be included in the crossover design. It is important to note that there were no side effects noted in any of the animals following any of the three doses or the long term 100 IU/animal study. Future studies might consider lower doses (e.g., 50 IU/animal) as their initial dose to determine dosing efficacy.

The results of this study confirmed that weekly injections of hCG at 100 IU/animal could be used to maintain elevated plasma testosterone concentrations over a month-long period. This study was also the first to demonstrate that the interval between hCG injections could be increased to weekly and still maintain elevated plasma testosterone concentrations.

The third hypothesis tested in this study was not proven, as hCG administration was not directly associated with an increase in testicular volume. However, a treatment and time interaction was identified, suggesting that some effect was occurring over the duration of the study. The authors elected to use ultrasound because it is a non-invasive method for measuring testicle size. This technique was selected because the ultimate goal of the authors is to develop clinical, ante-mortem methods that can be used to assess the reproductive cycle of male reptiles. Historically, post-mortem studies with histology have been used to measure the effects of exogenous hormones on testicle size. While useful, these types of studies cannot be conducted on threatened species. Studies that have evaluated the effect of hCG, or other hormones, on testicle size and function and used post-mortem testicle measurements have found variable results. A study evaluating serial dosing of hCG in the New Guinea bockadam during the quiescent phase of the gonadal cycle found that 50 IU/day for 14 and 28 days did not have any stimulatory effect on the seminiferous tubules in the regressed testicles but did have an effect in the epididymis.
(Jadhav et al., 2010). The animals treated for 28 days demonstrated an accumulation of lipid in the seminiferous tubules but no spermatogenic activity. These results demonstrated that hCG could significantly increase Leydig cell stimulation in this species after 28 days treatment. The results observed in the epididymal epithelial height was considered secondary to increased Leydig cell androgen secretion. Little brown skinks treated with 1 IU of ovine LH, hCG, ovine FSH, FSH and LH, and PMSG intracoelomically during the quiescent stage of the reproductive cycle and necropsied 15 days after treatment were found to have increased interstitial cell numbers, stimulated interstitial cell hypertrophy, cytoplasmic granulation, and increased epididymal and sexual segment epithelial height (Jones, 1973). These authors did not measure testosterone concentrations in these animals, although the gonadal data suggests there was a physiological stimulation of testosterone. Male oriental garden lizards (Calotes versicolor) treated with hCG and PMSG during the quiescent phase were found to have increased testicular weight and diameter. The seminiferous tubule diameter increased, and spermatids were the abundant germ cell elements observed in these animals. Steroidogenesis was initiated, and the weight and protein content of the epididymis increased. However, spermatogenesis was not complete since spermatozoa were not observed in the lumen of the seminiferous tubules (Sonar and Patil, 1994). Pregnant mare serum gonadotropin may have FSH-like activity in reptiles based on these findings since there was evidence of spermatogenesis in the gonads; this suggests that FSH stimulation is secondary prior to the preparation of the male reproductive tract with hCG. A histological and histochemical study of the epididymides of Indian wall lizards treated with mammalian gonadotropins (LH, ovine FSH), hCG, PMSG, and testosterone propionate found considerable variation in cell height and luminal diameter during the different phases of the reproductive cycle, and the investigators came to the conclusion that FSH by itself was capable
of stimulating the growth and secretory activity of the epididymides (Prasad and Sanyal, 1969; Reddy and Prasad, 1970a, b; Haider and Rai, 1987). Similar results have been observed in other species where hCG also stimulates the interstitial cells, sex accessory ducts, epididymides, and renal sexual segments in green anoles, broadhead skinks, and common wall lizards. (Herlant, 1933; Evans, 1935; Turner, 1935). The results of this study suggest that ultrasound may be a sensitive enough to measure ante-mortem morphometric changes associated with testicular measurements for volume.

The fourth hypothesis was not proven in this study, as there was no difference in electroejaculation collection success or ejaculate quality (concentration, motility, and volume) between the animals treated with the hCG and saline injections after 4 weekly injections of hCG 100 IU. However, this should not be surprising based on the results of hypothesis three and the findings in other studies (Jadhav et al., 2010; Jones, 1973; Prasad and Sanyal, 1969, Reddy and Prasad, 1970a, b; Haider and Rai, 1987). These previous studies and the current findings are evidence that hCG alone does not promote the full spermatogenic cycle in the reptile testicle, based on the length of time the studies measured this effect (<4 weeks) and that additional input is likely necessary from FSH or some FSH-like activity from drugs such as PMSG (Sonar and Patil, 1994). Unfortunately, we do not sufficiently understand the inner workings of the spermatogenic cycle of reptiles to comprehend the full endocrine and paracrine interactions between the cells. It is possible that higher levels of the endocrine system could be responsible for this, such as the hypothalamus, and that it no longer becomes responsive to elevated plasma testosterone levels, which reduces the likelihood that FSH is being stimulated (either tonically or in a pulse like fashion). We evaluated these animals towards the end of their reproductive season (Chapter 4), when we would have expected an increased likelihood to observe a clinical
response, and while there was a change in testicular volume, there was no difference in ejaculate success. Further work is needed in order to develop a better understanding of the male reproductive cycle so that scientists can stimulate the testicle to induce testicular recrudescence.

The development of safe and consistent methods for collecting ante-mortem semen samples from reptiles is essential to gaining control over biobanking and planned reproduction, such as artificial insemination. The techniques used in this study have been used previously in chameleons with good success (Perry et al., 2019); however, how electroejaculation affects the male reproductive tract, and more specifically where the semen is dispelled from, is unknown. If hCG has an impact on spermatogenesis within the epididymides as with previous studies, it is possible that the electroejaculation method will not facilitate expulsion of the spermatozoa from that level. Instead, manual manipulation, or a combination of manual manipulation and electroejaculation, or chemical means, may be needed to facilitate an ejaculation. For example, oxytocin and PGF$_{2\alpha}$ may be useful for increasing ejaculate concentration and quality, as well as smooth muscle contraction prior to or after electroejaculation. Additional research into chemical ejaculation in reptiles, including the use of $\alpha_2$ agonists, imipiramine, oxytocin and prostaglandin (PGF$_{2\alpha}$), should be considered to improve semen collection.

There were several limitations to consider in this study. While the results confirm an association between hCG administration, a rise in testosterone concentrations, and an increase in testicular volume, histology or immunohistochemistry were not done to confirm this was happening at the level of the testicle. The chameleons from this study were being used for additional reproductive studies, and thus could not be sacrificed. Additionally, the goal of this research is to develop clinical, ante-mortem methods of assessing the reproductive status of male reptiles and euthanasia is not an option for protected species.
In conclusion, hCG can be used to increase circulating testosterone concentrations in male veiled chameleons, and serial injections can maintain these elevated concentrations. Additionally, injections of hCG can be given weekly to achieve these results. Human choronic gonadotropin dosing in veiled chameleons found that 100 IU/animal was as effective as doses two-to three times as high. A one-month washout period is recommended for hCG dose determination studies to limit an additive effect that was observed in the dose determination study. Ultrasound can be used to safely and consistently measure testicle size in veiled chameleons. Finally, hCG and increased testosterone concentrations do not impact semen production after 30 days. Longer term studies are required to further elucidate temporal relationships.
CHAPTER 6. MEASURING THE LEVEL OF AGREEMENT BETWEEN OSMOMETER AND CALCULATED PLASMA OSMOLALITIES IN TWO SPECIES OF CHAMELEONS, *FURCIFER PARDALIS* AND *CHAMELEO CALYPTRATUS*

6.1. INTRODUCTION

Fluid therapy selection is an important consideration for managing hemodynamically unstable patients. Crystalloid therapy allows for acute reversal of shock syndromes and fluid replenishment based on a patient’s true and relative fluid deficits. Having an accurate assessment of plasma osmolality can guide clinicians in choosing an appropriately formulated fluid therapy to prevent life threatening shifts in fluid balance. Commercial crystalloid solutions are formulated based on the plasma osmolality of mammals. When working with reptiles and other non-mammalian species, limited information is available for species-specific plasma osmolality. A further understanding of reptile fluid balance and plasma osmolality is needed to select the most appropriate fluid for each situation.

In mammals, total body water constitutes approximately 60% of the body weight. Comparatively, reptiles have a higher total body water composition, with water constituting 75% and 66% of non-chelonian and chelonian species, respectively (Minnich, 1979). Within the body, total body water is distributed into two compartments, intracellular fluids (ICF) and extracellular fluids (ECF). The ECF compartment can be further divided into subcategories, including blood, intercellular fluid, third space (e.g., transcellular fluid: coelomic cavity, retrocoelomic, and pericardial), and the gastrointestinal tract (George and Zabolotzky, 2011). In reptiles, total body water distribution differs compared to mammals. Reptiles have higher ICF volumes (80%) and lower ECF volumes (20%) (Thorson, 1968), with plasma only accounting for 3.3-7% of the reptile’s total body weight (Thorson, 1968).
Serum osmolality can be used to measure the ECF osmolality and aid in selecting the most appropriate fluid therapy for a species. Osmolality measures the number of particles of solute per kilogram of solution, while osmolarity refers to the number of particles of solute per liter of solvent (Dibartola, 2011). Sodium (Na\(^+\)), potassium(K\(^+\)), chloride (Cl\(^-\)), and bicarbonate are the largest contributing solutes to serum osmolality, representing approximately 94% of the total solutes in mammals. The contributing solutes to the remaining 6% of the osmolality include blood urea nitrogen (BUN), glucose, phosphate, total calcium, and magnesium (George and Zabolotzky, 2011). Most body fluids are dilute aqueous solutions, so the difference between osmolality and osmolarity is negligible (Dibartola, 2011).

Plasma osmolality is typically measured using a freezing point depression osmometer; however, this method is often not available in clinical practice (Dibartola, 2011). In cases where the instrumentation is not available, serum osmolality can be estimated using formulas (calculations) that consider the different particles of solute in the fluid, including the electrolytes, glucose, and BUN (or the primary end product of protein catabolism). Although estimates are not an exact replacement for an osmometer, equation-based estimates have been developed that can approximate osmometer values. In mammals, the most commonly used formulas are:

- \(2[Na^+ + K^+(mmol/L)]\) for samples with normal glucose and BUN concentrations,
- \(2[Na^+ + K^+(mmol/L)] + (\text{glucose(mg/dL)/18} + (\text{BUN(mg/dL)/2.8})\) for samples with elevated glucose or BUN concentrations.

Over 14 equations have been developed to estimate plasma osmolality in mammals (Weisberg, 1971; George and Zabolotzky, 2011), while in reptiles there is a dearth of these types of estimates and they are species specific (Dallwig et al., 2010; Sanchez-Migallon et al., 2011; Nevarez et al., 2012). The objectives of this study were to 1) measure plasma osmolalities of male panther chameleons (\textit{Furcifer pardalis}) and male veiled chameleons.
(Chamaeleo calyptratus) using a freezing point osmometer, and 2) determine if calculated plasma osmolality using different formulas would be in good agreement with the measured plasma osmolality. The specific hypotheses tested in this study were: 1) that measured plasma osmolality would be different between the two species, and 2) that there would be poor agreement between the measured and calculated plasma osmolalities.

6.2 MATERIALS AND METHODS

This study was performed in compliance with the regulations set forth by the Institutional Animal Care and Use Committee at Louisiana State University (protocols 16-095, 16-096). Twelve adult, captive-bred, male panther chameleons and 12 adult, wild caught, male veiled chameleons were used for this study. Sample size was determined based on the following a priori expectations: an alpha=0.05, a power=0.8, an expected difference in measured osmolality between species of 10 mOsm/L, and a standard deviation of 7.5 mOsm/L for each species. Animals were individually housed at Louisiana State University in 46 cm x 46 cm x 92 cm (18 in x 18 in x 36 in) screen cages (Zoo Med, Paso Robles, CA, USA). A MistKing misting system (Jungle Hobbies Ltd., Ontario, Canada) was incorporated into the caging and used to mist the animals for three minutes, three times a day. Animals were provided a 12-hour photoperiod. Daytime ambient room temperature and a basking spot in each enclosure were maintained at 27.8-28.8°C (82-84°F) and 35-37.8°C (95-100°F) utilizing central heating and incandescent lighting (Fluker Farms, Port Allen, LA 70767, USA), respectively. Nighttime room temperature was 23.3-25.5°C (74-78°F). Animals were exposed to ultraviolet lighting 12 hours per day (Reptisun 10.0, Zoo Med). Bulb output was evaluated within the cages using a Solar meter 6.2 UV meter (Solarlight Company, Glenside, PA, USA). A combination of Repta-Vines (Fluker Farms), treated sand blasted grape vines (Fluker Farms), and Southern magnolia tree branches
Magnolia grandiflora) were used to provide cover and climbing surfaces within the cages. Chameleons were fed a diet consisting of gut-loaded house crickets (Acheta domestica) and superworms (Zophobas morio). Insects were gut loaded 24 hours prior to being offered to the chameleons with gut-loading diets (Fluker Farms) and vegetables.

The panther chameleons were born in captivity and acquired from a breeder (Panther-Ranch, Matiland, FL, USA), while the veiled chameleons were acquired from a reptile distributor (The Turtle Source, Ft. Meyers, FL, USA) that collected the animals in southwest Florida. A physical examination was performed on each chameleon at initial intake to determine they were healthy. All animals were acclimated to the new laboratory environment for one month prior to the start of the study. During this quarantine, fecal samples were collected and the endoparasite burden of the chameleons assessed using direct saline smears and fecal flotations using zinc sulfate. The fecal samples for the veiled chameleons were found to harbor numerous non-speciated flagellates, Strongyloides, and trematodes. Based on these findings, the chameleons were all treated with fenbendazole (Panacur; Merck Animal Health, Madison, NJ, USA) at 25 mg/kg PO SID for 3 days, praziquantel (Bimeda, Inc., Le Suer, MN, USA) 6 mg/kg IM. every 7 days for 2 treatments, and metronidazole (compounded metronidazole 50 mg/ml; Louisiana State University, Baton Rouge, LA, USA) at 20 mg/kg PO SID for 3 days.

For blood collection, the chameleons were held in dorsal recumbency using manual restraint. The blood samples were collected from the ventral coccygeal vein using a 25-gauge needle fastened to a 1-mL syringe. The blood sample was immediately placed in a lithium heparin microtainer tube (BD Biosciences, Franklin Lakes, NJ, USA) after collection. Within 90 minutes of sample collection, 0.1 mL of whole blood was removed from the microtainer to measure the clinical biochemistries using the VetScan Classic analyzer and Vetscan Avian-
Reptile Profile- Plus rotors (Abaxis Inc., Union City, CA, USA). The rotors provided results for Na⁺, K⁺, Cl⁻, uric acid (UA), and glucose. The lithium heparin microtainers were then centrifuged at 1,900 g for 10 minutes, the plasma removed and placed into cryogenic vials, and the samples frozen at -80°C. Within 90 days, the plasma was thawed and the osmolality measured in duplicate using a commercial freezing point depression osmometer (MicroOsmette; Precision Systems, Natick, MA, USA). All measurements were performed by one individual (MJA), and the osmometer was calibrated based on the manufacturer’s specifications. The two osmolality measurements from each sample were averaged to calculate a single value for analysis. Duplicate values had to have < 5% variance between measurements to be included in the study.

Calculated osmolality were determined using seven different equations that have been evaluated in mammals and other reptiles: 2 x Na⁺; 2 ([Na⁺ + K⁺]); 2 ([Na⁺ + K⁺])+(glucose/18); 2 ([Na⁺ + K⁺])+(uric acid/16.8 +(glucose/18)); 1.85(Na⁺ + K⁺); 1.86(Na⁺ + K⁺); and (1.86[Na⁺ + K⁺]+[glucose/18]+[uric acid/16.8]+9) (Weisberg, 1971; Dallwig et al., 2010; George and Zabolotzky, 2011; Sanchez-Migallon et al., 2011; Nevarez et al., 2012).

6.2.1. STATISTICAL METHODS

Statistical analyses were performed using SPSS 25.0 (IBM Statistics, Armonk, NY, USA) and MedCalc (Ostend, Belgium). A P≤0.05 was used to determine statistical significance. The distributions of the continuous data (biochemistry results and osmolality) were evaluated using the Shapiro-Wilk test, skewness, kurtosis, and q-q plots. Normally distributed data are reported by the mean, standard deviation (SD), and minimum-maximum (min-max) values, while non-normally distributed data are reported as the median, 25-75 quartiles (%), and min-max values. A Mann-Whitney U test was used to determine if there was a significant difference.
in the measured osmolalities between the panther and veiled chameleons. A single samples Wilcoxon test was used to determine if the measured osmolality of the chameleons differed from published osmolality for other species of reptiles. Canine and feline osmolality were also compared at both the high and low end of the published value for analysis. Calculated and measured osmolality were analyzed for agreement using Bland-Altman plots (Bland Altman, 1986). Bias was defined as the mean difference between the two methods, and limits of agreement were calculated as the bias +/- (1.96xSD). Good agreement was defined as a bias and limits of agreement that varied by <5% of the mean of the plasma osmolality. Passing-Bablok regression was done to determine whether there were systematic or proportional errors between the calculated and measured osmolality. The Cusum test was used to assess the potential for a violation in linearity. Systematic or proportional errors were confirmed when the 95% confidence interval for the intercept or slope did not contain the value 0 or 1, respectively. Multiple linear regression was performed to determine is new predictive formulas for calculated osmolality could be developed for veiled and panther chameleons using Na^+, K^+, glucose, and UA concentrations as predictors.

6.3. RESULTS

The plasma biochemistry results for both species are reported in Table 6.1, and all distributions were normal, except Na^+ in panther chameleons, this was due to a single outlier in the analysis that skewed the data to the right.
Table 6.1. Descriptive statistics of biochemical analytes for veiled chameleons and panther chameleons. Means and standard deviations (SD) should be used for normally distributed data and medians and 25-75% for non-normally distributed data (*).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Species</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>25-75%</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>Veiled</td>
<td>145.25</td>
<td>6.73</td>
<td></td>
<td></td>
<td>129-155.00</td>
</tr>
<tr>
<td></td>
<td>Panther*</td>
<td></td>
<td></td>
<td>140.5</td>
<td>135.25-142.0</td>
<td>133-164</td>
</tr>
<tr>
<td>Potassium</td>
<td>Veiled</td>
<td>6.52</td>
<td>1.21</td>
<td></td>
<td></td>
<td>4.80-8.5</td>
</tr>
<tr>
<td></td>
<td>Panther</td>
<td>6.23</td>
<td>1.00</td>
<td></td>
<td></td>
<td>4.4-7.60</td>
</tr>
<tr>
<td>Uric Acid</td>
<td>Veiled</td>
<td>8.9</td>
<td>7.04</td>
<td></td>
<td></td>
<td>1.5-25</td>
</tr>
<tr>
<td></td>
<td>Panther*</td>
<td>2.05</td>
<td>0.67</td>
<td></td>
<td></td>
<td>1.1-3.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>Veiled</td>
<td>273.5</td>
<td>22.83</td>
<td></td>
<td></td>
<td>231-313.0</td>
</tr>
<tr>
<td></td>
<td>Panther</td>
<td>275.3</td>
<td>26.3</td>
<td></td>
<td></td>
<td>227-307</td>
</tr>
</tbody>
</table>

Measured and calculated plasma osmolality for both species are reported in Tables 6.2 (veiled chameleons) and 6.3 (panther chameleons).

Table 6.2. Measured and calculated plasma osmolality for veiled chameleons. All values are reported in mOsm/kg.

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>Min</th>
<th>Max</th>
<th>25%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured osmolality (mOsm/kg)**</td>
<td>326.77</td>
<td>16.03</td>
<td>330.50</td>
<td>285</td>
<td>346.50</td>
<td>323</td>
<td>334.5</td>
</tr>
<tr>
<td>2 x Na**</td>
<td>291.27</td>
<td>13.83</td>
<td>290</td>
<td>258</td>
<td>310</td>
<td>286</td>
<td>302</td>
</tr>
<tr>
<td>2 x Na**+ K**</td>
<td>304.07</td>
<td>13.23</td>
<td>304.60</td>
<td>275</td>
<td>324.60</td>
<td>299.6</td>
<td>314.8</td>
</tr>
<tr>
<td>2 ([Na**+ K**]+(glucose/18)*</td>
<td>319.34</td>
<td>13.21</td>
<td>320.48</td>
<td>290.11</td>
<td>337.43</td>
<td>313.54</td>
<td>329.91</td>
</tr>
<tr>
<td>2 ([Na**+ K**]+(uric acid/16.8)+(glucose/18))</td>
<td>319.89</td>
<td>13.10</td>
<td>320.47</td>
<td>291.19</td>
<td>338.11</td>
<td>313.80</td>
<td>330.42</td>
</tr>
<tr>
<td>1.85(Na**+ K**)*</td>
<td>281.26</td>
<td>12.24</td>
<td>281.75</td>
<td>254.38</td>
<td>300.26</td>
<td>277.13</td>
<td>291.19</td>
</tr>
<tr>
<td>1.86(Na**+ K**)*</td>
<td>295.70</td>
<td>12.76</td>
<td>295.46</td>
<td>265.13</td>
<td>311.06</td>
<td>289.60</td>
<td>305.48</td>
</tr>
<tr>
<td>(1.86[Na**+ K**]+[glucose/18]+[uric acid/16.8]+9)*</td>
<td>307.60</td>
<td>12.18</td>
<td>308.25</td>
<td>280.94</td>
<td>324.38</td>
<td>301.83</td>
<td>317.38</td>
</tr>
</tbody>
</table>

Normal distributed*, Non-Normally distributed**
Table 6.3. Measured and calculated plasma osmolality for panther chameleons. All values are reported in mOsm/kg.

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>Min</th>
<th>Max</th>
<th>25%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured osmolality (mOsm/kg)**</td>
<td>311.77</td>
<td>16.98</td>
<td>308</td>
<td>294</td>
<td>358.50</td>
<td>306.5</td>
<td>334.5</td>
</tr>
<tr>
<td>2 x Na***</td>
<td>283.27</td>
<td>17.69</td>
<td>282</td>
<td>266</td>
<td>328</td>
<td>270</td>
<td>284</td>
</tr>
<tr>
<td>2 x Na⁺⁺ K⁺⁺</td>
<td>295.74</td>
<td>17.91</td>
<td>292.6</td>
<td>279.60</td>
<td>342</td>
<td>282.2</td>
<td>298.4</td>
</tr>
<tr>
<td>2 (/[Na⁺⁺ K⁺⁺])+(glucose/18)**</td>
<td>310.97</td>
<td>17.65</td>
<td>307.48</td>
<td>292.81</td>
<td>355.28</td>
<td>297.25</td>
<td>314.25</td>
</tr>
<tr>
<td>2 (/[Na⁺⁺ K⁺⁺])+(uric acid/16.8+(glucose/18))**</td>
<td>311.10</td>
<td>17.64</td>
<td>307.61</td>
<td>292.88</td>
<td>355.37</td>
<td>297.36</td>
<td>314.32</td>
</tr>
<tr>
<td>1.85(Na⁺⁺ K⁺⁺)**</td>
<td>273.56</td>
<td>16.57</td>
<td>270.65</td>
<td>258.63</td>
<td>316.35</td>
<td>261.03</td>
<td>276.02</td>
</tr>
<tr>
<td>1.86(Na⁺⁺ K⁺⁺)**</td>
<td>287.80</td>
<td>16.17</td>
<td>285.57</td>
<td>270.92</td>
<td>327.41</td>
<td>275.26</td>
<td>289.75</td>
</tr>
<tr>
<td>(1.86/[Na⁺⁺ K⁺⁺]+[glucose/18]+[uric acid/16.8]+9)**</td>
<td>299.40</td>
<td>16.39</td>
<td>296.13</td>
<td>282.26</td>
<td>340.43</td>
<td>286.61</td>
<td>302.52</td>
</tr>
</tbody>
</table>

Normal distributed*, Non-Normally distributed**

There was a significant difference (P=0.010) in the measured plasma osmolality between veiled and panther chameleons. There were also significant differences in the measured plasma osmolality between the panther and veiled chameleons and American alligators, bearded dragons, corn snake, desert iguana, and Gila monster. A significant difference in the measured osmolality was also found between panther chameleons and green iguanas, but not between veiled chameleons and green iguanas (Table 6.4). Bland Altman plots demonstrated poor agreement between the measured osmolality and the 7 calculated osmolality for the veiled and panther chameleons (Table: 6.5) (Figures: 6.1-6.14).

Multiple linear regression analyses found that Na⁺ could be used to predict the measured osmolality for veiled chameleons; parameter estimates were 2.098 (95%CI: 1.354-2.842, P<0.001). Based on this result, the following equation can be used to predict the osmolality of a clinically healthy male veiled chameleon: Osmolality = 2.098 (Na). Unfortunately, no
biochemistry (Na⁺, K⁺, UA, and glucose) was found to be a predictor of osmolality in the panther chameleons.

Table 6.4. Mean plasma osmolalities reported for different reptile species, FP=Panther chameleon, CC: veiled chameleon.

<table>
<thead>
<tr>
<th>Species</th>
<th>Mean</th>
<th>SD</th>
<th>Wilcoxon</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>American alligator</td>
<td>269.3</td>
<td>10.8</td>
<td>FP: p=0.003</td>
<td>Nevarez et al., 2011</td>
</tr>
<tr>
<td><em>Alligator mississippiensis</em></td>
<td></td>
<td></td>
<td>CC: p=0.004</td>
<td></td>
</tr>
<tr>
<td>Bearded dragon</td>
<td>295.4</td>
<td>9.35</td>
<td>FP: p=0.004</td>
<td>Dallwig et al., 2010;</td>
</tr>
<tr>
<td><em>Pogona vitticeps</em></td>
<td></td>
<td></td>
<td>CC: p=0.004</td>
<td></td>
</tr>
<tr>
<td>Corn snake</td>
<td>344.5</td>
<td>-</td>
<td>FP: p=0.004</td>
<td>Sanchez-Migallon et al., 2011</td>
</tr>
<tr>
<td><em>Pantherophis guttatus</em></td>
<td></td>
<td></td>
<td>CC: p=0.004</td>
<td></td>
</tr>
<tr>
<td>Desert iguana</td>
<td>300</td>
<td>-</td>
<td>FP: p=0.008</td>
<td>Nevarez, 2009</td>
</tr>
<tr>
<td><em>Dipsosaurus dorsalis</em></td>
<td></td>
<td></td>
<td>CC: p=0.004</td>
<td></td>
</tr>
<tr>
<td>Gila monster</td>
<td>292</td>
<td>5</td>
<td>FP: p=0.003</td>
<td>Davis and DeNardo, 2007</td>
</tr>
<tr>
<td><em>Heloderma suspectum</em></td>
<td></td>
<td></td>
<td>CC: p=0.004</td>
<td></td>
</tr>
<tr>
<td>Green iguana</td>
<td>327</td>
<td>3.3</td>
<td>FP: p=0.041</td>
<td>Fitzsimons and Kaufman, 1976</td>
</tr>
<tr>
<td><em>Iguana iguana</em></td>
<td></td>
<td></td>
<td>CC: p=0.533</td>
<td></td>
</tr>
</tbody>
</table>

*Wilcoxon test, p value <0.05

6.4. DISCUSSION

The median plasma osmolality of veiled and panther chameleons measured in this study using a freezing point depression osmometer were 330.5 and 308.0 mOsm/kg, respectively. The difference (6.8 %) in the osmolality between these two species was found to be statistically different, with the veiled chameleons having a higher osmotic pressure than the panther chameleons. This difference may be attributed to the environments these animals evolved. The veiled chameleon is native to the arid climates of the Yemen and Saudi Arabia, while the panther chameleon is native to the tropical climate of Madagascar.
Table 6.5. Bland Altman results for measured and calculated osmolality for veiled and panther chameleons. All values are reported in mOsm/kg.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Species</th>
<th>Bias</th>
<th>Limits of Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 x Na⁺</td>
<td>Veiled</td>
<td>35.5</td>
<td>-22.1 to 48.9</td>
</tr>
<tr>
<td></td>
<td>Panther</td>
<td>28.5</td>
<td>-27.2 to 84.2</td>
</tr>
<tr>
<td>2 ([Na⁺+ K⁺])</td>
<td>Veiled</td>
<td>22.7</td>
<td>6.7 to 38.7</td>
</tr>
<tr>
<td></td>
<td>Panther</td>
<td>16</td>
<td>-40.4 to 72.4</td>
</tr>
<tr>
<td>2 ([Na⁺+ K⁺])+(glucose/18)</td>
<td>Veiled</td>
<td>7.4</td>
<td>-8.1 to 23.0</td>
</tr>
<tr>
<td></td>
<td>Panther</td>
<td>0.8</td>
<td>57.5 to -55.9</td>
</tr>
<tr>
<td>2 ([Na⁺+ K⁺])+(uric acid/16.8</td>
<td>Veiled</td>
<td>6.9</td>
<td>-8.9 to 22.7</td>
</tr>
<tr>
<td>+ (glucose/18))</td>
<td>Panther</td>
<td>0.7</td>
<td>-56.0 to 57.4</td>
</tr>
<tr>
<td>1.85(Na⁺+ K⁺)</td>
<td>Veiled</td>
<td>45.5</td>
<td>-29.2 to 61.8</td>
</tr>
<tr>
<td></td>
<td>Panther</td>
<td>38.2</td>
<td>-16.0 to 92.4</td>
</tr>
<tr>
<td>1.86(Na⁺+ K⁺)</td>
<td>Veiled</td>
<td>31.1</td>
<td>-17.5 to 44.6</td>
</tr>
<tr>
<td></td>
<td>Panther</td>
<td>24.0</td>
<td>-30.0 to 78.0</td>
</tr>
<tr>
<td>(1.86[Na⁺+ K⁺]+[glucose/18]</td>
<td>Veiled</td>
<td>19.2</td>
<td>3.1 to 35.2</td>
</tr>
<tr>
<td>+ [uric acid/16.8]+9)</td>
<td>Panther</td>
<td>12.4</td>
<td>-42.4 to 67.1</td>
</tr>
</tbody>
</table>

The osmolality of both species of chameleons had osmolalities that were higher than most recorded lizard species (Table 4), except for green iguanas. When comparing the osmolality of each chameleon species, osmolalities to the published values for the other lizards there the green iguana showed no difference in osmolalities to the veiled chameleon. Differences were observed between both chameleon species and other lizard species (*Heloderma suspectum*, *Dipsosaurus dorsalis*, and *Pogona vitticeps*). This may be attributed to life history, as all of these lizard species live in extremely arid environments, such as the Sonoran/Mojave Desert and eastern/central Australian Desert. The osmolality of both chameleon species were also higher than American alligators (*Alligator mississippiensis*). There is only one example of a measured osmolality for a snake.
Figure 6.1. Equation 1: Panther chameleon (*F. pardalis*) Bias (solid horizontal line) was 28.5 mOsm/kg, and the limits of agreement were -27.2 to 84.2 mOsm/kg (dotted horizontal lines). P=0.0076

Figure 6.2. Equation 1: Veiled chameleon (*C. calyptratus*) Bias (solid horizontal line) was 35.5 mOsm/kg, and the limits of agreement were -22.1 to 48.9 mOsm/kg (dotted horizontal lines). (P<0.001)
Figure 6.3. Equation 2: Panther chameleon (*F. pardalis*) Bias (solid horizontal line) was 16.0 mOsm/kg, and the limits of agreement were -40.4 to 72.4 mOsm/kg (dotted horizontal lines). \( P=0.0945 \)

Figure 6.4. Equation 2: Veiled chameleon (*C. calyptratus*) Bias (solid horizontal line) was 22.7 mOsm/kg, and the limits of agreement were 6.7 to 38.7 mOsm/kg (dotted horizontal lines). \( P<0.001 \)
Figure 6.5. Equation 3: Panther chameleon (*F. pardalis*) Bias (solid horizontal line) was 0.8 mOsm/kg, and the limits of agreement were 57.5 to -55.9 mOsm/kg (dotted horizontal lines). P=0.9292

Figure 6.6. Equation 3: Veiled chameleon (*C. calyptratus*) Bias (solid horizontal line) was 7.4 mOsm/kg, and the limits of agreement were -8.1 to 23.0 mOsm/kg (dotted horizontal lines). P=0.0110
Figure 6.7. Equation 4: Panther chameleon (*F. pardalis*) bias (solid horizontal line) was 0.7 mOsm/kg, and the limits of agreement were -56.0 to 57.4 mOsm/kg (dotted horizontal lines). P=0.9403

Figure 6.8. Equation 4: Veiled chameleon (*C. calyptratus*) Bias (solid horizontal line) was 6.9 mOsm/kg, and the limits of agreement were -8.9 to 22.7 mOsm/kg (dotted horizontal lines). P=0.017
Figure 6.9. Equation 5: Panther chameleon (*F. pardalis*) bias (solid horizontal line) was 38.2 mOsm/kg, and the limits of agreement were -16.0 to 92.4 mOsm/kg (dotted horizontal lines). P=0.0010

Figure 6.10. Equation 5: Veiled chameleon (*C. calyptratus*) Bias (solid horizontal line) was 45.5 mOsm/kg, and the limits of agreement were -29.2 to 61.8 mOsm/kg (dotted horizontal lines).
Figure 6.11. Equation 6: Panther chameleon (F. pardalis) bias (solid horizontal line) was 24.0 mOsm/kg, and the limits of agreement were -30.0 to 78.0 mOsm/kg (dotted horizontal lines). P=0.0162

Figure 6.12. Equation 6: Veiled chameleon (C. calyptratus) Bias (solid horizontal line) was 31.1 mOsm/kg, and the limits of agreement were -17.5 to 44.6 mOsm/kg (dotted horizontal lines).

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Figure 6.13. Equation 7: Panther chameleon (*F. pardalis*) bias (solid horizontal line) was 12.4 mOsm/kg, and the limits of agreement were -42.4 to 67.1 mOsm/kg (dotted horizontal lines). P=0.1724

Figure 6.14. Equation 7: Veiled chameleon (*C. calyptratus*) Bias (solid horizontal line) was 19.2 mOsm/kg, and the limits of agreement were 3.1 to 35.2 mOsm/kg (dotted horizontal lines). P<0.0001
Table 6.6. Passing Bablok regression equations comparing the results of measured plasma osmolality to calculated plasma osmolality in both species of chameleons.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Species</th>
<th>Regression</th>
<th>Intercept (95%CI)</th>
<th>Slope (95%CI)</th>
<th>Cusum</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 x Na⁺</td>
<td>Veiled</td>
<td>y = 33.151515 + 0.787879 x</td>
<td>-144.6667-103.7143</td>
<td>0.5714-1.3333</td>
<td>P=0.77</td>
</tr>
<tr>
<td></td>
<td>Panther</td>
<td>y = -297.695652 + 1.869565 x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 ([Na⁺+ K⁺])</td>
<td>Veiled</td>
<td>y = 55.260000 + 0.760000 x</td>
<td>-211.2167-116.7121</td>
<td>0.5758-1.5833</td>
<td>P=0.83</td>
</tr>
<tr>
<td></td>
<td>Panther</td>
<td>y = -405.585714 + 2.257143 x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 ([Na⁺+ K⁺])+(glucose/18)</td>
<td>Veiled</td>
<td>y = 45.682506 + 0.837825 x</td>
<td>-313.5992-128.2056</td>
<td>0.5889-1.9437</td>
<td>P=1.00</td>
</tr>
<tr>
<td></td>
<td>Panther</td>
<td>y = -473.022221 + 2.525926 x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 ([Na⁺+ K⁺])+(uric acid/16.8)+ (glucose/18)</td>
<td>Veiled</td>
<td>y = 42.091574 + 0.848463 x</td>
<td>-322.5452-130.3118</td>
<td>0.5845-1.9730</td>
<td>P=1.00</td>
</tr>
<tr>
<td></td>
<td>Panther</td>
<td>y = -392.864938 + 2.266922 x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.85(Na⁺+ K⁺)</td>
<td>Veiled</td>
<td>y = 55.493232 + 0.690366 x</td>
<td>-162.6150-116.1584</td>
<td>0.5078-1.3632</td>
<td>P=1.00</td>
</tr>
<tr>
<td></td>
<td>Panther</td>
<td>y = -375.166786 + 2.087857 x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.86(Na⁺+ K⁺)</td>
<td>Veiled</td>
<td>y = 50.155706 + 0.752982 x</td>
<td>-198.4940-112.5660</td>
<td>0.5610-1.5131</td>
<td>P=0.98</td>
</tr>
<tr>
<td></td>
<td>Panther</td>
<td>y = -127.020051 + 1.346595 x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1.86[Na⁺+ K⁺]+[glucose/18]+[uric acid/16.8]+9)</td>
<td>Veiled</td>
<td>y = 58.635828 + 0.762327 x</td>
<td>-261.1286-143.8613</td>
<td>0.5051-1.7456</td>
<td>P=1.00</td>
</tr>
<tr>
<td></td>
<td>Panther</td>
<td>y = -271.925079 + 1.838372 x</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
species, the corn snakes (*Pantherophis guttatus guttatus*), and it was significantly (*P* = 0.004) higher than both chameleon values. The results of this study further reinforce that the osmolalities of reptiles are variable and that species-specific measurements are needed to best characterize the ECF osmolality of a reptile.

When compared to domestic mammals, the osmolality of the panther chameleons (Median: 308.0 mOsm/kg) were not significantly (310 mOsm/kg: *P* = .328) different from the values reported in healthy dogs and cats (290-310 mOsm/kg) (Macintire, 2005; DiBartola, 2011); however, panthers were significantly different when analyzed against the low end of the range (290 mOsm/kg: *P* = .003) and veiled chameleons (Median: 330.5 mOsm/kg) were higher (290 mOsm/kg: *P* = .033; 310 mOsm/kg: *P* = 0.004) than these mammalian species. Because the values are clinically similar, the commercial isotonic crystalloid fluids used for domestic mammals, such as Ringer’s solution (310 mOsm/L; Abbott, North Chicago, IL, USA), lactated Ringer’s solution (272 mOsm/L; Abbott), Plasma-Lyte A® (294 mOsm/L; Baxter Inc., Deerfield, IL, USA), and Normosol R® (295 mOsm/L; Abbott), would be appropriate for the chameleons, especially via the subcutaneous route. Care should be taken to not administer some of these fluids too rapidly (e.g., lactated Ringer’s) via intravenous or intraosseous routes, because the administration of large amounts of hypotonic fluids through these routes can lead to a decrease in plasma osmolality and a fluid shift resulting in intracranial swelling.

As hypothesized, there was poor agreement between the measured and calculated osmolality for both species. In all cases, the calculations underestimated the actual measured osmolality. The reason for the poor agreement suggests that either the factors used in the mammal-based formulas do not account for differences in the chameleons’ plasma, or that some
component of the chameleon plasma is not being measured but has an impact. Bicarbonate, phosphate, calcium, and magnesium are all additional contributors to osmolality, but weren’t measured or included for the analysis in this study. Attempts to further assess the contributions of these plasma biochemistries is warranted.

Because of the poor agreement between the calculated and measured osmolality, we used regression analysis to determine if we could develop a “better” species specific equation for plasma osmolality. Using this method, we were able to derive a specific equation for veiled chameleons: \(2.098 (\text{Na}^+) = \text{osmolality}\). This formula should be used by veterinarians for estimating normal plasma osmolality in a veiled chameleon. Once fluid deficit is corrected, multiplying the Na by 2.098 should derive a value within the reference reported herein (Table 2).

Unfortunately, a similar formula could not be derived for panther chameleons. The distribution of \(\text{Na}^+\) was not normally distributed for panther chameleons, but was normally distributed for veiled chameleons. It is possible that the limited sample size and an outlier impacted our ability to develop a species-specific formula. At this time, it is recommended to use an osmometer for measuring plasma osmolality in panther chameleons.

There are several limitations to this study. First, all study subjects were males. The animals used in this study were designated to a research study evaluating male reproduction in chameleons; thus, we were limited to a single sex. Additional research is needed to assess plasma osmolality in female chameleons. Sample size was also a limitation because the number of animals available was based on the primary reproductive study. Having noted this, it is important to recognize that sample size was only a limitation in testing the second hypothesis regarding the agreement between the different methods. Sample size was sufficient, based on our a priori assumptions, of proving our first hypothesis. Finally, the osmolality reported herein are for
healthy individuals. Animals experiencing physiological disturbances may develop alterations in their biochemical parameters that can affect the measurement of their osmolality. Ultimately, measuring osmolality of animals with specific diseases or treatments will be useful in guiding veterinarians responsible for managing these types of cases. A single study exists where plasma osmolality was measured in bearded dragons following treatment with furosemide at 5 and 10 mg/kg to evaluate the diuretic effect of this drug. No significant changes were measured in the osmolality of the bearded dragons despite an obvious diuretic effect (Parkison and Mans, 2018).

In conclusion, a freezing point osmometer is considered the best method for determining plasma osmolality of veiled and panther chameleons, as there was poor agreement using established mammalian formulas. When a freezing point osmometer is not available, a formula \(2.098 \times \text{Na}\) can be used to estimate the osmolality of veiled chameleons. Based on previous studies, there appears to be variability in the plasma osmolality of reptiles, with different equations derived for each species. These differences should not be unexpected in captive animals because of differences in life history, water acquisition methods, housing, nutrition, and rates of water loss. Ultimately, plasma osmolality should be measured for each species in captivity to provide a reference for veterinarians working with these animals.
CHAPTER 7. EVALUATION OF SEMEN EXTENDERS FOR SHORT-TERM REFRIGERATED STORAGE OF EPIDIDYMAL SPERMATOZOA IN TWO SPECIES OF SQUAMATES

7.1. INTRODUCTION

Successful spermatozoa collection, short-term extension, and long-term biobanking have become necessary for *in situ* and *ex situ* conservation programs for threatened and endangered species. Cryopreservation of gametes for endangered species is not a new concept, as the idea of a “Frozen Zoo” or Genome Resource Bank was established in the 1980’s; however, the focus of cryopreservation and biobanking has been primarily limited to higher vertebrates (Browne et al., 2011; Wildt et al., 1997, 2003, 2010; Benirschke et al., 1984; Clarke, 2009; Comizzoli et al., 2012, 2014, 2015; Lermen et al., 2009). The benefits of spermatozoa biobanking for threatened and endangered species include preserving genetic vigor and diversity; transporting valuable genes without the stress or expense of moving sensitive, fractious animals; and insuring existing genetic diversity that protects fitness and species integrity (Benirschke et al., 1984; Wildt et al., 2010; Clarke, 2009; Comizzoli et al., 2012, 2014, 2015; Lermen et al., 2009). These methods can be used to help sustain genetically diverse and sustainable populations of rare species and genotypes. Additionally, if proper field methods were developed, wildlife spermatozoa biobanking could bring new genetics into captive populations without depleting wild populations, while preserving genetics from specific populations that could be lost due to extinction.

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. In lizards, semen collection has only been reported in the green iguanas (*Iguana iguana*), veiled chameleon (*Chamaeleo calypttratus*), Spiny lava lizard (*Tropidurus spinulus*), Spiny lizard (*Sceloporus torquatus*) and
Grand Cayman blue iguana hybrids (*Cyclura lewisi x nubila*) (Zimmerman et al., 2013, Mitchell et al., 2015, López et al., 2018, Martínez-Torres et al., 2019, 2019, Perry et al., 2019) by electroejaculation, and the common house geckos (*Hemidactylus frenatus*) and the McCann’s skinks (*Oligosoma maccanni*) by coelomic massage (Molina et al., 2010). The investigators have also recently collected semen from veiled chameleons (*Chamaeleo calyptatus*) and panther chameleons (*Furcifer pardalis*) using electroejaculation (Perry et al., 2019; Chapter 4). In snakes, both coelomic massage and electroejaculation have also been employed successfully, coelomic massage has been successful in the corn snakes (*Elaphe guttata*), Angolan python (*Python anchiietae*), Timor python (*Python timoriensis*), Sinaloan milk snake (*Lampropeltis triangulum sinaloae*), Black rat snake (*Elaphe obsoletta obsolete*), Brazilian rattlesnake (*Crotalus durissus terrificus*), and the Argentine boa constrictor (*Boa constrictor occidentalis*) while electroejaculation has been successful in the Checkered garter snake (*Thamnophis marcianus*) (Watson, 1990; Fitch, 1960; Miller and Watson, 2001; Mengden et al., 1980; Samour, 1986; Fahrig et al., 2007; Zacariotti et al., 2007; Mattson et al., 2007; Tourmente et al., 2007). In cheloniens, electroejaculation has been used to successfully collect semen from olive Ridley turtles (*Lepidochelys olivacea*), hawksbill turtles (*Eretmochelys imbricata*), leopard tortoises (*Stigmochyles pardalis*), and ploughshare tortoise (*Astrochelys yniphora*) (Platz et al., 1980; Wood et al., 1982; Juvik et al., 1991; Tanasanti et al., 2009; Kimskulvech and Suttiyoti, 2012; Kawazu et al., 2014, Sirinarumitr et al., 2010). Most of these studies have focused on semen collection and quantification, with only a few studies further evaluating short-term extension or long-term biobanking. Semen extenders were specifically evaluated in the olive Ridley turtle, American alligator (*Alligator mississippiensis*), hawksbill turtle, McCann's skink (*Oligosoma maccanni*), green iguana, corn snake (*Elaphe guttata*), and leopard tortoise. An egg
yolk-based extender was found to be associated with the best results, and short-term success was found using refrigeration for samples collected from cornsnakes, green iguanas, and leopard tortoises (Fahrig et al., 2007; Zimmerman et al., 2013, Zimmerman and Mitchell, 2017). In O. maccanni spermatozoa-maintained motility greater than 70% following 5 days of storage when incubated at 4°C following dilution in a Ham’s F-10 (Molinia et al., 2010). Attempts at spermatozoa cryopreservation in reptiles are limited to a few species, the saltwater crocodile (Crocodylus porosus), American alligator (Alligator mississippiensis), argentine black and white tegu (Tubinambis merianae), Eastern water skink (Eulamprus quoyii), red diamond rattlesnake (Crotalus ruber) and Burmese python (Python bivittatus) (Johnston et al., 2014, 2017; Young et al., 2017, 2017; Hobbs et al., 2018; Mengden et al. 1980; Clulow and Clulow, 2016; Platz et al., 1980, Zacariotti et al., 2011). The most extensive work looking at cryopreservation has been performed in C. porosus. One limited report exists in chelonians, demonstrating no recovery of spermatozoa using pellet freezing. The freezing rate of this is estimated to be -20-30°C/min. (Platz et al., 1980). All these studies reported various methodologies; a concerted effort should be made by the individuals investigating these methods to standardize the approach and use similar methods so information can be accurately compared, shared, and transferred when the information is limited. Collaboration in this respect may advance the field at an accelerated rate, in a time where technology development is needed sooner rather than later. It is clear from the preliminary work that has been done that taxon differences likely exist, as lizards may need a slower cooling rate for cryopreservation and crocodilians likely benefit from a faster freeze rate. Motility recovery with cryopreservation is the goal but it appears across taxa that low motility following cryopreservation is common. These limited results suggest that more research is needed if we hope to develop sustainable assisted reproduction programs for reptiles.
The purpose of this study was to further develop our understanding of short-term extension of squamate spermatozoa using commercial semen extenders and crystalloid solutions. The specific objectives of this study were to: 1) determine the % survival of spermatozoa collected from green anoles (Anolis carolinensis) and banded water snakes (Nerodia fasciata) following treatment with commercial semen extenders and crystalloid solutions commonly used for mammals, and 2) measure the impact of these extenders on spermatozoa motility. Our hypotheses were that 1) spermatozoa motility would be higher with the commercial semen extenders compared with the crystalloids, and that 2) spermatozoa motility would be highest in samples stored in test yolk buffer, INRA 96, and sperm wash media (SWM).

7.2. MATERIALS AND METHODS

A longitudinal prospective experimental study was performed in accordance with the rules and regulations established by Louisiana State University’s institutional animal care and use committee (protocol # 18-034). A total of twelve animals, were used for this study, 6 green anoles and 6 water snakes.

All wild-caught animals were sourced from a reptile distributor. These species were selected as models because they are not considered threatened. The sample size determined for this study was based on the following a priori information: an alpha=0.05, a power=0.8, an expected difference in motility of 70% between egg yolk buffer and the other extenders, and a 1/1 ratio for subject comparison. All animals were transported to the Louisiana State University School of Veterinary Medicine during the months gametogenesis has been observed for each species (green anoles: April-June; water snakes: August- September). Upon arrival, each animal received a thorough physical examination to confirm it was in good health. Physical
measurements, including body weight, snout-vent length, and snout tail-length, were recorded at the time of the examination.

Animals were euthanized in order to collect epididymal spermatozoa; post-mortem sampling was done because ante-mortem techniques do not provide sufficient sample volumes for serial testing. For euthanasia, animals were restrained by hand, sedated with alfaxalone (Alfaxan, Jurox, Kansas City, MO) 20 mg/kg subcutaneously, and then euthanized using a 390 mg/kg dose of sodium pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) intracardiac. This was followed by cervical dislocation. Death was confirmed using a Doppler (Parks Medical, Aloha, OR). Aseptic surgical techniques were used to collect the reproductive tract immediately post-euthanasia. The complete male reproductive tract, including the testicles, epididymides, ductus deferens, and urodeal connection, was removed. Testicular weight and size were measured to calculate a gonadal somatic index (GSI). The epididymides were placed into a petri dish with 1 mL and 3 mL of SWM (Irvine Scientific, Irvine, CA) for the green anoles and water snakes, respectively. This was done to prevent desiccation; the volume differential was based on the larger size of the snake epididymides.

Both epididymides were elongated under a dissecting scope using micro tissue forceps and the semen released from the epididymides by squeezing the epididymal tubes; this was continued until all semen was released from within the tubule. This method allowed for a pure epididymal semen sample and avoided the blood/somatic cell contamination associated with macerating the epididymides. The spermatozoa collected from both epididymides were pooled. Baseline spermatozoa motility and concentration were measured to ensure adequate spermatozoa concentration and motility for testing. Samples were required to have at least 50% motility for inclusion. The pooled epididymal spermatozoa and SWM were pipetted into a 1.5 mL Eppendorf
microcentrifuge tube and centrifuged at 100 x G for 10 minutes. The SWM supernatant was removed and the volume of the pellet quantified with a micropipettor. The pellets were then aliquoted into six separate commercial extender treatments and three crystalloid treatments and extended by a 1:10 (v/v) dilution for green anoles or a 1:100 (v/v) dilution for the water snakes. The six different commercial semen extenders were Ham’s F-10 without albumin (F10; ThermoFischer Scientific, Waltham, MA), a commercial egg-yolk based extender (TEST-yolk buffer; Irvine Scientific, Irvine, CA), Hank’s balanced salt solution (HBSS; 0.137 M NaCl, 5.4 mM KCl, 1.0 mM MgSO\(_4\), 0.25 mM Na\(_2\)HPO\(_4\), 0.44 mM KH\(_2\)PO\(_4\), 4.32 mM NaHCO\(_3\), and 5.55mM glucose), a commercial milk-based extender (INRA 96; IMV Technologies, Maple Grove, MN), a Tris based extender (Andro pro chill LT, MoFa, industries), and sperm washing media (Irvine Scientific, Irvine, CA). The three crystalloid solutions used were 0.9% sodium chloride injection (0.9% NaCl, Baxter Healthcare, Deefield, IL), phosphate buffered saline (PBS; ThermoFischer Scientific, Waltham, MA), and lactated Ringer’s solution (LRS; Hospira, Lake Forest, IL).

Epididymal spermatozoa concentrations were measured using a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) on a light microscope (CX41, Olympus Corporation, Tokyo, Japan) at 200-x magnification. Photographic images of the spermatozoa were captured with the charge-coupled device (CCD) camera of a Computer-Assisted Sperm Analysis (CASA) system (HTM-CEROS, version 14 Build 013, Hamilton Thorne Bio- sciences, MA, US). Based on the total volume of pellet recovered from each animal, 0.5 µL and 2 µL of sample were used for each sample time point in the green anoles and water snakes, respectively. The total number of spermatozoa were counted in 10 squares; this was repeated three times and the numbers averaged. Concentrations were confirmed with video playback. Motility was
measured by placing 0.5 µL and 2 µL of sample for green anoles and water snakes, respectively, on the counting chamber and covering the sample with a coverslip. The motility and curvilinear velocity of the semen were measured at 10 second intervals in three separate fields and documented using the CASA. Cell detection was predetermined for each species and cell size set based on pixel numbers. Previous pilot testing demonstrated that spermatozoa detection should be set at a minimum contrast of 40 pixels and cell size at 4 pixels. A total of 100 frames were captured for each measurement at 60 frames per second. Spermatozoa with an average measured path velocity (VAP) of >20 µm/s were counted as motile. This was based on pilot trials demonstrating that sperm with a VAP <20 µm/s were not detected as motile by gross analysis by one of the investigators (SP). Static settings were pre-selected to analyze both of these species at an average path of detection (VAP) at < 5 µm/s and straight-line velocity (VSL) at < 2 µm/s. These settings were predetermined based on pilot data demonstrating that the CASA picked up > 90% of the spermatozoa in the samples. Three measurements with different viewing areas with 50-200 spermatozoa in each observation were measured and averaged for each observation. Upon completing each observation, videos were reviewed and tracks with any erroneous readings were deleted manually, these included immotile sperm or other cell types (red blood cells, somatic cells) detected by the CASA.

After the baseline measurements were collected, the samples were immediately stored in a commercial refrigerator at 4°C. Data collected from the CASA included the following: motile cells (%); progressive motility(%); rapid, medium, slow, and static velocities (%); path velocity (VAP, µm/s); progressive velocity (VSL, µm/s); track speed (VCL, µm/s); lateral amplitude (ALH, µm); beat frequency (BCF, Hz); straightness(STR, %); linearity (LIN, %); elongation(%)
and area (µm²). Samples were analyzed for all of these measurements at the following time points: T0: 0 hours, T1: 12 hours, T2: 24 hours, T3: 48 hours, and T4: 72 hours.

7.2.1. STATISTICAL METHODS

Continuous data were evaluated for normality using the Shapiro-Wilk test, skewness, kurtosis, and q-q plots. Normally distributed data are reported by the mean, standard deviation (SD), and minimum-maximum (min-max) values, while non-normally distributed data are reported as the median, 25-75 quartiles (%), and min-max values. Non-normally distributed data were log transformed prior to analysis. Mixed linear models were developed for green anoles and watersnakes. Animal served the random variable in the model, while extender type, extender type (commercial v. crystalloid), actual extender (TYB, F-10, AndroProChill, INRA, LRS, PBS, NaCl, HBSS, SWM), and time were the fixed variables. Akaike’s information criterion was used to assess model fit. A separate statistical analysis were done using SPSS 25.0 (IBM Statistics, Armonk, NY). A P ≤ 0.05 was used to determine statistical significance.

7.3. RESULTS

Green Anole (Anolis carolinensis)

Descriptive statistics for the body weights, testicular measurements, GSI, and total spermatozoa recovered from green anoles are reported in Table 7.1 and 7.2. Motility, progressive motility, and average path velocity (VAPµm/s) for spermatozoa over time is presented in Table 7.3. Mixed model results for the green anole can be found in Table 7.4. There were significant differences in time, extender, and the time*treatment interaction % motile cells, motility, rapid velocity, slow velocity, static velocity, VAP, VSL, VCL, BCF, STR, and LIN, STR, and BCF varied depending on time (all P<0.05, Table 7.4). Only time and extender were significantly
different for progressive motility and medium velocity (p<0.05, Table 7.4). Figures 7.1, 7.2, 7.3 and 7.4 show graphical representation of median motility over time based on extender type.

Banded water snake (Nerodia fasciata)

Descriptive statistics for the whole-body weights, testicular measurements, estimated testicular volumes, GSI, and total spermatozoa recovered from the watersnakes are reported in Table 7.1 and 7.2. Motility, progressive motility, and average path velocity (VAP $\mu$m/s) for spermatozoa over time is presented in Table 7.4. Mixed model results for the banded water snake can be found in Table 7.5. There were significant differences in time, extender, extender type, and the time*treatment interaction for all outcome variables (all P<0.05, Table 7.5). Figures 7.5, 7.6, 7.7 and 7.8 show graphical representation of median motility over time based on extender type.

7.4. DISCUSSION

The results of this study represent the first comprehensive evaluation of semen extenders and short-term cool storage for a lizard or snake. Additionally, this study was the first to use CASA to evaluate reptile spermatozoa and obtain objective motility data for reptiles longitudinally. This study demonstrated that epididymal spermatozoa from different species of squamates can differ in their survival under the same conditions. This finding is important, as there are >10,000 species of reptiles, and suggests that species will need to be evaluated on a case-by-case basis to develop assisted reproductive programs.

The first hypothesis was proven for the banded water snakes but not the green anoles. In the banded water snakes, spermatozoa motility was significantly higher with the commercial extenders compared with the crystalloid solutions (Figure 7.5), confirming that water snake epididymal spermatozoa can undergo short term cooled storage with the appropriate semen
Table 7.1. Descriptive statistics for body weights, testicular measurements, and spermatozoa collection for green anoles and banded water snakes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Species</th>
<th>Mean</th>
<th>Median</th>
<th>SD</th>
<th>25-75%</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td><em>Green anole</em></td>
<td>3.6</td>
<td>0.43</td>
<td>3.1</td>
<td>3.1-4.2</td>
<td>261.7-437.1</td>
</tr>
<tr>
<td></td>
<td><em>Water snake</em></td>
<td>350</td>
<td>64.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left Testicle</td>
<td><em>Green anole</em></td>
<td>0.036</td>
<td>0.005</td>
<td>0.03</td>
<td>0.03-0.04</td>
<td>1.43-2.07</td>
</tr>
<tr>
<td>Weight (g)</td>
<td><em>Water snake</em></td>
<td>1.78</td>
<td>0.27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume</td>
<td><em>Green anole</em></td>
<td>69.87</td>
<td>18.16</td>
<td></td>
<td>39.43-88.10</td>
<td></td>
</tr>
<tr>
<td>(mm³)</td>
<td><em>Water snake</em></td>
<td>0.23</td>
<td>0.089</td>
<td></td>
<td>0.10-0.34</td>
<td></td>
</tr>
<tr>
<td>Right Testicle</td>
<td><em>Green anole</em></td>
<td>0.022</td>
<td></td>
<td>0.018-0.022</td>
<td>0.02-0.33</td>
<td>1.15-2.12</td>
</tr>
<tr>
<td>Weight (g)</td>
<td><em>Water snake</em></td>
<td>1.77</td>
<td>0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume</td>
<td><em>Green anole</em></td>
<td>61.76</td>
<td>25.77</td>
<td></td>
<td>34.63-105.49</td>
<td></td>
</tr>
<tr>
<td>(mm³)</td>
<td><em>Water snake</em></td>
<td>0.282</td>
<td>0.17</td>
<td></td>
<td>0.17-0.40</td>
<td></td>
</tr>
<tr>
<td>Gonadal Somatic</td>
<td><em>Green anole</em></td>
<td>3.24</td>
<td>3.35</td>
<td></td>
<td>1.4-9.55</td>
<td></td>
</tr>
<tr>
<td>Index</td>
<td><em>Water snake</em></td>
<td>1.018</td>
<td>0.077</td>
<td></td>
<td>0.88-1.1</td>
<td></td>
</tr>
</tbody>
</table>

extenders. Further, these data demonstrate that water snake spermatozoa can be successfully stored for up to 72 hours under cooled storage, reducing the need to cryopreserve the semen if artificial insemination can be done promptly. The results obtained for this study mimic those reported in corn snakes, although motility in the banded watersnakes was >50% for the entire 72-hour sampling period while cornsnake motility was limited to 48 hours (Fahrig et al., 2007). Motilities of >50% are preferred for insemination studies (Fahrig et al., 2007; Mattson et al., 2007). Based on the results for the water snakes, the authors suggest using commercial extenders.
Table 7.2. Descriptive statistics for volume spermatozoa recovered, concentration of spermatozoa following dilution, and estimated number of spermatozoa collected for green anoles and banded water snakes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Species</th>
<th>Mean</th>
<th>SD</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of spermatozoa recovered</td>
<td>Green anole</td>
<td>98.2</td>
<td>17.49</td>
<td>71.0-120.0</td>
</tr>
<tr>
<td></td>
<td>Water snake</td>
<td>150.83</td>
<td>45.87</td>
<td>100-205</td>
</tr>
<tr>
<td>Concentration of spermatozoa</td>
<td>Green anole</td>
<td>$5.4 \times 10^7$</td>
<td>$1.98 \times 10^7$</td>
<td>$3.50 \times 10^7-8.0 \times 10^7$</td>
</tr>
<tr>
<td>following dilution (Spermatozoa</td>
<td>Water snake</td>
<td>$1.13 \times 10^9$</td>
<td>$6.35 \times 10^8$</td>
<td>$7.03 \times 10^7-2.03 \times 10^9$</td>
</tr>
<tr>
<td>(µL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estimated total Spermatozoa</td>
<td>Green anole</td>
<td>$5.15 \times 10^9$</td>
<td>$1.68 \times 10^9$</td>
<td>$3.50 \times 10^9-8.0 \times 10^9$</td>
</tr>
<tr>
<td>(Spermatozoa /ml)</td>
<td>Water snake</td>
<td>$1.54 \times 10^{10}$</td>
<td>$8.3 \times 10^9$</td>
<td>$1.46 \times 10^9-2.6 \times 10^{10}$</td>
</tr>
</tbody>
</table>

Table 7.3. Motility, progressive motility, and Average Path Velocity (VAP µm/s) for spermatozoa over time for both the green anole and water snake.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Species</th>
<th>Median</th>
<th>25-75%</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility</td>
<td>Green anole</td>
<td>94.33</td>
<td>71.33-97.33</td>
<td>24-100</td>
</tr>
<tr>
<td>Initial</td>
<td></td>
<td>25.19</td>
<td>18.16-30.5</td>
<td>10.33-69.67</td>
</tr>
<tr>
<td>12 hours</td>
<td></td>
<td>17.33</td>
<td>13.67-25.33</td>
<td>0-43.67</td>
</tr>
<tr>
<td>24 hours</td>
<td></td>
<td>16.00</td>
<td>6.91-25.33</td>
<td>0-46.33</td>
</tr>
<tr>
<td>48 hours</td>
<td></td>
<td>8.16</td>
<td>0-16.16</td>
<td>0-29.67</td>
</tr>
<tr>
<td>72 hours</td>
<td></td>
<td>79</td>
<td>33.3-83.58</td>
<td>0-90.67</td>
</tr>
<tr>
<td>Water snake</td>
<td></td>
<td>61.67</td>
<td>6-85.30</td>
<td>0-95</td>
</tr>
<tr>
<td>Initial</td>
<td></td>
<td>63.83</td>
<td>0-82.33</td>
<td>0-91.67</td>
</tr>
<tr>
<td>12 hours</td>
<td></td>
<td>30.67</td>
<td>0-77.7</td>
<td>0-89.33</td>
</tr>
<tr>
<td>24 hours</td>
<td></td>
<td>14.67</td>
<td>0-76.5</td>
<td>0-92.3</td>
</tr>
<tr>
<td>48 hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72 hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(table cont’d)
first when developing this type of study for a new snake species.

In the green anoles, spermatozoa motility was poor in both the commercial extenders and the crystalloids (Figure 7.1). This finding in the green anoles is concerning because, at this time, there is no apparent extender that can be used to store the spermatozoa for this species. While this is not ultimately a concern for green anoles because they are a common species, similar findings for other small lacertillans that are threatened or endangered could be a concern. It is possible that intrinsic spermatozoa mechanisms could be responsible for motility in this species.
Table 7.4. Mixed model results for the green anole (*Anolis carolinensis*). Significant models are bolded (p<0.05).

<table>
<thead>
<tr>
<th>Anolis carolinensis</th>
<th>Model</th>
<th>Akaike’s Information Criteria (AIC)</th>
<th>F-statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motile cells (%)</td>
<td>Time*Treatment</td>
<td>1710.856</td>
<td>5.885</td>
<td>( \leq 0.001 )</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>57.085</td>
<td></td>
<td>( \leq 0.001 )</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>4.449</td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Crystalloid v. Extender</td>
<td>2045.223</td>
<td>0.508</td>
<td>0.511</td>
</tr>
<tr>
<td>Progressive Motility (%)</td>
<td>Time*Treatment</td>
<td>1527.29</td>
<td>1.279</td>
<td>0.164</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>15.994</td>
<td></td>
<td>( \leq 0.001 )</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>2.313</td>
<td></td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>Crystalloid v. Extender</td>
<td>1705.77</td>
<td>0.708</td>
<td>0.411</td>
</tr>
<tr>
<td>Rapid velocity (%)</td>
<td>Time*Treatment</td>
<td>1595.773</td>
<td>1.561</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>15.411</td>
<td></td>
<td>( \leq 0.001 )</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>2.238</td>
<td></td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>Crystalloid v. Extender</td>
<td>1793.840</td>
<td>0.762</td>
<td>0.416</td>
</tr>
<tr>
<td>Medium velocity (%)</td>
<td>Time*Treatment</td>
<td>1715.779</td>
<td>2.653</td>
<td>0.692</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>3.805</td>
<td></td>
<td>( \leq 0.001 )</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>3.948</td>
<td></td>
<td>( \leq 0.001 )</td>
</tr>
<tr>
<td></td>
<td>Crystalloid v. Extender</td>
<td>1946.770</td>
<td>0.085</td>
<td>0.776</td>
</tr>
<tr>
<td>Slow velocity (%)</td>
<td>Time*Treatment</td>
<td>1847.711</td>
<td>3.903</td>
<td>( \leq 0.001 )</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>6.062</td>
<td></td>
<td>( \leq 0.001 )</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>3.948</td>
<td></td>
<td>( \leq 0.001 )</td>
</tr>
<tr>
<td></td>
<td>Crystalloid v. Extender</td>
<td>2172.619</td>
<td>0.085</td>
<td>0.093</td>
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</table>

(table cont’d)
<table>
<thead>
<tr>
<th>(table cont’d)</th>
<th>Model</th>
<th>Akaike’s Information Criteria</th>
<th>F-statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anolis carolinensis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Static velocity (%)</td>
<td>Time*Treatment</td>
<td>1901.277</td>
<td>7.571</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>87.511</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>6.405</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crystalloid v. Extender</td>
<td>2314.643</td>
<td>0.830</td>
<td>0.384</td>
</tr>
<tr>
<td>Path Velocity (VAP, µm/s)</td>
<td>Time*Treatment</td>
<td>1564.381</td>
<td>3.472</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>17.408</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>10.170</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crystalloid v. Extender</td>
<td>2314.643</td>
<td>0.830</td>
<td>0.384</td>
</tr>
<tr>
<td>Progressive velocity (VSL, µm/s)</td>
<td>Time*Treatment</td>
<td>1515.366</td>
<td>2.649</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>12.384</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>8.733</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crystalloid v. Extender</td>
<td>1783.393</td>
<td>0.157</td>
<td>0.701</td>
</tr>
<tr>
<td>Track Speed (VCL, µm/s)</td>
<td>Time*Treatment</td>
<td>1710.757</td>
<td>5.181</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>29.629</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>13.126</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crystalloid v. Extender</td>
<td>2080.843</td>
<td>0.655</td>
<td>0.430</td>
</tr>
<tr>
<td>Beat Frequency (BCF, Hz)</td>
<td>Time*Treatment</td>
<td>1565.741</td>
<td>4.544</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>26.069</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>15.326</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crystalloid v. Extender</td>
<td>2080.843</td>
<td>12.755</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Straightness (STR, %)</td>
<td>Time*Treatment</td>
<td>1793.233</td>
<td>8.808</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>54.302</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>35.720</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crystalloid v. Extender</td>
<td>2290.416</td>
<td>10.471</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Animal</td>
<td>Model</td>
<td>Akaike’s Information Criteria</td>
<td>F-statistic</td>
<td>P-Value</td>
</tr>
<tr>
<td>-----------------</td>
<td>------------------------------</td>
<td>-------------------------------</td>
<td>------------</td>
<td>---------</td>
</tr>
<tr>
<td><em>Anolis carolinensis</em> Straightness (STR, %)</td>
<td>Crystalloid v. Extender</td>
<td>2290.416</td>
<td>10.471</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time*Treatment</td>
<td>1717.348</td>
<td>4.889</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td></td>
<td>21.821</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td></td>
<td>15.262</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Crystalloid v. Extender</td>
<td>2091.439</td>
<td>0.3986</td>
<td>0.065</td>
</tr>
</tbody>
</table>

Table 7.5. Mixed model results for the watersnake (*Nerodia fasciata*). Significant models are bolded (p<0.05).

<table>
<thead>
<tr>
<th>Animal</th>
<th>Model</th>
<th>Akaike’s Information Criteria (AIC)</th>
<th>F-statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nerodia fasciata</em> Motile cells (%)</td>
<td>Time*Treatment</td>
<td>1710.856</td>
<td>5.842</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>26.018</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>36.052</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crystalloid v. Extender</td>
<td>2472.175</td>
<td>15.092</td>
<td>≤0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Animal</th>
<th>Model</th>
<th>Akaike’s Information Criteria (AIC)</th>
<th>F-statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nerodia fasciata</em> Progressive Motility (%)</td>
<td>Time*Treatment</td>
<td>1168.896</td>
<td>4.712</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>30.984</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>29.926</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crystalloid v. Extender</td>
<td>2179.775</td>
<td>15.322</td>
<td>≤0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Animal</th>
<th>Model</th>
<th>Akaike’s Information Criteria (AIC)</th>
<th>F-statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nerodia fasciata</em> Rapid velocity (%)</td>
<td>Time*Treatment</td>
<td>1906.857</td>
<td>4.760</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>26.330</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>31.209</td>
<td>≤0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crystalloid v. Extender</td>
<td>2413.669</td>
<td>15.848</td>
<td>≤0.001</td>
</tr>
</tbody>
</table>

(table cont’d)
### Medium velocity (%)

<table>
<thead>
<tr>
<th>Model</th>
<th>Akaike's Information Criteria (AIC)</th>
<th>F-statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time*Treatment</td>
<td>1392.962</td>
<td>5.954</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Time</td>
<td>8.091</td>
<td></td>
<td>≤0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>55.288</td>
<td></td>
<td>≤0.001</td>
</tr>
<tr>
<td>Crystalloid v. Extender</td>
<td>1865.656</td>
<td>110.406</td>
<td>≤0.001</td>
</tr>
</tbody>
</table>

### Nerodia fasciata

#### Slow velocity (%)

<table>
<thead>
<tr>
<th>Model</th>
<th>Akaike's Information Criteria (AIC)</th>
<th>F-statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time*Treatment</td>
<td>1596.216</td>
<td>8.547</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Time</td>
<td>6.106</td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>Treatment</td>
<td>16.788</td>
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<td>≤0.001</td>
</tr>
<tr>
<td>Crystalloid v. Extender</td>
<td>1954.306</td>
<td>18.361</td>
<td>≤0.001</td>
</tr>
</tbody>
</table>

#### Static velocity (%)

<table>
<thead>
<tr>
<th>Model</th>
<th>Akaike's Information Criteria (AIC)</th>
<th>F-statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time*Treatment</td>
<td>2009.051</td>
<td>5.868</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Time</td>
<td>29.682</td>
<td></td>
<td>≤0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>40.683</td>
<td></td>
<td>≤0.001</td>
</tr>
<tr>
<td>Crystalloid v. Extender</td>
<td>2491.538</td>
<td>256.989</td>
<td>≤0.001</td>
</tr>
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</table>

#### Path Velocity (VAP, µm/s)

<table>
<thead>
<tr>
<th>Model</th>
<th>Akaike's Information Criteria (AIC)</th>
<th>F-statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time*Treatment</td>
<td>1767.173</td>
<td>3.652</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Time</td>
<td>26.589</td>
<td></td>
<td>≤0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>30.293</td>
<td></td>
<td>≤0.001</td>
</tr>
<tr>
<td>Crystalloid v. Extender</td>
<td>2225.426</td>
<td>188.887</td>
<td>≤0.001</td>
</tr>
</tbody>
</table>

#### Progressive velocity (VSL, µm/s)

<table>
<thead>
<tr>
<th>Model</th>
<th>Akaike's Information Criteria (AIC)</th>
<th>F-statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time*Treatment</td>
<td>1557.399</td>
<td>3.546</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Time</td>
<td>29.892</td>
<td></td>
<td>≤0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>32.540</td>
<td></td>
<td>≤0.001</td>
</tr>
<tr>
<td>Crystalloid v. Extender</td>
<td>1992.242</td>
<td>221.592</td>
<td>≤0.001</td>
</tr>
</tbody>
</table>
(table cont’d)

<table>
<thead>
<tr>
<th>Model</th>
<th>Akaike’s Information Criteria (AIC)</th>
<th>F-statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Track Speed (VCL, µm/s)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time*Treatment</td>
<td>2055.471</td>
<td>3.294</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Time</td>
<td>23.290</td>
<td>1.982</td>
<td>≤0.001</td>
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<td>Treatment</td>
<td>24.460</td>
<td>14.037</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Crystalloid v. Extender</td>
<td>2512.473</td>
<td>5.950</td>
<td>≤0.001</td>
</tr>
<tr>
<td><strong>Beat Frequency (BCF, Hz)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time*Treatment</td>
<td>1570.553</td>
<td>5.113</td>
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</tr>
<tr>
<td>Time</td>
<td>19.282</td>
<td>1.914</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>28.770</td>
<td>13.011</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Crystalloid v. Extender</td>
<td>1882.581</td>
<td>13.011</td>
<td>≤0.001</td>
</tr>
<tr>
<td><strong>Straightness (STR, %)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time*Treatment</td>
<td>1856.524</td>
<td>5.244</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Time</td>
<td>30.312</td>
<td>1.914</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>45.859</td>
<td>13.011</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Crystalloid v. Extender</td>
<td>2293.834</td>
<td>13.011</td>
<td>≤0.001</td>
</tr>
<tr>
<td><strong>Linearity (Lin, %)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time*Treatment</td>
<td>1659.990</td>
<td>5.046</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Time</td>
<td>22.756</td>
<td>1.914</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>46.187</td>
<td>13.011</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Crystalloid v. Extender</td>
<td>2086.00</td>
<td>13.011</td>
<td>≤0.001</td>
</tr>
</tbody>
</table>
Figure 7.1. Median motility over 72 hours between commercial semen extenders and crystalloid solutions in the green anole (*Anolis carolinensis*) for epididymal spermatozoa at 4°C.

Figure 7.2. Median motility over 72 hours between all different solutions utilized to store epididymal spermatozoa at 4°C in (*Anolis carolinensis*).
Figure 7.3. Median progressive motility over 72 hours between all different solutions utilized to store epididymal spermatozoa at 4°C in *Anolis carolinensis*.

Figure 7.4. Median average path velocity (VAP-µm/s) over 72 hours between all different solutions utilized to store epididymal spermatozoa at 4°C in *Anolis carolinensis*.
Figure 7.5. Median motility over 72 hours between commercial semen extenders and crystalloid solutions in the banded water snake (*Nerodia fasciata*) for epididymal spermatozoa at 4°C.

Figure 7.6. Median motility over 72 hours between all different solutions utilized to store epididymal spermatozoa at 4°C in the banded water snake (*Nerodia fasciata*).
Figure 7.7 Median progressive motility over 72 hours between all different solutions utilized to store epididymal spermatozoa at 4°C in banded water snake (*Nerodia fasciata*).

Figure 7.8 Median average path velocity (VAP-µm/s) over 72 hours between all different solutions utilized to store epididymal spermatozoa at 4°C in banded water snake (*Nerodia fasciata*).
and that some substrate is absent or at too low a concentration in the tested extenders. Research assessing specific mechanisms of the sperm may be needed to develop more complete extenders.

Cold shock can impact mammalian spermatozoa motility and may have played a role in this study (Hammersteadt et al., 1990). The small sample volumes used for the green anoles may have predisposed them to cold shock, as the green anole samples were ¼ of the volume of the snake samples. Cold shock has not been tested extensively in reptiles, with a single study in saltwater crocodiles (*Crocodylus porosus*) simulating a rapid change in temperature (Johnston et al., 2014). In that study the crocodilian spermatozoa were found to be cold shock resistant; however, despite only 10 µL being used, these samples are similar to the samples used for the anoles. Reptiles are ectotherms and can withstand a wide range of physiological temperatures, and green anoles are no exception as these animals naturally encounter short periods of freezing in their range. However, based on the anole’s breeding cycle, spermatogenesis is not expected during this time and thus motility would not be expected during times of freezing under natural circumstances. The impact of extension ratio, sample concentration, and sample volume on spermatozoa motility over time are not well understood in reptiles, and these too may have impacted the green anole results. The limited number of studies in reptiles have focused on fixed pre-determined volumes rather than evaluating a dosing effect. One of the limitations with these studies, as was the case with the anoles, is limited sample volume. The authors elected to test a variety of extenders versus performing a more limited study on extenders and evaluating extension ratio and sample volumes to attempt to identify a small number of potential extenders for follow-up study to better characterize these secondary questions of volume, concentration, and extension. The latter type of study design requires each extended to be tested, and in the case of the green anole would have required significantly more resources to complete the study. The
banded water snake results further support our study design, as we now know several extenders that can be further focused on to better characterize their value.

The results from this study appear to confirm a pattern with other squamates, that lizard semen appears less stable in cooled refrigeration than snake semen. Green iguana spermatozoa motility was found to significantly decrease after 24 hours in a 1:1 ratio of modified Ham’s F-10 with albumin and refrigeration test yolk buffer, with median motilities of 60% at 24 hours and 33% and 0% at 48 and 72 hours, respectively (Zimmerman et al., 2013). The extender combination used in the green iguana study was similar to that used in corn snakes, which reported spermatozoa motilities of 65%, 58%, and 38% at 24, 28, and 72 hours (Fahrig et al., 2007). Preliminary work with Grand Cayman blue iguana hybrids (*Cyclura lewisi x nubila*) has found that spermatozoa motility was poor after 12 hours of refrigeration in both Test Yolk buffer and INRA 96 (Perry, unpublished data). Despite these poor results for lizards one study does exist where motility was able to be maintained. In *O. maccanni* spermatozoa maintained motility greater than 70% after 5 days when incubated at 4°C following dilution in a Ham-F 10 (Molina et al., 2010; Gist et al., 2000). These same extenders were found to be useful for short term extension and cooling in the banded water snakes from the current study. These findings further reinforce the need to further elucidate the mechanistic differences between reptiles to ensure our success with developing functional management plans.

The second hypothesis was again (partially) proven for the banded water snakes but not the green anoles. For the water snakes, INRA and SWM had sustained motility over the duration of the study, while test yolk buffer experienced a decline after 24 hours. Ham’s F10, like INRA and SWM, also was found to have sustained motility over the duration of the study. The crystalloids experienced rapid declines in motility in the water snake study. These products were
initially included because they represent the types of solutions commonly available to herpetological veterinarians. The authors’ intent was to show that, although considered physiologic, they would not be useful for reptile spermatozoa. All the following extenders, INRA, SWM, and test yolk buffer, as well as all crystalloids, performed poorly in the green anole study. To further characterize what might be contributing to the poor results with the crystalloid solutions, a more critical evaluation of the solutions is required. Tables 7.6 and 7.7 include the formulations for the crystalloids and extenders used in the study; it is important to note that some of the extenders are proprietary and thus the recipes may be incomplete. The majority of the osmolarities reported are within the same physiological range of 280-310 mOsm/L. Lower or higher osmolarities could lead to spermatozoa swelling or crenation, respectively. The pH range for the commercial extenders is between 6.9-7.4, while the crystalloids such as 0.9% saline and lactated ringers solution have pH’s of 5.5 and 6.5 respectively while PBS is buffered with a pH of 7.3-7.4. The lower pH values would not be expected to be physiologic for reptile spermatozoa, and thus contribute to the poor results noted. Another deficiency in the crystalloid solutions is that they don’t contain energy sources for the spermatozoa to maintain motility, which correlates with the rapid loss of motility noted with these solutions. Four of the commercial extenders (TEST-yolk buffer, INRA 96, Sperm wash media, and AndroPro Chill) were supplemented with proteins (egg yolk, casein, animal free protein, or human serum albumin) to protect the spermatozoa plasma membrane. All the extenders tested in this study provide some form of energy substrate for the spermatozoa. The three media found to have the greatest effect in the banded watersnakes contained a variety of compounds, including dextrose, glucose, lactose, sodium pyruvate, and sodium lactate as energy substrates. When considering extenders, these types of energy substrates should be considered
important. A buffering system is also a critical component to each extender. For the banded water snakes, the extenders that sustained the best motility used different buffering systems; HEPES for INRA 96, sodium bicarbonate and HEPES for SWM, and sodium bicarbonate for Ham’s F-10. These two buffering systems should be considered when evaluating new extenders. Antibiotics and antifungals are something that can be added to extenders but are not used in crystalloids. In this study, only the INRA 96 contained antibiotics and antifungals. These may be necessary for reptiles, as ejaculates are collected from the cloaca, which is a general receptacle for feces, urine, and reproductive secretions. While not evaluated in this study, this is something to consider in future studies to minimize sample contamination.

There were significant differences in the all spermatozoa motility parameters for the water snakes including path velocity (VAP), progressive velocity (VSL), track speed (VCL), beat frequency (BCF), straightness (STR), and linearity (Lin). While there were also some significant differences in these motility parameters by extender for the green anoles, the value of these differences is limited since overall motility was low. At this point in time we do not fully understand what values produced by the CASA may be important for evaluating spermatozoa when it comes to determining fertilizing ability. For these reasons we focus on motility and progressive motility in this paper. Further work needs to be performed evaluating what parameters are important when predicting fertility and conception rate in reptiles. In reptiles, our understanding of spermatozoa motility in both the male and female reproductive tract is limited. However, based on their gross and microscopic reproductive anatomy, it is suspected that male reptiles are comparable to the higher vertebrates (birds, mammals) rather than lower vertebrates (fish, amphibians). In the reproductive tract of male lizards, motile spermatozoa appear to only
Table 7.6. Electrolyte composition of commercially available crystalloid solutions.

<table>
<thead>
<tr>
<th>Fluid</th>
<th>NaCl (g/L)</th>
<th>Sodium lactate (g/L)</th>
<th>Sodium phosphate dibasic (g/L)</th>
<th>Na⁺ (mEq/L)</th>
<th>KH₂PO₄ (g/L)</th>
<th>Cl⁻ (mEq/L)</th>
<th>K⁺ (mEq/L)</th>
<th>KCL (g/L)</th>
<th>Calcium chloride (g/L)</th>
<th>Ca²⁺ (mEq/L)</th>
<th>Mg²⁺ (mEq/L)</th>
<th>Buffer</th>
<th>Osmolarity (mOsm/L)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl</td>
<td>4.5</td>
<td>-</td>
<td>-</td>
<td>154</td>
<td>-</td>
<td>154</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>None</td>
<td>308</td>
<td>5.5</td>
</tr>
<tr>
<td>Ringer’s Lactated Solution</td>
<td>6</td>
<td>3.1</td>
<td>-</td>
<td>130</td>
<td>-</td>
<td>109</td>
<td>4</td>
<td>0.3</td>
<td>0.2</td>
<td>3</td>
<td>-</td>
<td>Lactate</td>
<td>272</td>
<td>6.5</td>
</tr>
<tr>
<td>Phosphate buffered saline</td>
<td>9</td>
<td>-</td>
<td>0.795</td>
<td>0.144</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Phosphate</td>
<td>280-315</td>
<td>7.3</td>
</tr>
<tr>
<td>Extender</td>
<td>Buffer system</td>
<td>Salts and Ions</td>
<td>Protein</td>
<td>Antibiotics/Antifungals</td>
<td>Energy source</td>
<td>Osmolarity (mOsm/L)</td>
<td>pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>--------------------------------</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test Yolk Buffer (TYB)</td>
<td>TES Tris</td>
<td></td>
<td>Egg yolk 200 mg/ml</td>
<td>gentamicin sulfate 10µg/ml</td>
<td>Dextrose</td>
<td>310-330</td>
<td>7.0-7.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INRA 96</td>
<td>HEPES</td>
<td>Hanks’ salts</td>
<td>Native Phosphocaseinate</td>
<td>Penicillin 0.038 mg/ml Gentamicin 0.105 mg/ml Amphotericin 0.315 µg/ml</td>
<td>Glucose Lactose</td>
<td>310</td>
<td>6.9-7.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ham’s F-10</td>
<td>Sodium Bicarbonate 1 g/L</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Dextrose Sodium Pyruvate</td>
<td>270-320</td>
<td>7.2-7.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sperm Wash Media</td>
<td>HEPES-21 mM Sodium Bicarbonate-4mM</td>
<td>Sodium Chloride Potassium Chloride Magnesium Sulfate Potassium Phosphate Calcium Chloride</td>
<td>Human Serum Albumin 5 mg/ml</td>
<td>None</td>
<td>Dextrose anhydrase Sodium Pyruvate Sodium Lactate</td>
<td>272-288</td>
<td>7.3-7.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(table cont’d)
<table>
<thead>
<tr>
<th>Extender</th>
<th>Buffer system</th>
<th>Salts and Ions</th>
<th>Protein</th>
<th>Antibiotics/Antifungals</th>
<th>Energy source</th>
<th>Osmolarity (mOsm/L)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>AndroProChill LT</td>
<td>Tris</td>
<td>Unknown</td>
<td>Animal Free Protein</td>
<td>Gentamicin</td>
<td>Citric acid</td>
<td>Glucose Fructose</td>
<td>-</td>
</tr>
<tr>
<td>Hanks’ Balanced Salt Solution</td>
<td>Sodium Bicarbonate/Phosphate Potassium Phosphate</td>
<td>Sodium Chloride Potassium Chloride Calcium Chloride Magnesium Sulfate</td>
<td>None</td>
<td>None</td>
<td>Glucose</td>
<td>300</td>
<td>7.2</td>
</tr>
</tbody>
</table>
be found in the epididymides and ductus deferens (Depeiges and Dacheaux, 1985; Nirmal and Rai, 1997). Studies in northern house geckos (Hemidactylus flaviviridis) and green iguanas support this theory by confirming that the testicles in both species did not have motile spermatozoa (Zimmerman et al., 2013; Nirmal and Rai, 1997). Motility is initially gained as the spermatozoa progress through the epididymides, and motility patterns have been shown to differ based on the location within the epididymides. In northern house geckos, erratic and circular movements prevailed in the cranial region of the epididymis, zig-zag movements in the middle, and a wavy pattern in the caudal epididymis (Nirmal and Rai, 1997). In Zootoca (formerly Lacerta) vivipara, the percentage of motile spermatozoa also increases as they pass through the epididymal tubule with the highest percentages of motile spermatozoa and sperm velocities being reached in the distal segment of the epididymides where the spermatozoa accumulate (Depeiges et al., 1987). These findings further support our decision to collect epididymal samples to assess the effect of extenders on motility, as collecting post-mortem epididymal samples was key to collecting sufficient volumes and active, motile spermatozoa.

In reptiles, spermatozoa longevity may be influenced by female sperm storage capabilities and seasonality (Gist, 2011). The samples collected in this study were never exposed to the maternal environment, which is likely the best place to preserve sperm function and longevity. In green anoles, females can store sperm from multiple males for several months (Fox, 1963; Server, 2002). The maternal environment likely provides nutritional support for the spermatozoa irrespective of ovarian state. Sperm storage occurs at the utero-vaginal transition in highly specialized tubules that occur only in this section, and spermatozoa have been shown to embed in the tubules in all sides of this area of the oviducal wall (Conner, 1980). These tubules are discrete structures which are highly differentiated from the rest of the oviductal wall. Unlike
in some mammals, the spermatozoa of green anoles have not been found to attach to the walls of the oviduct. There have been no studies to evaluate spermatozoa storage in banded water snakes. Characterizing the chemical and physiologic conditions of the female reproductive tract may prove useful in developing extenders for these animals.

There were several limitations to this study. The authors considered the need to sacrifice animals as a limitation, as the ultimate goal of this work is to collect and extend ante-mortem samples from threatened and endangered reptiles. Unfortunately, the current methods used for collecting ante-mortem samples from reptiles, electroejaculation or digital massage, do not provide sufficient volumes of semen to conduct multi-layered studies. Another potential limitation of this study may be the number of extenders tested. For the green anoles, this reduced the overall volume of samples that could be tested. If reptilian spermatozoa motility is impacted by extender volume/ratio, then it is possible that the motility loss seen in this study could have been minimized by testing fewer extenders and using larger sample volumes. Sample size may also be observed as a limitation for the green anole study; however, the statistical results suggest that the risk of a type II error is low. The significant differences found with the diamond back water snakes confirm that the sample size was adequate, as no risk of a type II error exists since the alternative hypotheses were accepted.

Epididymal spermatozoa collected from green anoles and banded water snakes exhibit taxa and species differences for extending and storing spermatozoa samples at 4°C. In banded water snakes, epididymal spermatozoa were successfully stored at 4°C for 72 hours using Ham’s F-10, INRA 96, and SWM. However, it is important to note that declines in motility are expected, so samples should be screened prior to use to confirm their clinical value. In the green anole, clinically significant spermatozoa motility parameters, including motility, progressive
motility, VAP, VSL, and VCL, declined over the 72-hour study period regardless of extender type. Additionally, there was no difference in green anole spermatozoa motility between crystalloid solutions or commercial semen extenders. Poor short-term extension was observed no matter the treatment. As anthropogenic activities continue to impact reptile populations, it is important that we continue work to develop functional assisted reproduction programs for these animals. This research is a start at providing a direction for further evaluating different methods for short-term extension of squamate semen.
CHAPTER 8. EVALUATING DIFFERENT METHODS OF SPERMATOZOA STORAGE IN RED-EARED SLIDERS TRACHEMYS SCRIPTA ELEGANS

8.1. INTRODUCTION

Chelonians provide a unique challenge for conservation scientists due to their evolutionary history; they are slow to mature, long-lived, and particularly sensitive to ecological, anthropogenic, and natural disturbances. A total of 351 species of chelonians have been characterized and 258 of these animals are included on the IUCN Red List (reptile database, IUCN red list). Based on the IUCN data, over half (57%, 203/351) of all known chelonians are categorized as threatened (i.e., critically endangered, endangered, or vulnerable) or near threatened. Of these species, 9 species are classified as extinct, 50 species are critically endangered, 45 species are endangered, 67 species are vulnerable, and 32 species are near threatened. The remaining animals are either classified as least concern or there is insufficient data for them to be classified (IUCN, 2019). Unfortunately, anthropogenic activities (climate change, habitat destruction, extirpation, illegal food trade, and the pet trade) will continue to place significant pressure on these animals. If we hope to prevent additional species extinctions in this group, we need to develop conservation management plans to help counter or protect them from the forces acting on them. While strides have been made in chelonian conservation to counter some of these threats, limited research has been pursued to develop assisted reproductive technologies for chelonians. These technologies may become even more important if environmental pressures are not controlled and these animals are forced into captivity.

Assisted reproductive technologies have been developed that focus on spermatozoa collection, short-term extension, and long-term biobanking to help augment in situ and ex situ conservation programs for threatened and endangered species. Cryopreservation of gametes for
endangered species is not a new concept, as the idea of a “Frozen Zoo” or Genome Resource
Bank was established in the 1980’s; however, the focus of this work has been primarily limited
to fish, mammals, and birds (Browne et al., 2011; Wildt et al., 1997, 2003, 2010; Benirschke et
al., 1984; Clarke, 2009; Comizzoli et al., 2012, 2014, 2015; Lermen et al., 2009). The benefits of
spermatozoa bio-banking for threatened and endangered species include preserving genetic vigor
and diversity; transporting valuable genes without the stress/expense of moving sensitive,
fractious animals; and insuring existing genetic diversity that protects fitness and species
integrity (Benirschke et al., 1984; Wildt et al., 1997, 2003, 2010; Clarke, 2009; Comizzoli et al.,
2012, 2014, 2015; Lermen et al., 2009). These methods can be used to help sustain genetically
diverse and sustainable populations of rare species and genotypes. Additionally, if proper field
methods were developed, wildlife spermatozoa biobanking could bring new genetics into captive
populations without depleting wild populations, while preserving genetics from specific
population that could be lost due to extinction.

Currently, our working knowledge of assisted reproduction in male reptiles is limited to
our ability to collect semen from less than 0.2% of all reptile species. Of the few chelonian
studies, that are available, ante-mortem semen collection appears to be the most common method
used. Electroejaculation has been used to successfully collect semen from red-eared sliders
(Trachemys scripta elegans), Galapagos tortoises (Chelonoidis nigra), green sea turtles
(Chelonia mydas), olive Ridley turtles (Lepidochelys olivacea), hawksbill turtles (Eretmochelys
imbricata), leopard tortoises (Stigmochyles pardalis), black marsh turtles (Siebenrockiella
crassicollis), and ploughshare tortoises (Astrochelys yniphora) (Wood et al., 1982; Zimmerman
and Mitchell, 2017; Platz et al., 1980; Juvik et al., 1991; Tanasanti et al., 2009; Kimskulvech and
Suttiyotin, 2012; Kawazu et al., 2014). As this method appears to provide consistency for
spermatozoa collection, the next steps for a successful program are the development of short-term extension and biobanking. Historically, chelonian semen has been difficult to store short term to maintain motility and viability. Previous literature reports poor motility upon initial collection. Numerous species have reported to have mean or median values ranging from 5% to 90% following collection either postmortem or by electroejaculation (Wood et al., 1982; Gist et al., 2000; Zimmerman and Mitchell, 2017; Sirinarumitir et al., 2010; Tanasanti et al., 2009). In *Sternotherus odoratus, Trachemys scripta,* and *Chrysemys picta* initial motility was poor following postmortem collection, *Sternotherus odoratus* (28-52%), *Trachemys scripta* (3-4%), and *Chrysemys picta* (2-5%), was reported. These samples were incubated at two temperatures 23°C and 2°C in Ham’s F-10 with bovine serum albumin for 1-3 hrs prior to analysis. Cooler temperatures appeared to maintain motility better in *Sternotherus odoratus* as motility was 52% at 2°C while motility declined to 28% at 23°C. Despite poor motility preservation, samples from this study were determined to be viable and retained the capacity for motility when stored at 4°C for 40 days if methylxanthines such as 3-isobutyl-1-methylxanthine were added to stimulate the spermatozoa (Gist et al., 2000; Clulow and Clulow, 2016).

Semen extenders have been evaluated in olive Ridley turtles, hawksbill turtles, and leopard tortoises. In sea turtles, contradictory information exists on the viability of spermatozoa. In olive Ridley sea turtles (*Lepidochelys olivacea*) and hawksbill turtles (*Eretmochelys imbricata*), mean motility following electroejaculation was 28% in *L. olivacea* and 60% in *E. imbricata.* In *L. olivacea* sperm viability following collection was approximately 90 minutes; however, when extended at 4°C it ranged from 16-22 hours (Tanasanti et al., 2009). Eight semen extenders were evaluated in *L. olivacea* and *E. imbricata* following electroejaculation (EEJ), this included refrigeration medium test yolk buffer, Tyrode medium with albumin, lactate, pyruvate,
Beltsville poultry semen extender, 3% sodium citrate buffer, Phosphate buffered saline, European eel extender (EEL), 1% bovine serum albumin, and Ham’s F-10. Initial motility for this study was poor following collection, with a mean initial motility of 8.3% for *L. olivacea* and 14% for *E. imbricata*. Motility declined to zero at 4°C in all treatments within 6 hours of collection, and most treatments declined more than 50% of initial motility within 1 hour of collection. The TALP based extender and test yolk buffer appeared to be the best for these species of sea turtles (Sirinarumitr et al., 2010). Semen collection using electroejaculation in the leopard tortoise (*Stigmochelys pardalis*) demonstrated a higher mean motility in spermatozoa samples of 57.3% with a range of 10-80% based on individual following collection. Leopard tortoise spermatozoa stored at 4°C in modified Ham’s F10 and test yolk buffer (Irvine Scientific, Santa Ana, CA, USA) at a 1:10 ratio declined to a median motility of 0% within 24 hours. To date, there have been no reports of any attempts to cryopreserve chelonian spermatozoa.

The purpose of this study was to further develop our understanding of short-term extension and cryopreservation of chelonian spermatozoa. The objectives of this study were to: 1) identify semen extenders that could be used for sustaining chelonian spermatozoa under refrigerated conditions, 2) to assess the effects of cryoprotectants on chelonian spermatozoa, and 3) evaluate freezing rates and cryoprotectant concentrations on chelonian spermatozoa. The specific hypotheses tested in this study were: 1) chelonian spermatozoa would survive short term storage for up to 96 hours using INRA 96, Ham’s F10, and Sperm Wash Media (SWM) as semen extenders, 2) spermatozoa motility would decline significantly with increasing cryoprotectant concentrations and exposure times, and 3) chelonian spermatozoa would successfully survive cryopreservation.
8.2. MATERIALS AND METHODS

Animal handling

A longitudinal prospective experimental study was performed in accordance with the rules and regulations established by Louisiana State University’s institutional animal care and use committee (protocol # 18-034). A total of 17 red-eared slider turtles were used for this study. All of the animals were sourced from a commercial turtle farmer, who maintained them in an outdoor communal pond. This species was selected because the study required post-mortem sampling and red-eared slider turtles are considered common. The sample size determined for this study was based on the following a priori information: an alpha=0.05, a power=0.8, an expected difference in motility of 70% between egg yolk buffer and the other extenders, and a 1/1 ratio for subject comparison. All animals were transported to the Louisiana State University School of Veterinary Medicine during the months gametogenesis has been observed for this species (October-January). Upon arrival, each animal received a thorough physical examination to confirm that they were in good health. Physical measurements, including body weight and straight carapace length, were recorded at the time of the examination.

Epididymal collection

Animals were euthanized in order to collect epididymal spermatozoa; post-mortem sampling was done because ante-mortem techniques do not to provide sufficient sample volumes for serial testing. For euthanasia, animals were restrained by hand, sedated with alfaxalone (Alfaxan, Jurox, Kansas City, MO) 10 mg/kg intravenously, and then euthanized using an 85 mg/kg dose of sodium pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) intravenously. Death was confirmed by asystole (Doppler, Parks Medical, Aloha, OR) and the loss of the corneal reflex. Aseptic techniques were used to collect the reproductive tract
immediately after euthanasia. A Dremel rotary tool (Model 200, Dremel, Racine, WI) with a metal cutting bit was used to incise and remove the carapacial bridge (bilaterally) to fully expose the coelomic cavity and complete reproductive tract. The ventral coelomic membrane was entered with mayo scissors and then the testicles, epididymides, ductus deferens, and urodeal connections removed en bloc. Testicular weights and size were measured to calculate a gonadal somatic index (GSI). The epididymides were separated from the reproductive tract and placed into a petri dish with 3 mL of sperm washing media (SWM: Irvine Scientific, Irvine, CA) to prevent desiccation and allow for spermatozoa collection.

_Epididymal processing and spermatozoa recovery_

An incision was made across the epididymis using a #10 scalpel blade to expose the spermatozoa to the SWM. The samples were allowed to incubate at room temperature for 5 minutes to allow for any spermatozoa to swim out of the epididymides. The epididimydes were then massaged to empty any remaining spermatozoa. The spermatozoa collected from the right and left epididymides were pooled and baseline measures for motility and concentration determined to ensure adequate samples for experimentation. Samples were required to have at least 50% motility to be included within the study. Pooled epididymal spermatozoa and SWM were pipetted into a 1.5 mL Eppendorf microcentrifuge tube and then centrifuged at 100 x G for 10 minutes. The SWM supernatant was removed from the sample to quantify the volume of the pellet. Pellet volume was quantified with a micropipettor.

_Semen Extender Study_

The pelleted spermatozoa were randomly (random.org) aliquoted into treatments and resuspended with a 1:100 (v/v) dilution with each respective extender, including Ham’s F-10 without albumin (F10; ThermoFischer Scientific, Waltham, MA), a commercial egg-yolk based
extender (TEST-yolk buffer; Irvine Scientific, Irvine, CA), Hank’s balanced salt solution (HBSS; 0.137 M NaCl, 5.4 mM KCl, 1.0 mM MgSO$_4$, 0.25 mM Na$_2$HPO$_4$, 0.44 mM KH$_2$PO$_4$, 4.32 mM NaHCO$_3$, and 5.55 mM glucose), a commercial milk-based extender (INRA 96; IMV Technologies, Maple Grove, MN), electrolyte free media (0.33 M glucose, 3% bovine serum albumin [Saito et al., 1996]), and SWM.

Epididymal spermatozoa concentrations were measured using a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) on a light microscope (CX41, Olympus Corporation, Tokyo, Japan) at 200-x magnification. Photographic images were captured with the charge-coupled device (CCD) camera of a Computer-Assisted Sperm Analysis (CASA) system (HTM-CEROS, version 14 Build 013, Hamilton Thorne Biosciences, MA). Two μL sample volumes were used for each extender and respective time point. Total concentration was determined by counting the total number of spermatozoa in 10 squares on the Makler chamber; this was repeated three times and the numbers averaged. Total concentrations were confirmed with video playback. Motility was measured by placing 2 μL samples on the counting chamber and covering the sample with a coverslip. The motility and curvilinear velocity of the semen were measured at 10 second intervals in three separate fields and documented using the CASA. Cell detection was predetermined and cell size set based on pixel numbers. Pilot testing determined that cell detection should be set at a minimum contrast of 40 pixels and cell size at a minimum of 4 pixels. A total of 100 frames were captured in each measurement at 60 frames per second. Spermatozoa with an average measured path velocity (VAP) of >20 μm/s were counted as motile. This was based on pilot trials demonstrating that sperm with a VAP <20 μm/s were not motile by gross analysis by one of the investigators (SP). Static settings were pre-determined to analyze samples at an average path of detection (VAP) of <5 μm/s and straight-line velocity
(VSL) of <2 μm/s. These settings were selected based on pilot data confirming that the CASA identified > 90% of the spermatozoa in the samples. Three measurements with different viewing areas of 50-200 spermatozoa in each observation were collected and averaged for each observation. Upon completing each observation, videos were reviewed and tracks with any erroneous readings deleted manually; these included immotile sperm or other cell types (red blood cells, somatic cells) detected by the CASA.

After the baseline measurements were collected, the samples were stored in a commercial refrigerator at 4°C. Data collected from the CASA included the following: motile cells (%); progressive motility(%); rapid, medium, slow, and static velocities (%); path velocity (VAP, μm/s); progressive velocity (VSL, μm/s); track speed (VCL, μm/s); lateral amplitude (ALH, μm); beat frequency (BCF, Hz); straightness(STR,%); linearity (LIN,%); elongation(%); and area (μm²). Samples were analyzed for all of these measurements at the following time points: T0:0 hours, T1: 6 hours, T2: 12 hours, T3:24 hours, T4:48 hours, T5:72 hours, and T6: 96 hours.

Cryotoxicity

Six additional turtles were used to evaluate the cryotoxicity of penetrating cryoprotectants on red-eared slider spermatozoa incubated at room temperature (20 °C) over 40 minutes. Animals were euthanized and the epididymal spermatozoa collected as described previously. All samples were re-suspended 1:100 (v/v) with INRA 96. INRA 96 was chosen based on the results of the previous experiment. 0.5 mL of the extended sample was then aliquoted into nine treatments in 1.5 mL microcentrifuge tubes. Stock solutions of INRA 96 were mixed with the cryoprotectants: mixed Dimethyl-sulfoxide (DMSO) (Thermo-scientific, Waltham, MA), glycerol (Sigma Aldrich, St Louis, MO), and methanol (Fischer Scientific, Hampton, NH). Final concentrations of stock solutions were 10%, 20%, and 30%, respectively.
These solutions were allowed to equilibrate to room temperature over 4 hours. 0.5 mL of the stock extender cryoprotectant solution was used to further extend the spermatozoa samples at a ratio of 1:1 (v/v), with final cryoprotectant concentrations for all three cryoprotectants at 5%, 10%, and 15%, respectively. Samples were analyzed for motility using the CASA as described above. Motility analysis was performed prior to exposure to the cryoprotectant (baseline), immediately following exposure to cryoprotectant, and then every 10 minutes for 40 minutes at room temperature. Samples were briefly vortexed immediately after exposure and prior to analysis to ensure adequate mixing. Samples were analyzed as described above.

Five additional turtles were used to evaluate the effect of cryopreservation temperatures on red-eared slider spermatozoa. The turtles were euthanized and processed as described previously. Pelleted spermatozoa samples were extended to a ratio of 1:100 (v/v) in INRA 96. Extended semen was spermatozoa were allowed to cool at refrigeration temperature (4°C) for 2 hours in 10 mL conical tubes prior to being exposed to cryoprotectant and packaged in French straws (0.25 mL). A stock solution of INRA 96 was mixed with DMSO or glycerol to make final cryoprotectant stock solutions of 4%, 8%, and 12%. DMSO and glycerol were chosen as the cryoprotectants based on the previous experiment. These solutions were allowed to equilibrate to refrigeration temperature (4°C) prior to being mixed with the extended spermatozoa. Extended spermatozoa and the cryoprotectant stock solutions were combined at a 1:1 (v/v) ratio and hand mixed. The target spermatozoa concentration within the straws was 10-20 x 10⁶/ml. 250 µL French straws were hand loaded using a 1 ml syringe with a micropipette tip. Samples were allowed to incubate (4°C) for 20- minute to allow time for the cryoprotectants to penetrate the plasma membrane of the spermatozoa. Samples were collected to measure motility after this 20-minute incubation and prior to freezing. Straws were sealed individually with an ultrasonic straw.
sealer (Minitube, Verona, WI). A Minitube IceCube 14 S was used to freeze the samples at 5°C/min (Figure 8.1), 15 °C/min (Figure 8.2), and 30°C/min (Figure 8.3) from 4 to -80°C.

![Freezing curve](image)

**Figure 8.1.** Freezing curve demonstrating controlled rate freezing of spermatozoa samples packaged in 0.25 mL French straws at 5°C/min from 4 °C to -80°C.

Once the straws achieved a temperature of -80°C, they were held at that temperature for 5 minutes. Next, the samples were plunged into liquid nitrogen and held for 6 months. Straws were then thawed in a warm water bath (40°C) for 7 seconds and then transferred to 2 ml microcentrifuge tubes. Samples were immediately evaluated for motility following thawing using the methods described previously. Semen (10 µL) was then aliquoted into 490 µL of HBSS for flow
Figure 8.2. Freezing curve demonstrating controlled rate freezing of spermatozoa samples packaged in 0.25 mL French straws at 15°C/min from 4°C to -80°C.

cytometry analysis and to dilute the cryoprotectants. A 250 µL aliquot of the sample was stained with 1.25 µL of SYBR-14 and propidium iodine (PI), incubated for 15 minutes in a light deprived environment prior, and then analyzed using flow cytometry. Spermatozoa membrane integrity was evaluated using fluorescent dyes (live/dead sperm viability kit; Molecular Probes, Eugene, OR) by flow cytometry (Becton Dickinson Accuri; Becton Dickinson Biosciences, San Jose, CA). Flow cytometry measured spermatozoa plasma membrane integrity by analyzing 30 µL of the sample at a flow rate of 35 µL/min using CFlow® Plus software (Becton Dickinson Accuri; BD Biosciences). Green fluorescence (SYBR 14) was detected with a 530±15 nm bandpass filter, while red fluorescence (PI) was detected with a >670 nm longpass filter (Cuevas-
Figure 8.3. Freezing curve demonstrating controlled rate freezing of spermatozoa samples packaged in 0.25 mL French straws at 30°C/min from 4°C to -80°C.

Uribe et al., 2015). The proportion of intact spermatozoa are expressed as a percentage of the fluorescent population (i.e., sperm stained with SYBR 14, PI, or both) and exclude non-spermatozoa particles from the calculations.

8.2.1. STATISTICAL METHODS

Continuous data were evaluated using the Shapiro-Wilk test, skewness, kurtosis, and q-q plots. Normally distributed data are reported by the mean, standard deviation (SD), and minimum-maximum (min-max) values, while non-normally distributed data are reported as the median, 25-75 quartiles (%), and min-max values. To evaluate semen extenders, mixed linear
models were used to determine if extender, time, or the interaction of extender and time influenced the different outcome variables for spermatozoa motility and survival. Turtle was used as the random variable in the model, while extender (TYB, F-10, INRA, HBSS, SWM, and EFM), and time were used as the fixed variables. To evaluate cryoprotectant toxicity a mixed linear model was used to determine if cryoprotectant, time, cryoprotectant concentration and the interactions between the three variables influenced the spermatozoa motility upon exposure to cryoprotectants. Turtle was used as the random variable in the model while cryoprotectant (DMS, Glycerol, and Methanol), concentration (5%, 10%, and 15%), and time were used as the fixed variables. Akaike information criterion was used to assess model fit. To evaluate cryopreservation’s effects on spermatozoa motility, a mixed linear model was used to evaluate the effects of cryoprotectant type, cryoprotectant concentration, freezing rate, and time (initial motility, post-cryoprotectant exposure, and post thaw) on spermatozoa motility. Turtle served as the random variable in the model, while freezing rate temperature, cryoprotectant concentration, cryoprotectant type, and time were the fixed variables. Wilcoxon ranked sums tests were used to evaluate the influence of freezing on spermatozoa morphology. A mixed linear model was also used to evaluate the effects of cryoprotectant type, cryoprotectant concentration, and freezing rate on proportion of cells with intact plasma membranes following cryopreservation. Turtle served as the random variable in the model, while freezing rate temperature, cryoprotectant concentration, and cryoprotectant type were the fixed variables. Statistical analyses were performed using SPSS 25.0 (IBM Statistics, Armonk, NY, USA) and MedCalc (Ostend, Belgium). A p≤0.05 was used to determine statistical significance.
8.3. RESULTS

Descriptive statistics for the red-eared slider body weights, testicular measurements, estimated testicular volumes, GSI, and total spermatozoa are reported in Table 8.1 and 8.2. Mixed model results can be found in Table 8.3. There were significant differences in time, extender, and the time*extender interaction for % motile cells, progressive motility, rapid velocity, medium velocity, slow velocity, static velocity, VAP, VSL, VCL, BCF, STR, LIN varied depending on time (all p<0.05, Table 8.3). Motility, progressive motility, and average path velocity (VAP) over time are reported in Table 8.4. Figure 8.4 show the differences in motility between each extender type over time.

Table 8.1. Descriptive statistics for body weights and testicular measurements for red eared sliders (n=17).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>Median</th>
<th>SD</th>
<th>25-75%</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>804.22</td>
<td>327</td>
<td>340</td>
<td>1436</td>
<td></td>
</tr>
<tr>
<td>Left Testicle Weight (g)</td>
<td>0.68</td>
<td>0.39</td>
<td>0.13</td>
<td>1.21</td>
<td></td>
</tr>
<tr>
<td>Volume (mm$^3$)</td>
<td>820.34</td>
<td>531.2</td>
<td>40.76</td>
<td>1585.06</td>
<td></td>
</tr>
<tr>
<td>Right Testicle Weight (g)</td>
<td>0.93</td>
<td>0.69-1.4</td>
<td>0.24-9.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume (mm$^3$)</td>
<td>955.20</td>
<td>516.06</td>
<td>196.95</td>
<td>1873.94</td>
<td></td>
</tr>
<tr>
<td>Gonadal Somatic Index</td>
<td>0.20</td>
<td>0.14-0.31</td>
<td>0.11-2.99</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 8.2. Descriptive statistics for volume semen recovered, concentration of spermatozoa following dilution, and estimated number of spermatozoa collected for red eared sliders (n=17).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>Median</th>
<th>SD</th>
<th>25-75%</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of semen recovered (µL)</td>
<td>317.8</td>
<td>107.7</td>
<td>107.7</td>
<td>110-500</td>
<td>110-500</td>
</tr>
<tr>
<td>Concentration of spermatozoa following dilution (Spermatozoa /ml)</td>
<td>2.9 x 10^7</td>
<td>2.25 x 10^7</td>
<td>1.43 x 10^7</td>
<td>-4.56 x 10^7</td>
<td>1.63 x 10^10</td>
</tr>
<tr>
<td>Estimated total Spermatozoa (Spermatozoa/ml)</td>
<td>4.99 x 10^{10}</td>
<td>2.4 x 10^{10}</td>
<td>1.07 x 10^{9}</td>
<td>-1.23 x 10^{11}</td>
<td>-2.62 x 10^{13}</td>
</tr>
</tbody>
</table>

Figure 8.4. Median spermatozoa motility of red-eared slider samples mixed with extenders and stored at 4°C for 96 hours.
Table 8.3. Mixed model results for the red eared slider (*Trachemys scripta elegans*) semen extender study. Significant models are bolded (p<0.05).

<table>
<thead>
<tr>
<th>Model</th>
<th>Akaike’s Information Criteria (AIC)</th>
<th>F-statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motile cells (%)</td>
<td>Time*Treatment 1689.775</td>
<td>154.319</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>18.958</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>18.119</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Progressive Motility (%)</td>
<td>Time*Treatment 1472.317</td>
<td>3.779</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>14.458</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>11.295</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Rapid velocity (%)</td>
<td>Time*Treatment 1574.189</td>
<td>4.360</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>17.965</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>8.106</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Medium velocity (%)</td>
<td>Time*Treatment 1499.559</td>
<td>7.345</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>9.100</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>39.63</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Slow velocity (%)</td>
<td>Time*Treatment 1729.467</td>
<td>5.444</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>1.663</td>
<td>0.161</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>8.840</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Static velocity (%)</td>
<td>Time*Treatment 1761.952</td>
<td>10.772</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>15.180</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>30.783</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Path Velocity (VAP, µm/s)</td>
<td>Time*Treatment 1426.353</td>
<td>7.493</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>8.679</td>
<td>≤0.001</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>3.633</td>
<td>0.012</td>
</tr>
</tbody>
</table>

(table cont’d)
<table>
<thead>
<tr>
<th>Model</th>
<th>Akaike’s Information Criteria (AIC)</th>
<th>F-statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progressive velocity (VSL, μm/s)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Time*Treatment</td>
<td>1346.756</td>
<td>5.760</td>
<td>≤0.001</td>
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<tr>
<td>Time</td>
<td></td>
<td>4.305</td>
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</tr>
<tr>
<td>Treatment</td>
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<td>3.638</td>
<td>0.011</td>
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<tr>
<td>Track Speed (VCL, μm/s)</td>
<td></td>
<td></td>
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<tr>
<td>Time*Treatment</td>
<td>1643.434</td>
<td>8.207</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Time</td>
<td></td>
<td>10.965</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td>3.540</td>
<td>0.013</td>
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<tr>
<td>Beat Frequency (BCF, Hz)</td>
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<td></td>
<td></td>
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<tr>
<td>Time*Treatment</td>
<td>1401.457</td>
<td>10.185</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Time</td>
<td></td>
<td>7.629</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td>13.025</td>
<td>≤0.001</td>
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<tr>
<td>Straightness (STR, %)</td>
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<tr>
<td>Time*Treatment</td>
<td>1620.339</td>
<td>9.624</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Time</td>
<td></td>
<td>14.166</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td>40.217</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Linearity (Lin, %)</td>
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<td></td>
<td></td>
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<tr>
<td>Time*Treatment</td>
<td>1466.700</td>
<td>7.995</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Time</td>
<td></td>
<td>6.987</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td>25.727</td>
<td>≤0.001</td>
</tr>
</tbody>
</table>
Table 8.4. Motility, progressive motility, and average path velocity (VAP µm/s) for spermatozoa over time red eared sliders (n=6).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time</th>
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<th>25-75%</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility</td>
<td>Initial</td>
<td>66.67</td>
<td>53.3-72.16</td>
<td>31.33-79.00</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>63.33</td>
<td>53-72.9</td>
<td>27.33-87.67</td>
</tr>
<tr>
<td></td>
<td>12 hours</td>
<td>50.5</td>
<td>46.08-65.8</td>
<td>0-83.67</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>52.83</td>
<td>33.0-65.83</td>
<td>0-86.67</td>
</tr>
<tr>
<td></td>
<td>48 hours</td>
<td>41.33</td>
<td>27.25-61.41</td>
<td>0-82.00</td>
</tr>
<tr>
<td></td>
<td>72 hours</td>
<td>40.50</td>
<td>19.25-60.5</td>
<td>0-92.33</td>
</tr>
<tr>
<td></td>
<td>96 hours</td>
<td>40.16</td>
<td>9.5-59.33</td>
<td>0-87.33</td>
</tr>
<tr>
<td>Progressive Motility</td>
<td>Initial</td>
<td>18.33</td>
<td>12.75-22.58</td>
<td>2-38.33</td>
</tr>
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<td></td>
<td>6 hours</td>
<td>20.67</td>
<td>12.33-26.83</td>
<td>2.33-41.33</td>
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<td></td>
<td>12 hours</td>
<td>13.16</td>
<td>8.66-23.16</td>
<td>0-43.67</td>
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<tr>
<td></td>
<td>24 hours</td>
<td>12.16</td>
<td>6.08-22.5</td>
<td>0-39.33</td>
</tr>
<tr>
<td></td>
<td>48 hours</td>
<td>10.33</td>
<td>3.83-14.83</td>
<td>0-42</td>
</tr>
<tr>
<td></td>
<td>72 hours</td>
<td>9.33</td>
<td>2.75-16.16</td>
<td>0-53.67</td>
</tr>
<tr>
<td></td>
<td>96 hours</td>
<td>7.83</td>
<td>0.33-13.0</td>
<td>0-48.33</td>
</tr>
<tr>
<td>Average Path</td>
<td>Initial</td>
<td>21.43</td>
<td>19.29-26.3</td>
<td>14.10-32.30</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
<td>25.63</td>
<td>20.75-28.37</td>
<td>14.33-40.67</td>
</tr>
<tr>
<td>Velocity (VAP µm/s)</td>
<td>12 hours</td>
<td>24.8</td>
<td>20.52-27.20</td>
<td>0-44.57</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>24.3</td>
<td>16.70-27.94</td>
<td>0-44.03</td>
</tr>
<tr>
<td></td>
<td>48 hours</td>
<td>23.91</td>
<td>18.00-26.39</td>
<td>0-34.43</td>
</tr>
<tr>
<td></td>
<td>72 hours</td>
<td>22.18</td>
<td>12.34-27.56</td>
<td>0-37.93</td>
</tr>
<tr>
<td></td>
<td>96 hours</td>
<td>17.65</td>
<td>8.55-26.16</td>
<td>0-50.40</td>
</tr>
</tbody>
</table>

Spermatozoa motility following cryoprotectant exposure was found to change over time. Significant differences were noted for each time point compared with baseline (Table 8.4) however pairwise comparison shows that there was no significant decline within the first 10 minutes (P=0.52) following exposure to cryoprotectant. Significant differences in spermatozoa motility were detected by cryoprotectant, concentration, time, cryoprotectant*concentration, concentration*time, concentration*cryoprotectant*time. No significant difference was observed between the interaction of cryoprotectant*time, indicating all cryoprotectants led to declines in motility (Table 8.4). For cryoprotectants, pairwise comparison showed no difference between DMSO and Glycerol (P=0.135) with a significant difference between DMSO and methanol.
(P≤0.001) and glycerol and methanol (P≤0.001). For concentrations pairwise, comparison showed a difference between 5%, 10% and 15% cryoprotectant concentration (P≤0.001), while no difference was observed between 10% and 15% (p=0.797) (Figure 8.4 and Figure 8.5).

![Motility graph](image)

Figure 8.4. Percent motility prior to cryoprotectant exposure (DMSO, glycerol, and methanol) and following cryoprotectant exposure while incubated at 20 °C for 40 minutes.

Linear mixed model results can be found in Table 8.6. A significant reduction in motility was observed over time. Motility decreased following exposure to cryoprotectant with a 20-minute incubation period (P≤0.001) (Table 8.7.). Additionally, motility declined significantly following freezing compared to initial motility and motility following cryoprotectant and incubation (P≤0.001). A significant difference was observed in cryoprotectant concentration as a higher cryoprotectant concentration had a negative impact on motility (P≤0.001). Additionally, a
A significant difference was observed between the interaction of time and cryoprotectant concentration ($P \leq 0.001$). There was no significant difference in spermatozoa motility by freezing rate temperature or cryoprotectant or any of the other interactions tested (all $P > 0.123$).

Spermatozoa undergoing cryopreservation demonstrated significant changes in morphology for the following categories between baseline and post-thaw: normal spermatozoa ($Z = -7.9$, $P \leq 0.001$), proximal droplets ($Z = -3.83$, $P \leq 0.001$), distal droplets ($Z = -7.918$, $P \leq 0.001$), kinked tails ($Z = -3.209$, $P = 0.001$), coiled tails ($Z = -2.428$, $P = 0.015$), and detached heads ($Z = -4.533$, $P \leq 0.001$) (Table 8.8).

Significant differences in proportion of intact spermatozoa membranes were detected by cryoprotectant, concentration, and freezing rate. Plasma membrane integrity was found to
significantly decrease with a slower freezing rate temperature. Spermatozoa plasma membrane integrity was significantly higher (p= 0.009) at 30°C compared with 5°C. Cryoprotectant concentration had a significant effect on plasma integrity, with higher concentrations demonstrating a greater proportion of intact plasma membranes (p=0.003). No difference was observed in plasma membrane integrity between cryoprotectant types. There were also no significant differences in any of the interactions in the model (all P > 0.124) (Tables 8.9-8.12).

8.4. DISCUSSION

The results of this study represent the first comprehensive approach to evaluating multiple spermatozoa extenders in a chelonian, and confirmed our first hypothesis: that red-eared slider spermatozoa can be successfully stored using INRA 96, Ham’s F-10, and SWM at 4°C for at least 96 hours without observing a significant decline in motility. None of these extenders have been evaluated in other chelonians, so whether similar results would be achieved will require testing by species. Only three other studies have evaluated short-term spermatozoa storage in chelonians, and they reported poor results (Gist et al., 2000, Zimmerman and Mitchell, 2017, Sirinarumitr et al., 2009). In Sternotherus odoratus, Trachemys scripta, and Chrysemys picta initial motility was poor following postmortem collection, Sternotherus odoratus (28-52%), Trachemys scripta (3-4%), and Chrysemys picta (2-5%). These spermatozoa samples were incubated at two temperatures 23°C and 2°C in Ham’s F-10 with bovine serum albumin for 1-3 hrs prior to analysis. Cooler temperatures appeared to maintain motility better in Sternotherus odoratus as motility was 52% at 2°C while motility declined to 28% at 23°C. Despite poor motility preservation, samples from this study were determined to be viable and retained the capacity for motility when stored at 4°C for 40 days if methylxanthines such as
Table 8.5. Mixed model results for the red eared slider (*Trachemys scripta elegans*) cryoprotectant toxicity study. Significant models are bolded (p<0.05).

<table>
<thead>
<tr>
<th>Model</th>
<th>Akaike’s Information Criteria (AIC)</th>
<th>F-statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motile cells (%)</td>
<td>Cryoprotectant</td>
<td>1876.990</td>
<td>28.20</td>
</tr>
<tr>
<td></td>
<td>Concentration</td>
<td>180.82</td>
<td>35.12</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>19.614</td>
<td>1.263</td>
</tr>
<tr>
<td></td>
<td>Cryoprotectant*Concentration</td>
<td>20.604</td>
<td>0.046</td>
</tr>
<tr>
<td></td>
<td>Cryoprotectant *Time</td>
<td>2.657</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>Concentration*Time</td>
<td>19.614</td>
<td>2.657</td>
</tr>
<tr>
<td></td>
<td>Concentration<em>Cryoprotectant</em>Time</td>
<td>2.657</td>
<td>0.014</td>
</tr>
</tbody>
</table>

Table 8.6. Mixed model results for the red eared slider (*Trachemys scripta elegans*) cryopreservation study. Significant models are bolded (p<0.05).

<table>
<thead>
<tr>
<th>Model</th>
<th>Akaike’s Information Criteria (AIC)</th>
<th>F-statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motile cells (%)</td>
<td>Temperature</td>
<td>1565.206</td>
<td>0.193</td>
</tr>
<tr>
<td></td>
<td>Cryoprotectant</td>
<td>0.046</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>Concentration</td>
<td>22.762</td>
<td>62.509</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>23.002</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Time*Concentration</td>
<td>23.002</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Table 8.7. Spermatozoa motility before and after incubation a 4-6°C for 20 minutes with cryoprotectant in red eared sliders (n=5).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cryoprotectant</th>
<th>Concentration</th>
<th>Mean</th>
<th>Median</th>
<th>SD</th>
<th>25-75%</th>
<th>Min-Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motile cells (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>55.6-64.3</td>
<td>50-76.6</td>
</tr>
<tr>
<td>Initial</td>
<td>Glycerol</td>
<td>2%</td>
<td>41.3</td>
<td>20.6</td>
<td>6-82</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4%</td>
<td>30.1</td>
<td>16.1</td>
<td>3-66</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6%</td>
<td>12.1</td>
<td>9.17</td>
<td>0-28</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
<td>2%</td>
<td>44.7</td>
<td>15.5</td>
<td>18-76</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4%</td>
<td>24.0</td>
<td>17.2</td>
<td>4-62</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6%</td>
<td>14.2</td>
<td>10.8</td>
<td>3-46</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 8.8. Spermatozoa morphology prior to and after cryopreservation.

<table>
<thead>
<tr>
<th>Morphological spermatozoa characteristics</th>
<th>Before Cryopreservation</th>
<th>After Cryopreservation</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Median: 17 % 25th-75th: 10-23% Min-Max: 3-63%</td>
<td>Median: 37 % 25th-75th: 24-66.25% Min-Max: 7-91%</td>
<td>$P \leq 0.001$</td>
</tr>
<tr>
<td>Proximal Droplet</td>
<td>Median: 0 % 25th-75th: 0% Min-Max: 0%</td>
<td>Median: 0 % 25th-75th: 0-0% Min-Max: 0-7%</td>
<td>$P \leq 0.001$</td>
</tr>
<tr>
<td>Distal Droplet</td>
<td>Median: 79 % 25th-75th: 76-86% Min-Max: 35-90%</td>
<td>Median: 60.5 % 25th-75th: 29-74% Min-Max: 12-88%</td>
<td>$P \leq 0.001$</td>
</tr>
<tr>
<td>Kinked tail</td>
<td>Median: 2 % 25th-75th: 1-4% Min-Max: 0-11%</td>
<td>Median: 1 % 25th-75th: 0-3 % Min-Max: 0-23%</td>
<td>$P=0.001$</td>
</tr>
<tr>
<td>Coiled tail</td>
<td>Median: 0 % 25th-75th: 0% Min-Max: 0%</td>
<td>Median: 0 % 25th-75th: 0-0% Min-Max: 0-2%</td>
<td>$P=0.015$</td>
</tr>
<tr>
<td>Detached head</td>
<td>Median: 0 % 25th-75th: 0% Min-Max: 0%</td>
<td>Median: 0 % 25th-75th: 0-1 % Min-Max: 0-6%</td>
<td>$P \leq 0.001$</td>
</tr>
</tbody>
</table>

3-isobutyl-1-methylxanthine were added to stimulate the spermatozoa (Gist et al., 2000; Clulow and Clulow, 2016). In both olive Ridley and hawksbill sea turtles, semen extension was done using test yolk buffer and Tyrode’s medium (combined with albumin, lactate, and pyruvate). The authors reported that motility declined in samples extended in test yolk buffer to 28% and 25% within 24 hours in the olive Ridley and hawksbill sea turtles, respectively. The results for the Tyrode’s media were worse, with a total motility of 14% after 24 hours for both species. Other media that were utilized in this investigation were, Ham’s F-10, Beltsville poultry semen extender, 3% citrate buffer, phosphate buffered saline, EEL extender, and 1% bovine serum albumin, all these media resulted in poor semen preservation. Similar poor results were reported for leopard tortoise samples extended in Ham’s F10 with albumin and test yolk buffer at refrigeration temperature. The leopard tortoise motility averaged 57% at baseline but was 0%
Table 8.9. Mixed model results for the red eared slider (*Trachemys scripta elegans*) cryopreservation study. Significant models are bolded (p<0.05).

<table>
<thead>
<tr>
<th>Model</th>
<th>Akaike’s Information Criteria (AIC)</th>
<th>F-statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact plasma Temperature</td>
<td>540.287</td>
<td>4.146</td>
<td><strong>0.031</strong></td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryoprotectant</td>
<td>0.158</td>
<td>0.705</td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td>22.136</td>
<td>0.527</td>
<td>0.609</td>
</tr>
<tr>
<td>Cryoprotectant*Concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryoprotectant *Temp.</td>
<td>0.667</td>
<td>0.530</td>
<td></td>
</tr>
<tr>
<td>Concentration*Temp.</td>
<td>0.844</td>
<td>0.514</td>
<td></td>
</tr>
<tr>
<td>Concentration<em>Cryoprotectant</em>Temp</td>
<td>2.055</td>
<td>0.124</td>
<td></td>
</tr>
</tbody>
</table>
Table 8.10. Proportion of spermatozoa membrane integrity following Live/Dead staining with SYBR 14 and Propidium Iodine.

<table>
<thead>
<tr>
<th>Cryoprotectant</th>
<th>% Intact membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>Median: 13.93%</td>
</tr>
<tr>
<td></td>
<td>25\textsuperscript{th}-75\textsuperscript{th}: 8.3-24.18 %</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 2.18-52.02%</td>
</tr>
<tr>
<td>DMSO</td>
<td>Median: 14.72%</td>
</tr>
<tr>
<td></td>
<td>25\textsuperscript{th}-75\textsuperscript{th}: 5.92-20.30%</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 0.29-44.37%</td>
</tr>
</tbody>
</table>

Table 8.11. Proportion of spermatozoa membrane integrity following Live/Dead staining with SYBR 14 and Propidium Iodine.

<table>
<thead>
<tr>
<th>Freezing temperature</th>
<th>% Intact membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>5\textdegree C</td>
<td>Mean: 11.44%</td>
</tr>
<tr>
<td></td>
<td>SD: 6.12%</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 1.94-24.19%</td>
</tr>
<tr>
<td>15\textdegree C</td>
<td>Median: 15.43%</td>
</tr>
<tr>
<td></td>
<td>25\textsuperscript{th}-75\textsuperscript{th}: 7.25-20.20%</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 2.06-46.23%</td>
</tr>
<tr>
<td>30\textdegree C</td>
<td>Mean: 20.06%</td>
</tr>
<tr>
<td></td>
<td>SD: 13.06%</td>
</tr>
<tr>
<td></td>
<td>Min-Max: 0.29-52.02%</td>
</tr>
</tbody>
</table>

within 24 hours. Variability between tortoise samples was observed and motility remained in some cases, no sample was over 50% 24 hours after sampling. Test yolk buffer was used in the current study and red-eared slider spermatozoa motility was found to be greater than that reported in the other three species of chelonians at 48 hours. There are several potential reasons for this difference. First, samples were collected via post mortem in the current study, which is
Table 8.12. Proportion of spermatozoa membrane integrity following Live/Dead staining with SYBR 14 and Propidium Iodine.

<table>
<thead>
<tr>
<th>Cryoprotectant</th>
<th>Temperature</th>
<th>Cryoprotectant concentration</th>
<th>Mean (%)</th>
<th>SD (%)</th>
<th>Min-Max (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>5</td>
<td>2 %</td>
<td>8.7</td>
<td>3.27</td>
<td>4.46-12.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 %</td>
<td>10.34</td>
<td>2.75</td>
<td>7.11-13.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 %</td>
<td>18.09</td>
<td>6.10</td>
<td>10.25-24.188</td>
</tr>
<tr>
<td>Glycerol</td>
<td>15</td>
<td>2 %</td>
<td>9.52</td>
<td>4.5</td>
<td>4.94-15.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 %</td>
<td>16.27</td>
<td>6.3</td>
<td>7.22-25.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 %</td>
<td>32.4</td>
<td>11.47</td>
<td>19.7-46.22</td>
</tr>
<tr>
<td>Glycerol</td>
<td>30</td>
<td>2 %</td>
<td>13.9</td>
<td>9.47</td>
<td>2.18-26.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 %</td>
<td>19.17</td>
<td>11.94</td>
<td>5.35-33.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 %</td>
<td>26.56</td>
<td>18.81</td>
<td>2.24-52.02</td>
</tr>
<tr>
<td>DMSO</td>
<td>5</td>
<td>2 %</td>
<td>8.41</td>
<td>6.86</td>
<td>2.14-18.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 %</td>
<td>9.11</td>
<td>6.94</td>
<td>1.94-20.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 %</td>
<td>13.92</td>
<td>4.65</td>
<td>6.96-16.63</td>
</tr>
<tr>
<td>DMSO</td>
<td>15</td>
<td>2 %</td>
<td>5.57</td>
<td>5.82</td>
<td>2.06-16.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 %</td>
<td>13.94</td>
<td>9.92</td>
<td>4.96-30.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 %</td>
<td>21.04</td>
<td>11.22</td>
<td>7.2-38.61</td>
</tr>
<tr>
<td>DMSO</td>
<td>30</td>
<td>2 %</td>
<td>11.68</td>
<td>11.34</td>
<td>4.67-28.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 %</td>
<td>15.94</td>
<td>9.87</td>
<td>0.28-27.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 %</td>
<td>31.43</td>
<td>7.71</td>
<td>24.48-44.36</td>
</tr>
</tbody>
</table>

similar to the methods used evaluating *Sternotherus odoratus*, *Trachemys scripta*, and *Chrysemys picta*; however, the initial motilities in this study were poor *C. picta* and *T. scripta* suspended in Ham’s F-10 media showed low motility at 3-6%, while *S. odoratus* had an initial motility of 40%. These samples were only evaluated over 1 hour at 2°C. Ante-mortem methods were utilized in the sea turtle and tortoise studies. In these two studies, electroejaculation was
Electroejaculation tends to result in mixed fecal-urine-semen samples. This contamination could lead to alterations in semen survival because of organics that impede motility. In the current study, sterile semen samples were collected, so this was not a concern. Another issue to consider is osmolarity of the samples. Freshwater turtles and tortoises have different proportions of fluids in their intracellular and extracellular spaces as overall their total body water is less than other reptile species at 66%, which will also impact their plasma osmolarities (Petritz and Tina Son, 2019). These differences could impact spermatozoa survival and motility, as non-physiologic osmolarities could lead to crenation or cellular swelling. Individuals attempting to review extenders for a new species of chelonian should consider INRA 96, Ham’s F10, and SWM. Additionally, these extenders need to be evaluated using ante-mortem semen samples collected via electroejaculation to assess their performance under these conditions.

The results of the cryoprotectant study confirmed our second hypothesis, that dose and time dependent declines in motility would occur following exposure to three different cryoprotectants. Motility was found to decrease by 37% for DMSO and 40% glycerol at 5% concentrations during the initial mixing of the DMSO and glycerol with spermatozoa, with even lower motilities noted for methanol. Because the motility results were found to be the highest with DMSO and glycerol, the cryopreservation study was done with these cryoprotectants. Again, DMSO and glycerol were toxic at all times, with a significant decline noted directly after mixing the samples and both cryoprotectants. Similar declines in spermatozoa motility and motility rate were observed in saltwater crocodiles (Crocodylus porosus) following exposure to dimethylacetamide (DMA), DMSO, and glycerol at 0.68M, 1.35 M, and 2.7M concentrations when incubated at 4°C for 2 hours (Johnston et al., 2014). In the current study, significant gross changes to the media were seen following the addition of methanol to the stock INRA 96. This
resulted in a rapid loss in motility. The results of this study suggest that less than 10 minutes is the preferred time for incubating samples with the cryoprotectants used in this study. However, in this study, we used 20 minutes because of the limitations associated with packing the number of straws used when freezing occurred. This is a limitation to our study which likely resulted in lower pre-freeze motilities than we would have liked. Moreover, 10 minutes may not be enough time for the cryoprotectants to penetrate into the cellular membrane and displace water. Further studies need to be performed to evaluate cryoprotectant penetration into reptile spermatozoa.

Based on the sharp declines in motility noted with the DMSO, glycerol, and methanol at 5%, 10% and 15% in this study, all the cryoprotectants appeared to have a significant effect on the spermatozoa. However, methanol showed the most toxic effects compared to DMSO and glycerol. There was no difference between the toxic effects of DMSO and glycerol so these cryoprotectants were selected for the cryopreservation study. Additionally, the cryoprotectants selected for this study are commonly used for cryopreserving spermatozoa in humans, domestic animals, and fish. We thought this to be a good starting point.

Following the observation that cryoprotectants impacted motility at high concentrations prior to freezing, Cryopreservation freezing rates were tested with DMSO and glycerol at 2 %, 4% and 6% concentrations for the third part of our study. These concentrations were selected because of the toxicity noted at 5% and above, with 6% serving as a toxicity control. This would allow us to determine if these concentrations could have the chance to preserve motility while maintain morphology and plasma membrane integrity.

Investigations into cryopreservation of reptile spermatozoa have been limited, despite cryopreservation being well-developed in other species. There is a critical need for gamete biobanking to preserve the genetic material of threatened and endangered reptiles, especially
chelonians. To date, only seven studies have evaluated cryopreservation in reptiles, no full study has evaluated chelonian spermatozoa. In chelonians one limited report exists (Platz et al., 1980). Thus, this study represents the first comprehensive attempt a cryopreservation in a chelonian, the previous report demonstrated no recovery with pellet freezing. Unfortunately, the third hypothesis of this study, that red-eared slider spermatozoa would survive cryopreservation, was only partially achieved.

Similar results were initially found in saltwater crocodiles, where motility and rapid motility were not observed in their spermatozoa post-thawing (Johnson et al., 2014). In saltwater crocodile spermatozoa exposure to cryoprotectants and subsequent cryopreservation using DMSO, DMA, and glycerol resulted in significant declines in motility. Glycerol, DMSO, and DMA demonstrated a negative impact on motility and rate of progression following exposure of spermatozoa to cryoprotectants at 4°C for 1 hour. A concentration dependent effect was observed with increasing concentrations of all three cryoprotectant agents, 0.68M (5%), 1.35 M (10%), and 2.7 M (20%) respectively and a significant decline in motility. No change was observed in plasma membrane integrity during the equilibration phase despite a significant loss of motility. Cryopreservation was performed in this study using 0.25 mL French straws at a freezing rate of 6°C/min from 4°C to -86°C then plunged into liquid nitrogen (-196°C) and stored for 6 months. Despite a significant loss of motility and rate of progression was observed following thawing, plasma membrane integrity was preserved in about 25 % of sperm. Higher concentrations of cryoprotectant improved plasma membrane preservation (Johnston et al., 2014). In a subsequent study in saltwater crocodiles non-penetrating cryoprotectants trehalose, raffinose, or sucrose at 0.3M were compared to glycerol at concentrations of 0.3M, 0.68M, 1.35M, and 2.7M. It was determined that glycerol at the highest concentration maintained the plasma membrane the best
and limited DNA fragmentation following thawing, although motility and rate of movement were poor post thaw. From this information, cytotoxicity of glycerol and sucrose was evaluated prior to cryopreservation and revealed no loss of survival when sperm was diluted with 0.68 M glycerol or 0.2-0.3M sucrose when cryoprotectants were washed out with PBS or Briggers, Whitten and Whittingham medium containing sperm capacitation agents (BWWCAP), this combination allows for adequate pre-freeze survivability before cryopreservation by preserving motility, rate of movement, plasma membrane integrity, and plasma membrane integrity. Additionally, freezing rate was evaluated combining the use of penetrating (0.68 and 1.35 M glycerol) and non-penetrating cryoprotectants (0.2 or 0.3 M sucrose). These were exposed to a fast freeze (21°C/min) and slow freeze (6 °C/min) from 5°C to -80°C. Post thaw survival was highest with a combination of 0.2 M sucrose and 0.68 M glycerol followed by washing of BWWCAP independent of freezing rate (Johnston et al., 2017).

Two studies exist in assessing cryopreservation methods in a lizard species. The first attempt was made in the Argentine black and white tegu (Tubinambis merianae), Post-mortem spermatozoa samples were collected in order to evaluate two cryoprotectants, DMSO and glycerol, at three different concentrations 8%, 12%, and 16%, with three different freezing rates, 0.3°C/min, 1°C/min, and 6.3°C/min. Samples were frozen in cryovials in a Test-Yolk- M199 with HEPES combination at a concentration of 50 x 10⁶ mL. Overall the slowest freezing rate had the higher cryosurvival. Additionally, DMSO provided better plasma membrane integrity and acrosome integrity compared to glycerol. To determine the best cryopreservation method a sperm quality index was developed to equally give equal weight to the three measured indicators including motility, plasma membrane integrity, and acrosome integrity. Based on this metric, a slower freezing rate of 0.3°C/min with a 12 % DMSO concentration had the highest sperm
quality index. Additionally, in this study a significant male effect was observed for post thaw sperm quality parameters. This phenomenon has been repeatedly observed in mammals and birds, and now has been observed repeatedly in reptiles (Young et al., 2017, 2017; Johnston et al., 2017).

In the eastern water skink (*Eulamprus quoyii*), sperm was collected via ventral coelomic massage to examine sperm sensitivity to cryopreservation. Wild-caught males were collected and sperm volume, concentration, progressive and total motility, and membrane integrity were assessed. It was observed that motility could be maintained up to 70% with spermatozoa up to 16 hours diluted in PBS, TLHepes, or Ham’s F-10 at room temperature. Cryotoxicity was examined using PBS and the following cryoprotectants; DMSO, DMA, and glycerol at 4°C for 2 hrs. Spermatozoa diluted in 1.35 and 2.7 M dimethyl sulfoxide in PBS had higher motility and proportion of live sperm than control or dimethyl acetamide. Cryopreservation was evaluated, using 1.35 M of cryoprotectant in Tris-yolk buffer, Beltsville poultry semen extender and PBS. There was no benefit to the complex extenders compared to PBS for skinks. Post-thaw progressive motility was significantly higher, 5%, with 1.35 M dimethyl sulfoxide Tris-yolk buffer than all other treatment groups. They concluded, dimethyl sulfoxide may yield promise as a cryoprotectant for skink species. Spermatozoa samples were frozen in 0.2 mL French straws with a controlled rate freezer at −6°C/min, then plunged into liquid nitrogen throughout this study (Hobbs et al., 2018).

There is a single report in corn snakes that demonstrated that reptile spermatozoa could be cryopreserved with some success, with fair to poor post-thaw motility (Mattson et al., 2008). Bilady A with 20% egg yolk with 17% glycerol was used to extend and cryoprotect the snake spermatozoa. Mean post-thaw motility in the corn snakes was 27.1%, with a range of 17.8% to
50.2%. While these values are considered poor for insemination, they do prove at least one group of reptile spermatozoa can survive cryopreservation. The snake samples were frozen by cooling them for one hour, suspending the straws one inch over liquid nitrogen for 10 minutes, and then plunging them into liquid nitrogen. The authors attributed their success to using density gradient centrifugation with a percoll media to enhance the number of usable spermatozoa with greater motility and a higher rate of progression. Percoll media with density centrifugation was not utilized in this study due to a good proportion of motile sperm following collection. Since then, cryopreservation methods in *C. ruber* and *P. bivittatus* have been investigated. In *C. ruber*, Lake’s extender with DMSO (2% and 4%) did not show any post thaw survival, no matter the cryopreservation method or cryoprotectant concentration. All semen frozen in Test-Yolk- M199 with HEPES combination exhibited motility post-thaw. 8% glycerol frozen in cryovials in a controlled-rate freezer and cooled at 1°C min⁻¹ to −20°C, then 50°C min⁻¹ to −80°C resulted in the greatest post-thaw motility in all treatments (Zacariotti et al., 2011). Further investigation using *P. bivittatus* as a model species evaluated cryopreservation methods using sperm extended in TEST-yolk buffer with final DMSO or glycerol (GLY) concentrations of 8, 12, or 16%, or combinations of DMSO and GLY with final concentrations of 4:4, 6:6, or 8:8%. Samples were frozen in vials at 0.3°C/min to −40°C before exposure to liquid nitrogen. Sperm frozen in the 6%DMSO:6%GLY and 4%DMSO:4%GLY treatments resulting in the highest post thaw motility. Spermatozoa frozen in a DMSO/GLY combination showed significantly higher % initial motility score (IMS) than all treatments of DMSO or GLY. These same protocols exhibited highest plasma membrane integrity following thawing. A sperm quality index was used to determine the best overall freeze method for *P. bivittatus*, which was then applied to the Argentine black and white tegu (*Tubinambis merianae*) study discussed previously. This analysis
showed Burmese python sperm frozen at 0.3°C/min in either 6%DMSO:6%GLY or 4%DMSO:4%GLY exhibited significantly higher post-thaw viability (Young et al., 2017). Ultimately, success with cryopreservation is measured in recovering motile spermatozoa. At this time, the cryoprotectants appear to present a major hurdle to success, and additional study is needed.

Despite the poor results in motility preservation, red-eared slider spermatozoa plasma membrane integrity was found to survive the process of cryopreservation with the appropriate cryoprotectant concentration. Higher concentrations of glycerol and DMSO resulted in a higher proportion of intact plasma membranes. Based on the results of this study, the cryoprotectant appears to be the primary culprit in the loss of motility, and the impact of the cryoprotectant appears to be time and dose dependent. Saltwater crocodiles similarly experienced good plasma membrane integrity with poor motility post-thaw. Glycerol and PBS, were used as the cryoprotectant and extender, respectively, in the crocodiles. Because of the effect of the cryoprotectant on motility, it may be possible to achieve better results by reducing the incubation time, and thus the time the spermatozoa are exposed to the cryoprotectant. In the turtles, the incubation time was limited to 20 minutes; this was done to allow the cryoprotectants to equilibrate and cross into the spermatozoa. However because of experimental design and the number of treatments it was not possible to fill straws and load the controlled rate freezer within less than 10 minutes to maintain maximal spermatozoa motility. Future research should investigate a shorter incubation time. The limitation to a shorter incubation is that it could limit the amount of cryoprotectant that enters the spermatozoa, and thus increase the risk of cryoinjury following freezing and thawing; however, this could be evaluated by measuring plasma membrane integrity. If reptile spermatozoa cannot be reliably cryopreserved and thawed with
appropriate motility, then techniques of timed artificial insemination may require high intra-ovidoctual delivery for cryopreserved spermatozoa. Additionally, further investigation to motility activation needs to be investigated with phosphodiesterase inhibitors. One report in *C. picta* 40-day old refrigerated non-motile sperm was able to regain motility with the addition of methylxanthines such as 3-isobutyl-1-methylxanthine to stimulate the spermatozoa (Gist et al., 2000). Using phosphodiesterase inhibitors post thaw, may be better to evaluate the spermatozoa’s motility ability, and may be needed prior to insemination. Non-motile cryopreserved spermatozoa may still be useful. For example, in-vitro fertilization or intracytoplasmic sperm injection of the ova could be performed with non-motile spermatozoa. While this is feasible in mammals, it has not been evaluated in reptiles. This technique would pose certain challenges not experienced with mammals, as the ova would need to be removed surgically because of their size and replaced surgically to ensure that the egg shell develops correctly. If this process was possible, then the cryopreservation methods as outlined could be considered successful, since the spermatozoa plasma membrane was intact in the spermatozoa tested using both DMSO and glycerol at different concentrations. In addition to re-assessing incubation time, non-penetrating cryoprotectants could be evaluated. In this study we wanted to only evaluate penetrating cryoprotectants as they have been consistently used across species. Other potential penetrating cryoprotectants to consider are ethylene glycol, formamide, propylene glycol, and dimethylacetamide, while non-penetrating cryoprotectants include bovine serum albumin, sucrose, and trehalose. This list is not extensive however these should be further investigated for cryopreserving turtle spermatozoa.

The concentration of spermatozoa being frozen is an important consideration when cryopreserving semen. Cryopreservation concentrations range depending on species from 10
x10^6 - 100 x 10^6 spermatozoa/mL. The target concentration for the red-eared sliders was 10-20 x 10^6/mL. We selected this concentration because it mimicked those used for humans as 20 x 10^6/ml has been shown to limit lipid peroxidation (Wang et al., 1997). Only one reptile paper reports a target freezing concentration, which was 50 x 10^6 spermatozoa/mL in cryovials (Young et al., 2017). It is unknown what the ideal freezing concentration is in reptiles. The concern is that if spermatozoa are frozen at too low of a concentration, loss of some sperm surface components may occur and this could impact plasma membrane permeability and ultimately lead to sperm senescence (Johnston et al., 2014; Watson et al., 2000). The receptacle that the sample is frozen in is also an important consideration. The turtle samples in the current study, as well as the crocodile, and skink studies, used French straws, while the tegu, corn snake, Burmese python, and red-diamondback rattlesnake used cryovials (Mattson et al., 2008; Johnston et al., 2014, 2017; Young et al., 2017; Hobbs et al., 2018; Zacariotti et al., 2011). Although both are appropriate storage units for cryopreserved spermatozoa, the thermodynamics of the freezing process is likely different based on size, shape, and material.

Further studies are desperately needed to develop consistent methods for cryopreserving reptile spermatozoa with a goal of motile spermatozoa post-thaw. In mammals, motility remains an important parameter for predicting potential success when performing timed artificial insemination. At this time, the exact role spermatozoa motility plays in the female reptile reproductive tract is unknown. It is possible that the microenvironment of the female reproductive tract has evolved to store sperm with zero motility until the appropriate reproductive conditions occur (Gist, 2011). Further, stimulation or capacitation of the spermatozoa within the female reproductive tract could occur that ultimately leads to
hyperactivation (Nixon et al., 2016). Ultimately, these types of questions must be answered if we hope to develop successful assisted reproductive plans for these threatened animals.
CHAPTER 9. CONCLUSIONS AND FUTURE DIRECTIONS

9.1. CONCLUSIONS

This dissertation project was pursued to fulfill a life-long dream, to ensure reptiles exist for future generations. There has long been a need to develop ART for reptiles, as numerous other taxa have received the attention, to ensure that we can protect them from the pressures being placed on them during the Anthropocene era. The goal of this research was to systematically transfer and apply ART to male reptiles. This approach could be used for all male reptiles to further initiate the collection of evidence-based data for managing these animals ex situ. I believe that the results of this work confirm that we have made progress, but there remains much to be done.

The first question, “Can we collect semen repeatedly in reptiles without harming the individual?”, was addressed using veiled chameleons and electroejaculation. The veiled chameleon was used for two reasons: 1) chameleons are one of the most endangered groups of animals in the world, and 2) chameleons are considered “fragile”. It is important to develop models for endangered species, and who better to evaluate “stress” than a group of animals considered to be “fragile” and susceptible to stress. This study indeed confirmed that serial electroejaculation and anesthesia could be performed on these “fragile” animals.

The second question, “Does understanding the normal reproductive physiology help us predict when we can collect semen?”, was also pursued with chameleons because of the need to gain some insight into their reproduction. Prior to this work, no study has ever assessed the reproductive cycle of a male chameleon. For this study we selected two different species, the veiled chameleon and the panther chameleon. These two were selected because they originate from different habitats, and for this reason it was thought they could serve as models for different
chameleons. This decision was proven to be a good choice as they use different reproductive strategies. The results confirmed that veiled chameleons have a pre-nuptial reproductive strategy, while panther chameleons have a post-nuptial reproductive strategy. Additionally, this study demonstrated that chameleons can undergo repeated electroejaculations under anesthesia, twice monthly, for a year. So much for “fragile”.

The third question focused on “Can we exogenously (hormonally) stimulate a reptile to control its reproductive system, and thus when it produces semen?”. First, a study was done to determine an effective dose of hCG to increase plasma testosterone concentrations. Three doses (100 IU, 200 IU, 300 IU) were selected based on the literature. All three doses achieved the same result, increased plasma testosterone. It is likely that the minimum effective dose is lower, but I was restricted to the study results. Next, the 100 IU dose was given weekly to evaluate its effect on testosterone, testicle size, and semen collection. When 100 IU hCG was given weekly for a month, plasma testosterone concentrations were sustained over the study period. However, weekly administration of 100 IU hCG was not able to increase the likelihood of semen collection, nor did it have an effect on testicle size. These results coincide with others in reptiles, that other hormones, such as FSH, likely play a role in gonad maturation.

To answer the fourth question, “When semen is successfully collected, can we preserve it? If so, what semen extenders should we use to handle the semen and how long can reptile semen be stored at refrigeration temperatures?”, two temperate squamates, the green anole and banded water snake, and one chelonian, the red eared slider, were used as model species. Post-mortem epididymal spermatozoa was collected from all three species, and they exhibited taxa and species-based differences. In banded water snakes, epididymal spermatozoa were successfully stored at 4°C for 72 hours using Ham’s F-10, INRA 96, and SWM. In the green anole,
epididymal spermatozoa declined dramatically in the same extenders and under the same cooled storage conditions. The last model tested, the red-eared slider, was found to have the best results, with spermatozoa being successfully stored using INRA 96, F-10, and SWM at 4°C for at least 96 hours. These findings confirm that reptiles are indeed unique and that we need to assess each species individually to determine best practices.

Finally, the last question focused on a desire to preserve reptile genetic material for eternity, “Can we preserve (biobank) reptile spermatozoa indefinitely?” Using the knowledge gained from answering question four, attempts were made to cryopreserve red-eared slider spermatozoa. First, the effects of three different cryoprotectants (DMSO, glycerol, and methanol) at three different cryoprotectant concentrations (5%, 10%, and 15%) were evaluated. 5% DMSO was found to provide the best results, although this was a relative term as motility was poor, similar to other reptiles. Because of concerns with cryotoxicity, the percentages attempted for the cryopreservation trial were changed to 2%, 4% and 6%. Motility declined prior to the cryopreservation process, which was expected; however, cryopreservation effectively eliminated motility in all treatments. Despite the elimination of motility, when evaluating plasma membrane integrity, red-eared slider spermatozoa was found to survive the process of cryopreservation with the appropriate cryoprotectant concentration. Higher concentrations of glycerol and DMSO resulted in a higher proportion of intact plasma membranes. Further studies are desperately needed to develop consistent methods for cryopreserving reptile spermatozoa, with a goal of motile spermatozoa post-thaw.

Ultimately, we achieved our goal to systematically transfer and apply ART to male reptiles. To develop ART for a novel male reptile, the methods and procedures outlined here can be utilized as a road map. First, one needs to understand the animals basic physiology and
reproductive cycle, second it is vital to determine how to collect semen safely and repeatedly without harming the sexually mature individual, third we need to be able to handle semen and store the samples short term, while finally we need to be able to store the samples indefinitely to preserve extant genetics. The approach and methods utilized here could be used for any reptile species increasing our species knowledge to successfully preserve these evolutionary gems.

9.2. FUTURE DIRECTION

So where can we take these technologies in reptiles? At this point in time, we have proven we can successfully collect semen repeatedly and safely from reptiles, and that the ejaculates can be successfully stored in the short term for transport. Further work needs to be completed in order to routinely cryopreserve reptile semen and begin biobanking extant reptile populations. However, the question does arise, “What is the minimum amount of motility needed to fertilize an ovulated oocyte in a reptile?”. In order to answer this question, artificial insemination methods need to be established for reptiles. Fundamentally, the next step is to also perform a systematic evaluation of female reptile reproduction, characterizing the hormonal cycle for females, following follicular dynamics with minimally invasive methods, determining ovulation timing, developing a method to induce ovulation, and then determining the peak time for insemination. Energies must be directed towards this research if we hope to prevent reptiles from going the way of the dinosaurs. Long live the reptile!
APPENDIX A. COPYRIGHT INFORMATION
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Nancy Lung, Editor, JZWM

On Sun, Feb 16, 2020 at 3:08 PM Sean Perry <seanmperry87@gmail.com> wrote:

2/16/2020

Dear Editor Lung:

I am completing a doctoral dissertation at Louisiana State University entitled “CAN ASSISTED REPRODUCTIVE TECHNOLOGIES HELP CONSERVE 300 MILLION YEARS OF EVOLUTION? A FIRST ATTEMPT AT DEVELOPING ASSISTED REPRODUCTIVE TECHNOLOGIES FOR MALE REPTILES”

I would like your permission to reprint the following materials in my dissertation which is in preparation for my graduation in May 2020:


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Sincerely,

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“There is no better high than discovery”

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REFERENCES


51) Boretto JM, Ibargüengoytía NR, Acosta JC, et al. 2007. Reproductive biology and
sexual dimorphism of a high-altitude population of the viviparous lizard from the

pituitary gonadotropins and evidence of a hormone-induced desensitization in the


54) Browne RK, Kaurova SA, Uteshev VK, et al. 2015. Sperm motility of externally

through gene banking and other reproduction technologies. Russ J Herpetol, 18:165–
174.

56) Burke RL. 1990. Conservation of the world’s rarest tortoise. Conserv Biol, 4: 122–
126.

57) Callard GV, Ryan KJ. 1977. Gonadotropin action and androgen synthesis in enzyme
dispersed testicular cells of the turtle (Chrysemys picta). Gen Comp Endocrinol,

58) Callard IP, Bayne CG, McConnell WF. 1972. Hormones and reproduction in the


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<th>Details</th>
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302) Kneidinger N. 2009. GnRH implant in green iguana (Iguana iguana) [Thesis]. Vienna (Austria): University of Veterinary Medicine


397) McKinney ML. 2002. Urbanization, Biodiversity, and Conservation The impacts of urbanization on native species are poorly studied, but educating a highly urbanized human population about these impacts can greatly improve species conservation in all ecosystems. Bioscience. 52 10:883-90.


Sirinarumnitr K, Patthong Y, Jaimjaturong P, et al. 2010. Extender for sperm dilution in olive ridley turtle (Lepidochelys olivacea) and hawksbill turtle (Eretmochelys imbricata) semen. Proc 5th International Symposium on Southeast Asia Sea Turtle Associative Research and Bio-logging Science: 7-10


TARGET 12 - Technical Rationale extended (provided in COP/10/INF/12/Rev.1)[Internet] [cited 2020 January 23]. Available from https://www.cbd.int/sp/targets/rationale/target-12/


588) Volsøe H. 1944. Structure and Seasonal Variation of the Male Reproductive Organs of *Vipera Berus* (L.)B. Lunos bogtrykkeri a/s.


VITA

Sean Michael Perry was born and raised in California by his parents Kathie Lee Perry and Timothy Craig Perry. He graduated from the University of California Santa Cruz with a Bachelor of Science degree in Neuroscience and Behavior in 2009. He completed the requirements for the degree of Doctor of Veterinary Medicine in 2013 from the College of Veterinary Medicine at Western University of Health Sciences, Pomona, California. He was then selected as a small animal medicine and surgery intern at the University of Illinois, Urbana, Illinois for 2013-2014. In 2014, he went into private practice in the Florida Keys at Marathon Veterinary Hospital. He was then recruited to be an emergency and critical care intern at the University of Illinois, Urbana, Illinois from 2015-2016. In 2016, he was accepted into the graduate program in the Department of Veterinary Clinical Sciences, School of Veterinary Medicine, Louisiana State University. He is expected to graduate with a Doctorate in Philosophy in Veterinary Clinical Sciences in May 2020. He is expected to be employed by…