

5-2018

The Evolution of Testis Architecture and Sperm Morphology in *Anolis* Lizards

Hannah R. Hall

Trinity University, hhall@trinity.edu

Follow this and additional works at: https://digitalcommons.trinity.edu/bio_honors

Recommended Citation

Hall, Hannah R., "The Evolution of Testis Architecture and Sperm Morphology in *Anolis* Lizards" (2018). *Biology Honors Theses*. 25.
https://digitalcommons.trinity.edu/bio_honors/25

This Thesis open access is brought to you for free and open access by the Biology Department at Digital Commons @ Trinity. It has been accepted for inclusion in Biology Honors Theses by an authorized administrator of Digital Commons @ Trinity. For more information, please contact jcostanz@trinity.edu.

THE EVOLUTION OF TESTIS ARCHITECTURE AND SPERM MORPHOLOGY IN *ANOLIS*
LIZARDS
HANNAH R. HALL

A DEPARTMENT HONORS THESIS SUBMITTED TO THE DEPARTMENT OF BIOLOGY AT
TRINITY UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
GRADUATION WITH DEPARTMENTAL HONORS

DATE APRIL 13, 2018

Michele A. Johnson, Ph.D
THESIS ADVISOR

Jonathan M. King, Ph.D
BIOLOGY DEPARTMENT CHAIR

Gerard M. J. Beaudoin III, Ph.D
COMMITTEE MEMBER

Ariel F. Kahrl, Ph.D
COMMITTEE MEMBER

Tim O'Sullivan, AVPAA

Student Agreement

I grant Trinity University ("Institution"), my academic department ("Department"), and the Texas Digital Library ("TDL") the non-exclusive rights to copy, display, perform, distribute and publish the content I submit to this repository (hereafter called "Work") and to make the Work available in any format in perpetuity as part of a TDL, Institution or Department repository communication or distribution effort.

I understand that once the Work is submitted, a bibliographic citation to the Work can remain visible in perpetuity, even if the Work is updated or removed.

I understand that the Work's copyright owner(s) will continue to own copyright outside these non-exclusive granted rights.

I warrant that:

- 1) I am the copyright owner of the Work, or
- 2) I am one of the copyright owners and have permission from the other owners to submit the Work, or
- 3) My Institution or Department is the copyright owner and I have permission to submit the Work, or
- 4) Another party is the copyright owner and I have permission to submit the Work.

Based on this, I further warrant to my knowledge:

- 1) The Work does not infringe any copyright, patent, or trade secrets of any third party,
- 2) The Work does not contain any libelous matter, nor invade the privacy of any person or third party, and
- 3) That no right in the Work has been sold, mortgaged, or otherwise disposed of, and is free from all claims.

I agree to hold TDL, Institution, Department, and their agents harmless for any liability arising from any breach of the above warranties or any claim of intellectual property infringement arising from the exercise of these non-exclusive granted rights."

I choose the following option for sharing my thesis (required):

Open Access (full-text discoverable via search engines)

Restricted to campus viewing only (allow access only on the Trinity University campus via digitalcommons.trinity.edu)

I choose to append the following [Creative Commons license](#) (optional): Attribution-NonCommercial-ShareAlike

Table of Contents

Abstract	3
Acknowledgments	4
Introduction	6
Introduction to this Thesis	15
Materials & Methods	22
Results	28
Discussion	33
Conclusion	37
References	38
Appendix	49

Abstract

The major function of the testes is sperm production. Sperm morphology is incredibly diverse both within and across species, and this variation results in part from differences in the duration and patterns of cell division during sperm development, or spermatogenesis. To produce this diversity, testis architecture may evolve in correlation with sperm morphology, as for example, sperm length is correlated with the evolution of thicker epithelia of the seminiferous tubules in birds. In this study, I examined relationships between sperm morphology and testis size and architecture in a group of 18 species of *Anolis* lizards from the Dominican Republic and Puerto Rico. Using cryosectioned testis tissues, I measured the cross-sectional area (CSA) of each testis, the seminiferous tubules within the testis, and the lumina of the tubules, and used the latter two measures to calculate the CSA of the epithelium. Moreover, I used a grid to identify the proportions of cells belonging to the lumen, epithelium, and interstitial space. Lastly, I obtained measures of sperm head, midpiece, and tail lengths for each species (Kahrl 2017, PhD dissertation, University of Virginia). After controlling for body size, I found that species with larger testes had larger tubules with larger lumina (not epithelia) and that larger testes produced sperm with longer heads. Additionally, seminiferous tubules with larger lumina and thicker epithelia produced sperm with longer tails. Lastly, I found that the proportion of epithelial cells within the testis was not associated with any measure of sperm length or testis size or architecture. These results show that future studies of sperm morphology should consider measurements of sperm tail, midpiece, and head lengths (instead of only studying overall sperm length), as these components show differing relationships with testis architecture, and that the evolutionary relationships between testis architecture and sperm morphology differ between birds and lizards.

Acknowledgments

When I first joined the Johnson Lab in the fall of 2015 as a sophomore, I looked up to senior Jake Stercula and junior Maria Jaramillio as I tried to figure out how to balance my lab responsibilities with my academic work and extracurricular commitments. Jake and Maria were the first to inspire me, unknowingly and each in their own way, to work harder than I had ever worked before and to view each obstacle as a chance to improve myself—both in the lab and in my role as a student. Now that I am a senior, I feel a deep responsibility to act as a similar source of inspiration for the younger students in the lab and am so privileged that I have been able to serve as a mentor for Victoria Gonzalez and Isabela Carson. Victoria helped me finish my data collection last semester and Bela has helped cryosection the remaining testes in our freezer so that this work can be expanded, and for this assistance I am incredibly grateful.

I'd also like to express the utmost appreciation for Brittney Ivanov, our lab technician, who trained me in the lab and taught me how to use the cryostat, stain my slides, navigate NIS-Elements, and measure different aspects of testis architecture. She has always been there to help me troubleshoot problems that arose in NIS-Elements or with the two microscopes I used to examine my slides, but more importantly, she has provided an immeasurable amount of support and guidance as both a colleague and a dear friend.

Dr. Johnson, my research mentor and biggest advocate, is the reason I am who I am today. Her passion for equality, diversity, and inclusivity in STEM; her role as young woman in science and academia; and her dedication to the well-being and success of all students (elementary through graduate) continues to inspire and guide me as I attempt to follow in her footsteps. She has taught me how to handle criticism, perform under pressure, and think on my

feet; she has trained me to be a confident leader, scientist, and public speaker; and she has instilled within me the grave importance of science communication and outreach work.

I have an immense appreciation for every single person I have gotten to meet and work with as a result of working in Dr. Michele Johnson's lab. Thanks to Brittney Ivanov, Chris Robinson, Jake Stercula, Leah Selznick, Jamal Murray, Miguel Webber, Maria Jaramillo, Adam Zeb, Faith Deckard, Amy Payne, Jesus Vega, Marzieh Rouzbehani, Daisy Horr, and the newly joined Bailey Charles, Isabela Carson, and Shelby Irwin, the lab has always felt like a home to me.

Finally, I'd like to acknowledge my thesis committee members: Dr. Ariel Kahrl for sharing her sperm morphology data set in this study and for providing consistent support and feedback throughout the duration of this project, and Dr. Gerard Beaudoin for offering a fresh perspective on my thesis and for believing in me as I've explored my passion for research.

Introduction

An Overview of Sexual Selection

Sexual organisms often exhibit enhanced traits that have evolved to increase their likelihood of reproductive success. In many taxa, organisms choose mates based on traits that reflect overall fitness or genetic quality (reviewed by Andersson & Simmons 2006). These traits—which include sexual behaviors, physiology, and morphology—are involved in each portion of the reproductive process, from courtship to mate acquisition, through copulation and fertilization. In some taxa, animals compete against one another for access to mates (intrasexual selection; generally male-male competition for females). Further, some individuals choose their mates from among the pool of available mates (intersexual selection; generally females choosing males). Intrasexual selection with respect to the male can be further broken down: In addition to male-male competition occurring before copulation (precopulatory sexual selection), it also plays a role after copulation in polyandrous species via sperm competition (postcopulatory sexual selection). Thus, there are many traits at play when examining sexual selection; males may evolve larger bodies or weaponry such as sharp teeth or horns to enhance their ability to win in male-male combat, or they may evolve elaborate ornamentation such as colorful feathers or songs to stand out and be chosen by a female. Moreover, genitalia may evolve characteristics that enhance female stimulation or allow sperm to be deposited closer to the female's ova, and sperm may evolve specialized morphology to improve swimming speed or strategy in the face of sperm competition. Additionally, female cryptic choice, a poorly understood mechanism by which a female influences paternity after copulation, may drive the selection of those pre- or postcopulatory traits. Finally, because sperm develop in the testes, selection on sperm morphology may also have implications on testis composition.

The complex nature and variety of modes by which sexual selection can act on a set of traits, as well as how those traits are related to each other (e.g., via energetic tradeoffs), will be explored in this Introduction. However, the core of this thesis assesses the evolutionary relationship between testis composition and sperm morphology in a group of *Anolis* lizard species. This is the first study to consider these relationships using multiple components of sperm length, and how each of these measures may have independent relationships with various aspects of testis composition.

Precopulatory Sexual Selection and Associated Phenotypes

Sexual Size Dimorphism

Precopulatory sexual selection occurs across animal taxa and can be driven by male-male competition or female-female competition, female or male mate preference, or some combination of these factors. In male-male competition, a form of intrasexual selection, the reproductive success of the male may be determined by a single characteristic or a suite of traits that allows him to outcompete other males. Similarly, such traits may attract a potential female mate. Body size is one of the traits most strongly associated with precopulatory competition (reviewed by Andersson 1994), and selection for enhanced body size often leads to male-biased sexual size dimorphism (mbSSD; male body size is greater than female body size) across a variety of taxa (e.g. mammals: Linderfors et al. 2007; birds: Szekely et al. 2007; lizards: Cox et al. 2007; amphibians: Kupfer 2007). Andersson (1994) presented several scenarios in which mbSSD is advantageous: physical male-male combat (e.g. lizards: reviewed by Stamps 1983; mammals: Linderfors et al. 2007), forceful matings, in which males mount and subdue a female to copulate (e.g., insects: Sih & Krupa 1992; birds: McKinney et al. 1983), and endurance

activities, in which males that copulate longer have greater paternity success (mammals: Higham et al. 2010). In male-male combat, mbSSD can also aid in territory defense. When mating success is determined by territory ownership and defense, larger males that are better able to defend their territory also have greater opportunities to reproduce (e.g., American rubyspot bird: Serrano-Meneses et al. 2007).

However, females can also exhibit enhanced sexual size dimorphism (female-biased SSD; fbSSD). In females, egg production generally increases directly with female body size (e.g., insects: Thornhill & Alcock 2014; non-insect invertebrates: Ridley & Thompson 1979; fish: Perrone 1978; amphibians: Tilley 1968) because of an increased volume of internal storage space (e.g., Williams 1966) or a greater availability of stored energy. A model for this relationship between size and fecundity was first introduced by Darwin (1874; critiqued by Shine 1988) and later bolstered by evidence showing that the capacity for increased energy storage is favorable because it compensates for size-related metabolic demands (e.g., Millar and Hickling 1990). In brief, larger females may also be better at parenting and obtaining food and territory. Thus, the phenomenon of fbSSD can also be advantageous to reproductive success, as seen throughout most invertebrates and some vertebrates (e.g., baleen whales: Sears & Perrin 2009; rabbits: Lindenfors et al. 2007; horned lizards: Zamudio 1998; spiders: Foellmer & Moya-Larano 2007), but can be disadvantageous in other taxa (yellow-pine chipmunks: Schulte-Hostedde et al. 2002).

Alternatively, fbSSD can arise from the prevalence of small males, just as mbSSD can be attributed to relatively small females. In the former, some species exhibit protandry, the state at which males emerge from development before females in an attempt to secure a mate. In this case, having a small body size allows for faster maturation and a greater chance at being the first

male to inseminate a female, when compared to larger males that take longer to mature (e.g., spiders: Maklakov and Bilde 2004). Similarly, in species with small females, smaller females can begin breeding after a shorter maturation period than that of larger females, as seen in female weasels *Mustela nivalis*, which are half the size of males (Erlinge 1979; Ralls & Harvey 1985).

Ornamentation and Behavior

Beyond sexual size dimorphism, a variety of other traits arising from sexual selection exists. Sexually selected traits are often condition-dependent, meaning that they develop in relation to an individual's overall fitness; only males of highest health and condition, and thus with high quality genes, are able to dedicate resources to the development of secondary features (Williams 1996; Andersson 2006). Some exhibit hyper-sensitive responses to the organism's nutritional and/or physiological state, such that in certain conditions, growth of secondary sexual features is exaggerated in comparison to non-sexual traits (rhinoceros beetles: Johns et al. 2014). This allows secondary sexual features to serve as a clear signal to potential mates about the fitness of the individual (Johns et al. 2014).

Most secondary sexual traits are those directly associated with male phenotype or behavior, such as ornamentation (e.g., elaborate feather coloration), weaponry (e.g., horns or teeth), or song. Other sexually selected behavioral traits include those associated with athleticism, such as stamina or courtship dances. Athletic or acrobatic behaviors are those that utilize muscle movement patterns not typical of routine, non-sexual behaviors. For example, males of the golden-collared manakin perform strong muscle contractions that result in a loud snapping sound, a tactic used to both court females and compete with males (Fuxjager et al. 2016). Moreover, males of some species build structures or elaborate nests that influence female

mate choice, and thus the quality of those structures also function as sexually selected traits. These external, architectural traits are seen across taxa, from hooded burrow entrances and pillars built by male fiddler crabs (Christy et al. 2002), to the bowerbirds bowers (reviewed by Marshall 1954), and sand craters made by some cichlid fish (Schaedelin & Taborsky 2010).

Postcopulatory Sexual Selection

Copulation alone does not always guarantee reproductive success in polyandrous species, as paternity studies using molecular (DNA) data have long revealed (reviewed by Hughes 1998). Instead, there are a variety of female-mediated roadblocks that may inhibit sperm from reaching and fertilizing ova, thus influencing paternity outcomes after copulation. Several of these roadblocks and their associated implications in postcopulatory sexual selection are explored here.

Cryptic Female Choice and Sperm Competition

Many females can select which sperm they will use to fertilize their ova, although evidence for *how* paternity is determined is unclear—hence the phrase “cryptic female choice.” One mechanism through which cryptic female choice occurs involves accumulating sperm from multiple males before determining paternity. To do this, females of many species have developed sperm storage tubules within their oviduct (e.g., mammals: Racey 1979; birds: Birkhead & Moller 1993; turtles: Gist & Jones 1989; *Anolis* lizards: Sever & Hamlett 2002; teleost fish: Gardiner 1978) as a means to temporally separate the act of copulation from fertilization, perhaps to optimize fitness or increase genetic diversity in their offspring by creating a larger pool of potential sires.

Other studies have examined how the order of mating events influences sperm competition. For example, in a small marsupial species, the second male to mate sires the majority of the offspring, but when a female is near ovulation, the first male to mate has greater paternity success (Kraaijeveld-Smit et al. 2002). In other species, the most recent male to mate has a paternity advantage. Moreover, the duration of sperm viability while in the sperm storage ducts may provide more information to the female about the quality of the male (Eberhard 1998).

Sperm competition is another pressure that drives the wide variability in sperm shape, size, and quantity across sexual organisms (Pitnick et al. 2009). Sperm competition occurs when sperm must compete against other males' sperm for storage positions or access to fertilization once inside the female. Possible mechanisms of sperm competition operate alongside cryptic female choice. For example, organisms with internal fertilization exhibit some degree of spatial separation between the ejaculate deposit site and the site of fertilization (Eberhard 1985), where sperm must move in order to reach the site of fertilization or secure a place in a sperm storage tubule (e.g., mammals: Suarez & Pacey 2006; birds: Birkhead & Moller 1993; spiders: Austad 1984). This presents the opportunity for faster sperm to reach the fertilization site or sperm storage tubules first. Other mechanisms involve direct or indirect displacement of sperm from sperm storage tubules (e.g., Gack & Peschke 1994). For example, dragonflies have evolved genitalia that can scoop or snag sperm from previous mates and either displace them to a site less likely to be used in fertilization or remove them completely (Cordoba-Aguilar et al. 2003), crickets use their own ejaculate to flush out sperm from previous mates (Ono et al. 1989), and certain sharks and rays use seawater to rid the cloaca of competitors' sperm (reviewed by Eberhard 1985). Another mechanism is sperm stratification, in which each sperm storage tubule contains only sperm from a single male, with the sperm from the last male to mate residing in the

sperm storage tubule closest to the site of fertilization (e.g., King et al. 2002). Regardless of the actual mechanism at play, cryptic female choice and/or sperm competition are strong selective pressures on male postcopulatory phenotypes.

The relationship between sperm design and function has been the focus of many studies. In investigating how the degree of sperm competition effects sperm design, Immler et al. (2008) found that high levels of sperm competition are associated with low intramale variability in sperm morphology, compared to high variability in species that face little sperm competition. This trend suggests that in species with strong postcopulatory sexual selection, there may be an optimal sperm design, resulting in stabilizing selection and reduced variation in sperm morphology within and among species. Moreover, relative testis size is greater in taxa that exhibit sperm competition (e.g., mammals: Harcourt et al. 1981; birds: Moller 1991).

Genitalia

In many species, male genitalia are ultimately responsible for delivering male gametes to the female, therefore genital morphology can play a critical role in determining paternity. In fact, male genitalia are one of the fastest evolving traits (reviewed in Eberhard 1985, Klaszco 2016) and exhibit high variability, even among closely related species that are otherwise nearly identical. There are several selective forces that can drive the evolution of highly specialized genitalia, for example: the coevolutionary arms-race between male and female genitalia in an attempt to control reproductive outcomes, female preference for males that are good stimulators, or female preference for males of high fitness. The former occurs in the face of forced copulations, where female genitalia rapidly evolve to obstruct insemination from males of poor quality, as exemplified by waterfowl ducks' spiral, maze-like vagina. From the female ducks'

perspective, only males with a similarly spiraled penis will be capable of penetrating far enough to ensure fertilization, thus deeming him fit enough to produce similarly fit offspring (Brennan et al. 2007). Indeed, the male waterfowl penis has been a focus of studies in sexual selection for its impressive and unusual cork-screw shape and explosive eversion mechanism (e.g., Brennan et al. 2010). A similar relationship occurs among seed beetles, where the degree of potential harm the males' spiked penis could cause to the female is positively related to the degree of protective reinforcement along the females' copulatory duct (Ronn et al. 2007).

Developmental and Energetic Trade-Offs Arising from Sexual Selection

The development and maintenance of any one organismal trait is limited by the resources and energy available to it. Thus, the enhancement of one trait may come at the expense of another. Indeed, many studies have examined this phenomenon among neighboring somatic tissues: for example, butterfly larvae of *Precis coenia* develop disproportionately large adult forewings relative to their body size when the precursor hindwing discs are surgically removed prior to their development (Nijhout and Emlen 1997), thoracic horned beetles present a tradeoff between horn and wing size (Kawano 1995), and head horned beetles exhibit tradeoffs between horn and eye size (Nijhout and Emlen 1997). Studies that have examined this phenomenon in non-neighboring tissues have found similar patterns; for example, some bat species exhibit increased testis mass at the expense of brain size when mated with promiscuous females (Pitnick et al. 2006).

Tradeoffs among precopulatory and postcopulatory sexual traits—that is, resource allocation strategies dedicated to either obtaining mates or succeeding in fertilization—have also been investigated, but are much more complex. Mathematical theory supports the possibility of

developmental tradeoffs between costly sexual traits (Parker et al. 2013). However, across taxa, only males that employ female monopolization strategies are candidates for tradeoffs between these two categories of traits (Lüpold et al. 2014). For example, in the leaf-footed cactus bug, the natural loss of a sexually selected precopulatory weapon leads to greater investment in the testes (Joseph et al. 2018) and in horned beetles, experimental restriction of horn development also yields greater testes size (Simmons & Emlen 2006). There are also tradeoffs between sexual size dimorphism (SSD) and relative testis size among promiscuous species (acanthocephalan worms: Poulin & Morand 2000; snakes and lizards: Kahrl et al. 2016) and harem breeders (pinnipeds: Fitzpatrick et al. 2012). Other tradeoffs occur between spawning coloration and sperm density (*Salvelinus alpinus* fish: Liljedal et al. 1999), breeding coloration and testes mass (*Pomatoschistus minutus* fish: Kvarnemo et al. 2010), and plumage ornamentation and sperm quantity (red-backed fairy-wren bird: Rowe et al. 2010), in addition to a host of other related tradeoffs (see Table 1 in Kvarnemo & Simmons 2013). These relationships illustrate the importance of considering any one trait as it may relate to other traits within the whole of an organism.

Introduction to this Thesis

Sperm Development in the Testes

The details of sperm development (spermatogenesis) differ across taxa, however, amniotes (reptiles, birds, and mammals) exhibit a general testis morphology and spermatogenic pattern that is distinct from nonamniotes (fish and amphibians). For amniotes, sperm development takes place within the highly convoluted seminiferous tubules within the testis. Specifically, spermatogenesis occurs within the seminiferous epithelial tissue that lines the walls of the tubules, beginning along the periphery and progressing inward as sperm mature. Mature sperm (spermatazoa) accumulate in the lumen and are eventually transported into the epididymis via the efferent ductuli where they are stored until ejaculation. This outline of events in the process of sperm development and excretion is conserved across amniotes, while more minute details vary across taxa (reviewed by Gribbins & Rheubert 2014). One critical difference among amniotic taxa lies in the mode of sperm development: Lizards (along with amphibians and other reptiles) exhibit temporal germ cell development, where germ cells develop as a single population, while birds and mammals exhibit spatial germ cell development, where germ cells occupy specific layers in the epithelium based on their stage of development (reviewed by Gribbins & Rheubert 2014).

Because this thesis focuses on *Anolis* lizards, here I describe an overview of what is currently known about the germ cell cycle in lizards. Within the lizard germinal epithelium, pre-meiotic spermatogonia function as precursor cells that replicate via asymmetric mitotic divisions to both maintain a population of germ stem cells and produce differentiable daughter cells. The daughter cells then undergo symmetric mitotic expansion before entering the meiotic stage of spermatogenesis, where the dividing spermatocytes are easily identified by their stage of meiosis.

The haploid daughter cells (spermatids) undergo the final stage of spermatogenesis (spermiogenesis) to form mature sperm. Spermiogenesis is characterized by acrosome formation followed by elongation and then spermiation of mature sperm into the lumina. Developing sperm cells are supported by the nutrient-supplying Sertoli cells in the seminiferous epithelia (reviewed by Gribbins & Rheubert 2014).

Many (but not all) lizards are seasonal breeders. Spermatogenesis generally begins near the end of the summer breeding season (in temperate, seasonally breeding species) after most of the sperm stored in the epididymis is depleted. At that point, and throughout the rest of the non-breeding season, the testis works to replenish its store of spermatocytes that will then continue developing when temperatures rise in the spring. By the start of the summer breeding season, spermiation events begin to occur and males are ready for copulation. Sertoli cells and the pre-meiotic spermatogonia cells, however, are consistently present in the seminiferous epithelium, regardless of seasonal changes (reviewed by Gribbins & Rheubert 2014).

Because lizards are seasonal breeders, they exhibit an increase in both testis size and seminiferous tubule size (but not seminiferous epithelial size) during the summer breeding season (Gribbins et al. 2011). Although, seminiferous epithelium width varies with season and the stage of spermatogenic cycle (Gribbins & Rheubert 2014). However, no studies have yet investigated how testis size and tubule size vary across taxa during the summer breeding season. Gribbins and Rheubert (2014) proposed that tubule size may become larger due to an increase in the size of the lumina, rather than the seminiferous epithelial tissue, possibly because of an accumulation of fluids in the lumina during this season (mammals: Zhou et al. 2001). Beyond this, little else is understood about *Anolis* testis architecture, especially as it relates to sperm morphology.

As reviewed earlier, there are many ways that postcopulatory sexual selection can act on sperm morphology (e.g., modes of cryptic female choice, sperm competition). Not yet discussed is how germ cell development strategies contribute to variation in sperm morphology. Germ cell development strategies are also highly variable and can differ greatly from one group to another. Currently, little is known about germ cell development strategies in lizards (especially in anoles), let alone how spermatogenesis is associated with sperm morphology in this taxon. Ramm and Schärer (2014) reviewed a variety of germ cell development strategies. For example, if ejaculates composed of high levels of mature sperm are associated with greater reproductive success, then that selective pressure will be reflected in rates of cell division during spermatogenesis. Moreover, changes in female reproductive anatomy may select for specialization in sperm morphology, such as tail length, that in turn require sperm to spend more time in the elongation stage of spermiogenesis. Furthermore, the outcome of spermatogenesis may vary based on the number of spermatogonia germ cells present at the start of sperm development, the number of mitotic cells produced, or the rate of both asymmetric and symmetric cell divisions (Ramm & Schärer 2014).

Trends in Testis Architecture and Sperm Morphology in Other Taxa

In birds, sperm length is positively correlated with the size of seminiferous tubules (Lüpold et al. 2009). Moreover, sperm output per unit of time is greater in those individuals with larger testes (e.g., invertebrates: Schärer et al. 2004). However, several studies have shown that testis size is not an appropriate proxy for sperm-producing tissue: in rodents with similar testis size and sperm output, seminiferous volume varied from 33-92% across individuals within the study (Russell et al. 1990); and in birds, seminiferous tissue comprised 88-96% of tubule volume

even when there were significant differences in measures of testis morphology across the study sample (Lüpold et al. 2009).

To better understand how testis morphology is implemented in the evolution of sperm design and production, Lüpold et al. (2009) measured the testicular cross sectional area, seminiferous epithelial thickness, testicular interstitial area, and the thickness of the outer lining of the testis, as well as sperm overall length in two families (28 species total). Among these traits, they found no relationship between absolute testis size and seminiferous tubule size, but found positive relationships between seminiferous tubule size and sperm length, as well as between seminiferous epithelial thickness and relative testis size (testis size corrected for body size). Moreover, there was a positive relationship between seminiferous epithelial thickness and both sperm length and tubule size, but no relationship between lumen area and sperm length or tubule size. The authors concluded that the size of seminiferous tubules is more closely linked with sperm length than the overall size of the testis. Because the authors used relative testis size as a proxy for sperm competition, they also concluded that increased sperm competition is positively associated with increased thickness of sperm-producing tissue (i.e., seminiferous epithelial tissue). In other words, testis size may be a good proxy for the strength of postcopulatory selection, but it is not the only measurement that should be examined in studies of postcopulatory sexual selection.

Anoles Lizards: A Model for Sexual Selection

The genus *Anolis* is an especially speciose group of lizards, with hundreds of species occupying a variety of habitats across a large geographical range (Losos and Schneider 2009). Anole species vary greatly in a number of sexually dimorphic traits, including morphology,

coloration, and behavior, and are a particularly useful study group for this thesis because they exhibit both pre- and postcopulatory sexual selection. Males perform a variety of visual display behaviors, including push-ups, head-bobs, and extension of a colorful throat fan called a dewlap, that typically precede physical combat and are a central component in courtship. While these behaviors—and associated characteristics like dewlap color—are implicated in male mating success, male body size may be a predominant predictor of success in aggressive male interactions, mate acquisition, and female choice (Cox & Kahrl 2014; but see Bush et al. 2016). The degree of male-biased sexual size dimorphism across species of anoles varies greatly, where some species' males have snout-vent-lengths (SVLs) that are up to 50% longer than their female counterparts, while some have females with SVLs that are 20% longer than males (Cox et al. 2007). Further, there is a widespread tradeoff between SSD and testis size across anoles and other squamates (Kahrl et al. 2016).

Anoles are also polygamous; males exhibit sperm competition, females have specialized sperm storage tubules that can store sperm for over a year (Birkhead & Moller 1993; Holt & Lloyd 2010), and females produce clutches of multiple paternity (Calsbeek et al. 2007). In experiments with sequential mating schemes, females show paternity bias towards the first fertilization (Duryea et al. 2013), yet it is unclear if this outcome is due to cryptic female choice, sperm competition, or both. To quantify rates of evolution among testis size and sperm morphology in *Anolis* lizards, Kahrl et al. (unpublished) collected testis volume, and sperm head, midpiece, and tail measurements of 15 mature sperm cells per male for 18 species (2-20 males per species, average = 15 individuals per species). They found that the sperm midpiece evolves 2-3 times faster than the sperm head or tail, while testis size evolves 10-30 times faster than any one measure of sperm morphology. They concluded that increased postcopulatory sexual

selection (i.e., sperm competition) may select for larger testes and therefore greater sperm production, rather than sperm morphology.

The relationships among testis composition, spermatogenesis, and sperm morphology remain poorly understood in lizards. Lizards are no exception to the widespread pattern of highly diverse sperm morphology, yet it is unclear what postcopulatory sexual selection mechanism drives such diversity in this group. Further investigation is needed to better understand how the machinery responsible for sperm production may influence the evolution of sperm morphology, or vice versa. In this thesis, I examine the relationships between SSD, absolute and relative testis size, testis composition, proportions of testicular cell types, and three components of sperm morphology to gain a greater understanding of 1) resource allocation strategies in the adult male and 2) the mechanism by which postcopulatory sexual selection acts on male reproductive features. Specifically, I aim to test whether some aspects of testis composition are more strongly associated with testis size than others, whether three components of sperm morphology (i.e., sperm head, midpiece, and tail lengths) are independently associated with different measures of testis composition, and whether there is a tradeoff between the proportion of spermatogenic cells and sperm length.

Hypotheses

Based on previous literature, I expect that the evolution of testis size and composition is associated with the evolution of sperm morphology across species. First, because it has been shown that both testis size and seminiferous tubule size increase during the summer breeding season (Gribbins & Rheubert 2014) most likely due to the accumulation of seminal fluids in the lumina, as seen in mammals (Zhou et al. 2001), I predicted that absolute testis size is exclusively

driven by seminiferous lumen size. However, I anticipated a different relationship when analyzing relative testis size. Because this aspect of testis size in relation to testis composition has not been explored in lizards, I based my prediction on the trend found in birds: that relative testis size is more strongly associated with seminiferous epithelial area (as opposed to seminiferous tubule size; Lüpold et al. 2009), such that males that invest more energy into developing larger testes relative to their body size have a greater capacity to produce sperm. Moreover, I expected to see a greater proportion of epithelial cells within the seminiferous epithelium in those individuals with larger relative testis size, since sperm production can be traced back to the number of germ cell divisions per spermatogenic cycle (Ramm & Schärer 2014). Based on the principle that there is a tradeoff between germ cell frequency and sperm length, I hypothesized that sperm length would be negatively associated with the proportion of testis epithelial cells within the testes. Lastly, I also expected to see a positive relationship between the size of the seminiferous epithelium and sperm length, since the development of longer sperm may require a thicker epithelium. (See Table 1 for a summary of these hypothesized relationships).

Table 1. Anticipated evolutionary relationships among morphological measurements of testes and sperm morphology. Predictions based on previous literature are denoted by footnotes, while relationships not found in the literature are my own hypotheses. Predictions of directionality are indicated by + or -. Question marks indicate that no clear predictions were made.

	Seminiferous Tubule Size	Seminiferous Epithelial Size	Seminiferous Lumen Size	Proportion of Testis Epithelial Cells	Sperm Length
Absolute Testis Size	+ ^	No ^	+ **	? ^	? ^
Relative Testis Size	No ^	+ *	? ^	+ ^	? ^
Sperm Length	+ *	+ *	No *	- ^	---

*birds: Lüpold et al. 2009; **mammals: Zhou et al. 2001; ^This study

Material and Methods

Specimen collection and tissue processing

In this study, 18 species of *Anolis* lizards from Puerto Rico and the Dominican Republic in 2010-2015 (Table 2) were collected for further examination. Lizards of each species were captured by noose or hand during the anole summer breeding season (May-July). In the field, a clear plastic ruler was used to measure snout-vent length (SVL) of 5-20 adult males and adult females per species to the nearest 1mm, and a Pesola spring scale was used to measure the mass of collected males to the nearest 0.1g. Within 2-3 days of capture, sperm samples were collected and a subset of males of each species (average = 7.9; Table 2) were transported to Trinity University in San Antonio, Texas where they were immediately euthanized via rapid decapitation. Following euthanasia, a midline incision was made in the abdomen, and before removing the testes from the body cavity, both the width and length of the right testis were measured with digital calipers (Mitutoyo; Kawasaki, Japan) to the nearest 0.1mm. Both testes were then surgically removed, flash-frozen on dry ice, and stored at -80°C until further processing. Each pair of testes was cryosectioned at 20µm in four alternate series on SuperFrost Plus microscope slides (Fisher Scientific; Hampton, New Hampshire USA) and stored at -80°C. One series for each individual was stained with hematoxylin and eosin (H&E).

Table 2. Capture sites and number of individuals collected for this study per each *Anolis* species.

Country	Species	n	Coordinates
Dominican Republic	<i>A. chlorocyanus</i>	13	18.525, -70.510
	<i>A. christophei</i>	8	19.042, -70.535
	<i>A. cybotes</i>	10	17.96, -71.19; 18.062, -71.111
	<i>A. distichus</i>	10	18.232, -70.347; 18.386, -70.442
	<i>A. etheridgei</i>	2	19.034, -70.542
	<i>A. insolitus</i>	2	19.041, -70.518; 18.386, -70.442
	<i>A. longitibialis</i>	10	17.836, -71.450
	<i>A. marcanoii</i>	8	18.386, -70.442
	<i>A. olsoni</i>	4	18.232, -70.347
	<i>A. semilineatus</i>	3	18.825, -70.689
Puerto Rico	<i>A. cristatellus</i>	11	18.342, -65.826
	<i>A. evermanni</i>	10	18.321, -65.820; 18.333, -65.817
	<i>A. gundlachi</i>	10	18.333, -65.817
	<i>A. krugi</i>	9	18.333, -65.817
	<i>A. occultus</i>	5	18.453, -66.597
	<i>A. poncensis</i>	10	26.949, -66.876
	<i>A. pulchellus</i>	9	18.321, -65.820; 18.331, -65.824
	<i>A. stratulus</i>	10	18.342, -65.826

Testis Volume and Gonadosomatic Index Calculations

I calculated testis volume for each individual by converting measurements of testis width and length into the radii of the width and length (a , b , respectively) and solving via the formula for an ellipsoid:

$$Volume = \frac{4}{3} \pi a^2 b$$

The average testis volume for each species was then \log_{10} transformed for statistical analyses. I calculated gonadosomatic index (GSI) by first transforming testis volume into testis mass through the density equation, where the density of lizard testes is approximately one:

$$Testis\ mass = \frac{Volume\ (mm^3)}{1\ \frac{mg}{mm^3}}$$

Then, testis mass and body mass were used to calculate GSI:

$$GSI = \frac{testis\ mass}{body\ mass}$$

Finally, individual GSI measurements were used to calculate species averages.

Histological Data Collection

Cross-sectional area of testis

I measured testis cross-sectional area by identifying the three largest, most histologically intact right and left testis sections for each individual with a Nikon SMZ18 stereomicroscope with a SHR Plan Apo 0.5x lens at 3x-8x magnification (determined by the size of each testis section). I used the area measurement tool in NIS-Elements (Nikon) to trace around the border of each selected section, for a total of six cross-sectional area measurements per individual. These measurements were then averaged to produce a single testis cross-sectional area value for each lizard. Individual data for each species were averaged together to create a single species average (Appendix).

Seminiferous tubule, lumen, and epithelium measurements

To create a high resolution, digital image of one tissue section per individual, I photographed one testis per individual using Nikon Eclipse Ni microscope with a Plan Fluor 10x/0.30 objective lens. Our desired resolution restricted the testis area that could be viewed in a single frame, so I created a composite image by stitching together different positional frames of the testis using the “Grab Large Image Free Shape” tool in NIS-Elements.

I acquired measurements of the cross-sectional area of seminiferous tubules by selecting only those tubules that were approximately circular—which indicates that the tubule was near perpendicular to the plane of sectioning—and excluding any tubules on the periphery of the testis, as they may have been obstructed during cryosectioning. I then measured the cross-

sectional area of each tubule that met these criteria and the corresponding lumen (average = 8.3 per lizard; Fig. 1). From these data, I also determined epithelial cross-sectional area, as the difference between tubule area and lumen area. Individual data for each species were averaged together to create a single species average for each measurement (Appendix).



Figure 1. Traces of approximately circular seminiferous tubules (outer trace) and their lumina (inner trace) in a cross-section of an *Anolis* testis tissue (20 μ m thick) at 10x magnification.

Proportions of testicular tissue types

To determine the relative distribution of each testicular cell-type, a colleague blind to the measured cell types cropped each composite image (the same images used to collect seminiferous tubule cross-sectional area) such that a 10x10 grid (1000 μ m x1000 μ m) could be positioned over the image (Fig. 2A). I then identified whether the grid point landed on a dark purple cell stained with hematoxylin, or the space in between cells stained light pink. The cell or

space at each gridpoint was recorded as either interstitial (connective tissue between tubules), epithelial (tissue lining the walls of the tubule), or lumen (innermost hollow space typically filled with mature sperm) (Fig. 2B). The quantity of each testicular cell type per individual testis was averaged to yield the proportion of testicular cell types for each species.

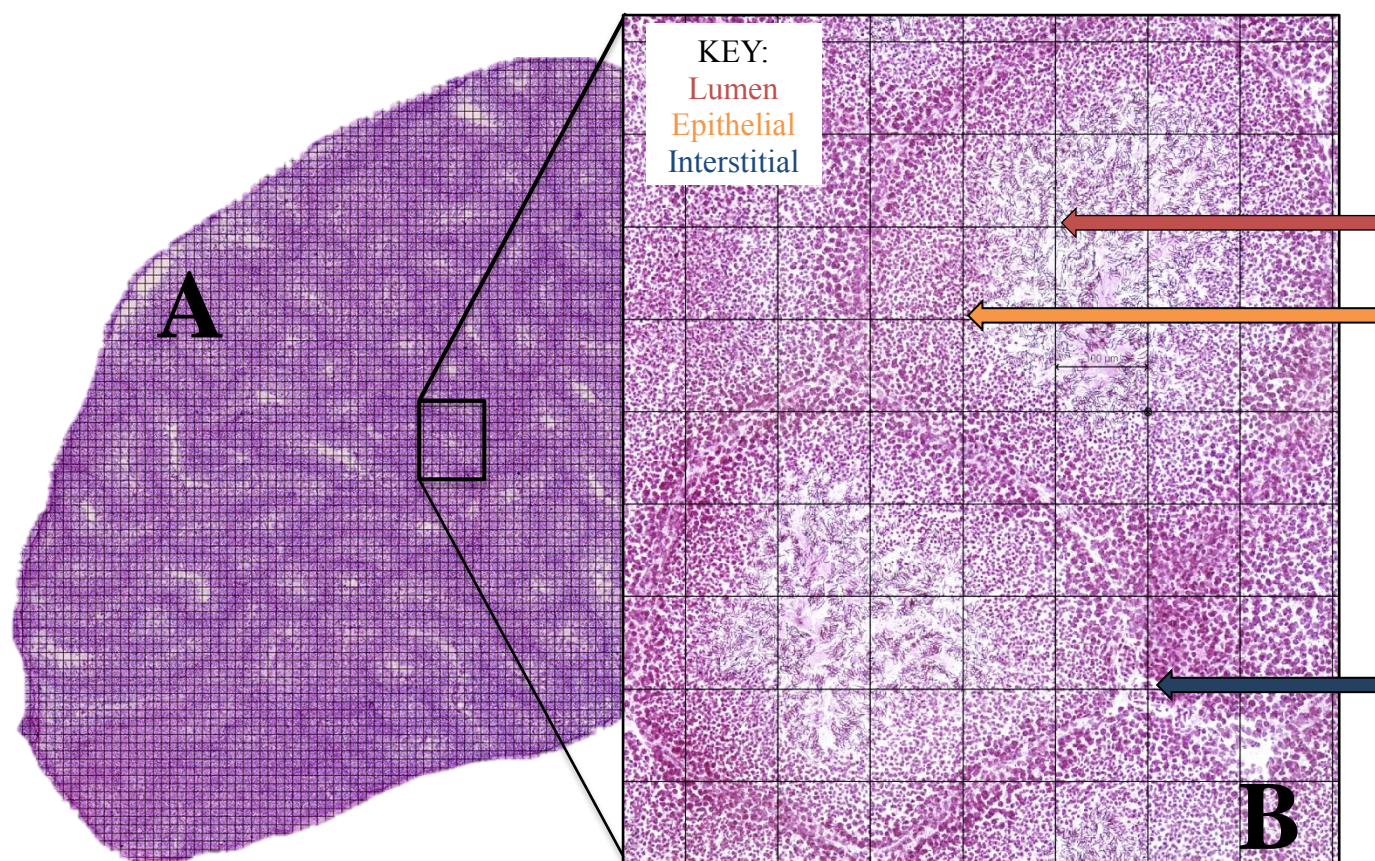


Figure 2. (a) “Stitched” 10x magnification image of an *Anolis* testis cross-section (20 μ m) underneath a 1mm x 1mm grid. (b) A closer look at the cell types identified under each gridpoint of a 9x8 grid. Each arrow points to an example of one type of cell identified in this study, either lumen, epithelial, or interstitial.

Data Analysis

The species averages for male and female SVL were used to calculate the average sexual size dimorphism of each species (SSD; $[(\text{length of larger sex}/\text{length of smaller sex}) - 1]$; Lovich et al. 1992). For all other traits, I calculated species averages from all individual data for analysis

in R (version 3.4.3; R Core Team 2017). The \log_{10} transformed values for the following data were used in all statistical analyses: testis volume; cross-sectional areas (CSA) of testis, seminiferous tubule, lumen, and epithelia; SVL; and sperm head, midpiece, and tail lengths. Proportions of testis cell types were not transformed. To determine relationships among measures of testis architecture and sperm morphology, I used the Phylogenetic Generalized Least Squares (PGLS) function in the *caper* package in R (Freckleton et al. 2002) using the squamate phylogeny from Pyron et al. (2013) pruned to include the species in this study. Measures of size (SVL and testis cross-sectional area) were included as covariates when either was significantly related with the variable in question. For example, because testis CSA and SVL are strongly associated ($p < 0.001$), SVL was included as a covariate when examining the relationship between testis CSA (independent variable) and seminiferous tubule CSA (dependent variable).

Results

Confirming GSI-SSD relationships in Anoles

First, I assessed whether our study populations exhibited a negative relationship between gonadosomatic index (GSI) and sexual size dimorphism (SSD) as reported by Kahrl et al. (2016). This trend was present among our 18 species, although it was not significant ($R^2 = 0.10$, $F_{1,16} = 2.91$, $p = 0.12$; Fig. 3). I also showed that males of species with larger bodies (as determined by snout-vent-length; SVL) have larger testes (Table 3a).

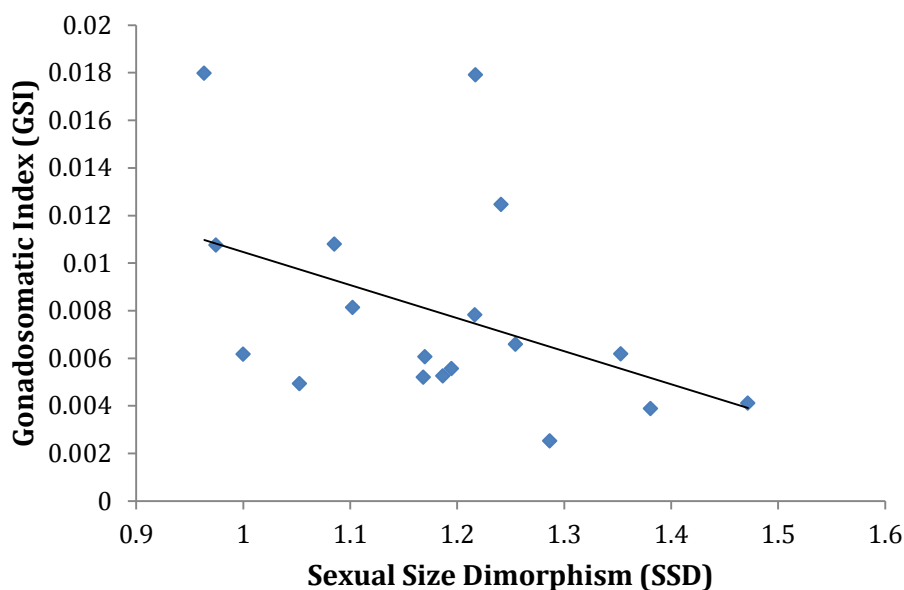


Figure 3. Negative relationship between sexual size dimorphism (male body size in relation to female body size) and gonadosomatic index (testis size relative to body size) across 18 species of *Anolis* lizards.

Testis size and testis architecture

I found a trend towards a positive relationship between testis CSA (or testis volume) and seminiferous tubule CSA, as well as a strong relationship between testis CSA and lumen CSA, such that larger testes have marginally larger tubules and larger lumina (Table 3b; Fig. 4). However, these relationships were driven by the relationship between tubule CSA and lumen

CSA; when testis CSA was included as a covariate with tubule CSA, only tubule CSA was associated with lumen CSA (Table 3f). Similarly, SVL was not associated with tubule CSA when included as a covariate with testis CSA (Table 3c). From this, lumen CSA increases in association with tubule CSA only.

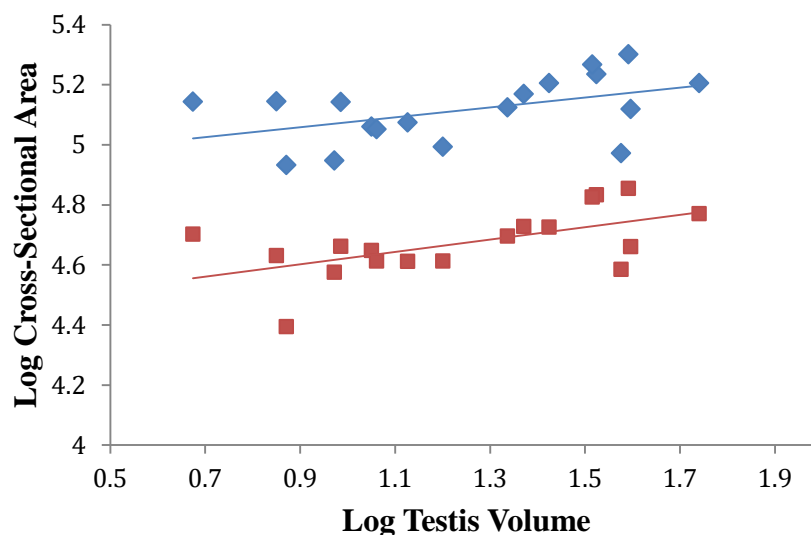


Figure 4. Relationship between \log_{10} transformed values of testis volume and cross-sectional area of seminiferous tubules (blue diamond) and seminiferous tubule lumina (red square) across 18 species of *Anolis* lizards.

Testis size and sperm morphology

Testes with greater CSA were positively associated with sperm with longer heads (Table 3b; this relationship is independent of body size: Table 3c; Fig. 5a). There were no relationships among absolute testis CSA and sperm midpiece or tail lengths (Table 3b). However, sperm midpiece length exhibited a nonsignificant trend towards a positive relationship with relative testis size (Fig. 5b), but not with any other measures of sperm length (Table 3d).

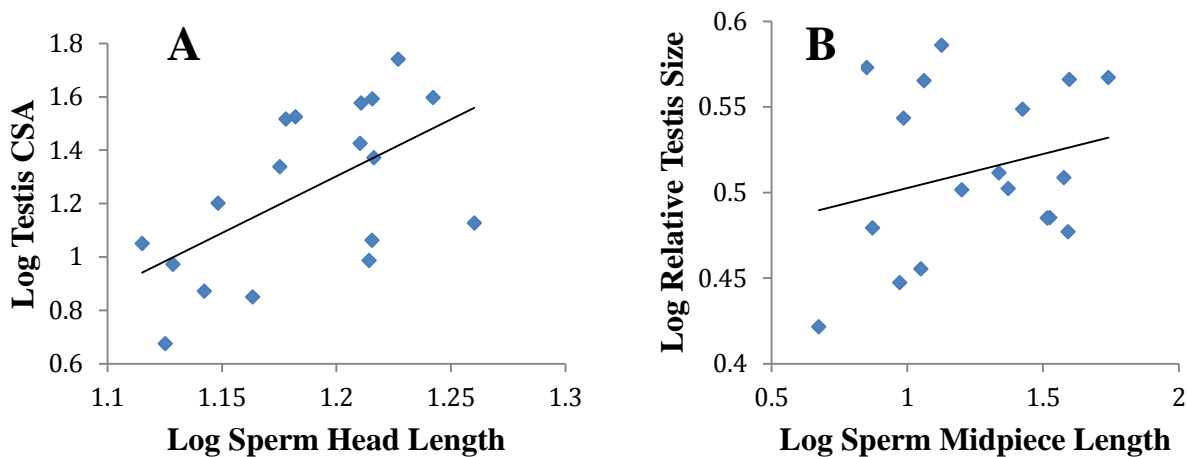


Figure 5. Relationship between log₁₀ transformed values of (A) testis cross sectional area and sperm head length and (B) relative testis size and sperm midpiece length across 18 species of *Anolis* lizards.

Testis architecture and sperm morphology

Sperm tail length was positively associated with tubule CSA (Table 3e), lumen CSA (Table 3g), and epithelial CSA (Table 3h; Fig. 6). No other measures of sperm morphology were associated with testis architecture.

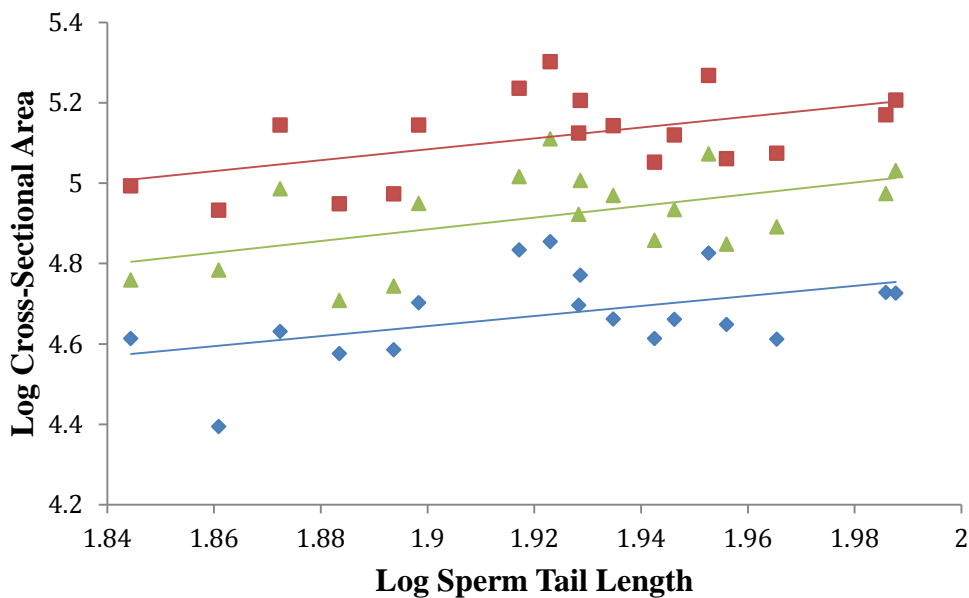


Figure 6. Log₁₀ transformed values of tubule cross sectional area (red square), epithelial cross sectional area (green triangle), and lumen cross sectional area (blue diamond) in relation to sperm tail length across 18 species of *Anolis* lizards.

Testis epithelial cell proportion

There was no significant relationship between the ratio of testis epithelial cells (Fig. 7) and any measure of sperm length (all $p > 0.1$). Moreover, larger epithelia do not necessarily have more epithelial cells (Table 3h).

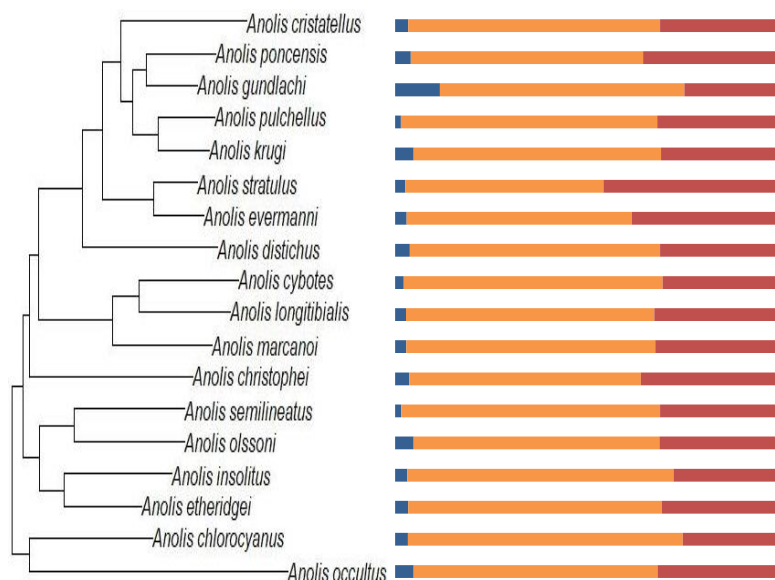


Figure 7. Average proportions of interstitial (blue), epithelial (yellow), and lumen (red) tissues within the testes per species, as shown by the phylogeny (left).

Table 4. Statistical relationships among traits associated with the evolution of pre- and postcopulatory sexual selection in *Anolis* lizards.

Independent Variable (+covariate) Dependent Variable Covariate	Adjusted R ²	F-statistic*	p-value
a. SVL			
Testis Size	0.54	21.01	<0.001
Testis CSA	0.59	25.15	<0.001
Sperm Head	0.009	1.15	0.30
Sperm Midpiece	-0.055	0.11	0.74
Sperm Tail	-0.008	0.86	0.37
b. Testis CSA			
Seminiferous Tubule CSA	0.12	3.27	0.089
Lumen CSA	0.24	6.37	0.02
Epithelial CSA	0.03	1.58	0.23
Sperm Head	0.20	5.20	0.037

Sperm Midpiece	-0.05	0.20	0.66
Sperm Tail	0.07	2.2	0.16
Proportion of Epithelial Cells	0.04	1.78	0.20
c. Testis CSA + SVL			
Seminiferous Tubule CSA	0.06	1.54	0.25
Testis CSA			0.32
SVL			0.92
Lumen CSA	0.24	3.74	0.048
Testis CSA			0.46
SVL			0.31
Sperm Head	0.19	3.03	0.078
Testis CSA			0.048
SVL			0.36
d. GSI (Relative Testis Size)			
Seminiferous Tubule CSA	-0.06	0.003	0.96
Lumen CSA	-0.06	0.06	0.82
Epithelial CSA	-0.06	0.003	0.96
Sperm Head	0.075	2.38	0.14
Sperm Midpiece	0.12	3.30	0.088
Sperm Tail	-0.056	0.097	0.76
Proportion of Epithelial Cells	0.027	1.47	0.24
e. Tubule CSA			
Lumen CSA	0.83	83.03	<0.001
Epithelial CSA	0.95	330.2	<0.001
Sperm Head	-0.01	0.81	0.38
Sperm Midpiece	-0.06	0.03	0.86
Sperm Tail	0.31	8.68	0.009
f. Tubule CSA + Testis CSA			
Lumen CSA	0.84	45.9	<0.001
Tubule CSA			<0.001
Testis CSA			0.15
Sperm Tail	0.27	4.17	0.036
Tubule CSA			0.045
Testis CSA			0.72
g. Lumen CSA			
Sperm Head	-0.02	0.69	0.42
Sperm Midpiece	-0.05	0.19	0.67
Sperm Tail	0.24	6.26	0.024
h. Epithelial CSA			
Sperm Head	-0.01	0.76	0.40
Sperm Midpiece	-0.06	<0.001	0.99
Sperm Tail	0.27	4.17	0.036
Proportion of Epithelial Cells	0.005	1.085	0.31

*F-statistic for df = 1, 16 unless covariate included, then F-statistic for df = 2, 15

Discussion

Most lizards are seasonal breeders that exhibit an increase in testis size and seminiferous tubule size during the breeding season. Beyond this, no details on the relationship between testis size and architecture in lizards are available. In this thesis, I show that seminiferous lumen size, rather than epithelial size, is associated with absolute testis size (Table 4). In birds, Lüpold et al. (2009) showed not only that testis size and seminiferous tubule size are not related, but that epithelial size is responsible for an increase in tubule size, while a study on mammals showed that lumina within the male reproductive tract increase in size in response to fluid accumulation (Zhou et al. 2001). Because lizards are phylogenetically more closely related to birds than mammals, this finding suggests that birds may have evolved this feature of their reproductive morphology separately from other amniotes.

Table 4. Summary of hypotheses presented in Table 1 (top row of each cell) with results in bold indicated by + (positive relationship), - (negative relationship), or no (no relationship).

	Seminiferous Tubule Size	Seminiferous Epithelial Size	Seminiferous Lumen Size	Proportion of Testis Epithelial Cells	Sperm Length
Absolute Testis Size	+ ^ +	No ^ No	+ ** +	? ^ No	? ^ + (head)
Relative Testis Size	+ ^ No	+ * No	? ^ No	+ ^ No	? ^ + (mid)
Sperm Length	+ * + (tail)	+ * + (tail)	No * + (tail)	- ^ No	---

*birds: Lüpold et al. 2009; **mammals: Zhou et al. 2001; ^This study

Further, I found that at least one aspect of sperm morphology was correlated with each measure of testis size or architecture (Table 4). First, sperm tail length was strongly associated

with seminiferous tubule, epithelial, and lumen size, indicating that there is a strong evolutionary relationship between these reproductive traits. Moreover, because only one measure of tubule size was significantly correlated with absolute testis size, while no measures of tubule size were correlated with relative testis size, it may be that sperm tail length is a stronger predictor of seminiferous tubule size, seminiferous epithelial size, and lumen size than any measure of absolute or relative testis size. Additionally, sperm head length correlated with absolute testis size, while sperm midpiece length correlated with relative testis size. Together, these findings suggest that there is selection for the evolution of testis size and morphology as a function of sperm length. The strength of this relationship may explain why there was no association between relative testis size and epithelial thickness, a relationship that has been previously described in the literature (Lüpold et al. 2009). Alternatively, it is possible that testis size is driven by the overall length of the seminiferous tubules, a feature that I could not measure, such that longer tubules require more space and thus a larger testis.

Since sperm develop in a manner that is perpendicular to the thickness of the seminiferous epithelium, it makes sense that sperm tail length, the longest component contributing to overall sperm length, is positively associated with larger epithelia; longer sperm require a larger epithelium within which to develop. Moreover, longer sperm are also positively associated with larger lumina within the seminiferous tubules, a relationship not exhibited in birds (Lüpold et al. 2009). From this, it may be that sperm production is more important in birds, whereas sperm storage may be more important in lizards.

Moreover, since both sperm head and midpiece lengths were associated with testis size while sperm tail length was not, it is critical to consider each of these measures separately. Previous studies have only used overall length as a measure of sperm morphology and

consequently may have missed different relationships for each component of sperm length. While it is not yet clear what mechanisms link certain aspects of sperm length to different features of testis size and architecture, it is evident that sperm head, midpiece, and tail lengths should be recognized as individual measures of sperm morphology.

As described by Ramm and Schärer (2014), spermatogenesis can yield two broadly different types of sperm: shorter sperm that are present in the ejaculate in higher quantities, or longer sperm that are present in lower quantities, where quantity reflects the number of cell divisions that took place during spermatocytogenesis. For this reason, I hypothesized that there would be a negative relationship between sperm length and the proportion of testis epithelial cells, however this was not the case (Table 4). There are several possible explanations for why there was no relationship among the proportion of testis epithelial cells and either sperm length or the thickness of the seminiferous epithelium across species. First, there are many different cell types within the epithelium, and thus it may have been inappropriate to lump all cells residing in the epithelium into a single category for statistical analysis. For example, Lüpold et al. (2011) examined the density of sperm cells at each stage of spermatogenesis, in addition to Sertoli cells, and found that round spermatids in particular varied significantly across ten species of birds. By focusing on this one cell type, Lüpold et al. found strong, positive relationships between the proportion of round spermatids/Sertoli cells and both residual testis mass and sperm length. Conversely, these authors found that as the ratio of elongated sperm cells/round spermatids increased, both relative testis mass and sperm length decreased. While ratios of various germ cell stages and Sertoli cells in relation to testis size and sperm length are beyond the data available in this thesis, this study highlights the possible directions for future study in the evolutionary relationship between testis composition and sperm morphology.

Second, it is possible that the overall proportion of testis cells residing in the seminiferous epithelium does indeed correspond with sperm morphology or testis composition, but that the methodology used for sampling the proportions of different cell types within each testis in this study could be improved. While it was easy to identify individual cells via the unmistakable bright purple hematoxylin stain, distinguishing cell types along the periphery of each tubule proved to be somewhat challenging, and thus some traces of interstitial cells may have been included in the total number of epithelial cells (and vice versa) for each individual. To improve upon this point of uncertainty in the continuation of this study, I suggest utilizing a much higher magnification. As a follow up to this study, I propose to use methodology similar to that of Lüpold et al. (2011)--the optical dissector method--in order to clearly visualize and count both Sertoli cells and spermatogenic germ cells within the epithelium, as well as accurately identify cell types along the border of each tubule. However, it is likely that even at high magnifications, the H&E stain is too general a stain to confidently distinguish epithelial cells from peritubular and/or interstitial cells along the periphery, and thus other histological methods must be considered. One possible alternative visualization method is transmission electron microscopy, an approach that involves an entirely different protocol for tissue preparation, but is excellent for high resolution images of testis cells (Rheubert et al. 2015).

Lastly, it is also possible that, as my data show, there truly is no relationship between the thickness of the seminiferous epithelium and the proportion of epithelial cells to other testis cells. This finding suggests that the germ cell development strategy in anoles is more complicated than what this thesis is able to address, and may require a higher resolution analysis of germ cell development as seen in Lüpold et al. (2011).

Conclusion

In *Anolis* lizards, the evolution of testis size and architecture, rather than germ cell density, is associated with the evolution of three components of sperm morphology. Specifically, sperm tail length is associated with seminiferous tubule, epithelium, and lumen size, while sperm head length is associated with absolute testis size. Absolute testis size is also associated with larger tubules and tubule lumina, which indicates that testis size may be more indicative of sperm storage, rather than production. On the other hand, relative testis size is not associated with any measure of testis architecture, and only exhibits a trend towards a positive relationship with sperm midpiece length. Ultimately, these findings provide previously unknown information on how postcopulatory sexual selection operates on two key morphological and physiological components of reproduction in lizards. Further, these findings indicate that subsequent studies should implement the use of multiple measures of sperm length (tail, midpiece, and head), as to not miss relationships with head and midpiece length that may be masked by only considering overall sperm length.

References

- Andersson, M. B. 1994. *Sexual Selection*. Princeton University Press.
- Andersson, M. B., and Simmons, L. W. (2006). "Sexual selection and mate choice." *Trends in Ecology & Evolution* 21: 296-302.
- Arak, A. 1983. "Sexual selection by male–male competition in natterjack toad choruses." *Nature* 306: 261-262.
- Austad, S. N. 1984. "Chapter 7: Evolution of sperm priority patterns in spiders." In: *Sperm Competition and the Evolution of Animal Mating Systems* pp. 223-247. (R. L. Smith, ed.) Orlando, FL: Academic Press.
- Balshine, S., B. J. Leach, F. Neat, N. Y. Werner, and R. Montgomerie. 2001. "Sperm size of African cichlids in relation to sperm competition." *Behavioral Ecology* 12: 726–731.
- Birkhead, T. R., and A. P. Møller. 1993. "Sexual selection and the temporal separation of reproductive events: sperm storage data from reptiles, birds and mammals." *Biological Journal of the Linnean Society* 50: 295-311.
- Brennan, P. L. R., C. J. Clark, and R. O. Prum. 2010. "Explosive eversion and functional morphology of the duck penis supports sexual conflict in waterfowl genitalia." *Proceedings of the Royal Society of London B: Biological Sciences* 277: 1309-1314.
- Brennan, P. L. R., R. O. Prum, K. G. McCracken, M. D. Sorenson, R. E. Wilson, and T. R. Birkhead. 2007. "Coevolution of male and female genital morphology in waterfowl." *PLoS One* 2: e418.
- Bush, J. M., M. M. Quinn, E. C. Balreira, and M. A. Johnson. 2016. "How do lizards determine dominance? Applying ranking algorithms to animal social behavior." *Animal Behavior* 118: 65-74.

- Calsbeek, R., C. Bonneaud, S. Prabhu, N. Manoukis, and T. B. Smith. 2007. "Multiple paternity and sperm storage lead to increased genetic diversity in *Anolis* lizards." *Evolutionary Ecology Research* 9: 495-503.
- Christy, J. H., P. R. Backwell, S. Goshima, and T. Kreuter. (2002). "Sexual selection for structure building by courting male fiddler crabs: an experimental study of behavioral mechanisms." *Behavioral Ecology* 13: 366-374.
- Córdoba-Aguilar, A., Uhía, E., and Rivera, A. C. (2003). "Sperm competition in Odonata (Insecta): the evolution of female sperm storage and rivals' sperm displacement." *Journal of Zoology* 261: 381-398.
- Cox, R. M., and A. F. Kahrl. 2014. "Chapter 4: Sexual selection and sexual dimorphism." In: *Reproductive Biology and Phylogeny of Lizards and Tuatara* 10: pp. 78-108. CRC Press, Boca Raton, FL (B. G. M. Jamieson, ed.)
- Cox, R. M., M. A. Butler, and H. B. John-Alder. 2007. "Chapter 4: The evolution of sexual size dimorphism in reptiles." In: *Sex, Size and Gender Roles: Evolutionary Studies of Sexual Size Dimorphism*: pp. 38-49. (D. J. Fairbain, W. U. Blanckenhorn, and T. Szekely, eds.)
- Darwin, C. 1871. *The Descent of Man, and Selection in Relation to Sex*.
- Duryea, M. C., A. D. Kern, R. M. Cox, R. Calsbeek. 2013. "A novel application for Approximate Bayesian Computation for detecting male reproductive advantages due to mating order." *Behavioral Ecology and Sociobiology* 67: 1867-1875.
- Eberhard, W. G. "Chapter 3: Female roles in sperm competition." In: *Sperm Competition and Sexual Selection*: pp. 91-116. (T. R. Birkhead and A. P. Moller, eds.) San Diego, CA: Academic Press.

- Eberhard, W. G. 1985. *Sexual Selection and Animal Genitalia* 244. Cambridge, MA: Harvard University Press.
- Erlinge, S. 1979. "Adaptive significance of sexual dimorphism in weasels." *Oikos* 33: 233-245
- Fitzpatrick, J. L., M. Almbro, A. Gonzalez-Voyer, N. Kolm, and L.W. Simmons. 2012. "Male contest competition and the coevolution of weaponry and testes in pinnipeds." *Evolution* 66: 3595-3604.
- Foellmer, M. W., and J. Moya-Larano. 2007. "Chapter 7: Sexual size dimorphism in spiders: patterns and processes." In: *Sex, Size and Gender Roles: Evolutionary Studies of Sexual Size Dimorphism*: pp. 71-84. (D. J. Fairbain, W. U. Blanckenhorn, and T. Szekely, eds.)
- Freckleton, R. P., P. H. Harvey, and M. Pagel. 2002. "Phylogenetic analysis and comparative data: a test and review of evidence." *The American Naturalist* 160: 712-726.
- Fuxjager, M. J., J. H. Lee, T. M. Chan, J. H. Bahn, J. G. Chew, X. Xiao, and B. A. Schlinger. 2016. "Research resource: hormones, genes, and athleticism: effect of androgens on the avian muscular transcriptome." *Molecular Endocrinology* 30: 254-271.
- Gack, C. and K. Peschke. 1994. "Spermathecal morphology, sperm transfer and a novel mechanism of sperm displacement in the rove beetle, *Aleochara curtula* (Coleoptera, Staphylinidae)." *Zoomorphology* 114: 227-237.
- Gage, M. J. G., and R. P. Freckleton. 2003. "Relative testis size and sperm morphometry across mammals: no evidence for an association between sperm competition and sperm length." *Proceedings of the Royal Society of London B: Biological Sciences* 270: 625–632.

- Gage, M. J. G., C. P. Macfarlane, S. Yeates, R. G. Ward, J. B. Searle, and G. A. Parker. 2004. "Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success." *Current Biology* 14: 44–47.
- Gardiner, D. M. 1978. "Cyclic changes in fine structure of the epithelium lining the ovary of the viviparous teleost, *Cymatogaster aggregata* (Perciformes: Embiotocidae)." *Journal of Morphology* 156.3: 367-379.
- Gist, D. H. and J. M. Jones. 1989. "Sperm storage within the oviduct of turtles." *Journal of Morphology* 199: 379-384.
- Gribbins, K. M., and J. L. Rheubert. 2014. "Chapter 11: The architecture of the testis, spermatogenesis, and mature spermatozoa." In: *Reproductive Biology and Phylogeny of Lizards and Tuatara* 10: pp: 340-424. (B. G. M. Jamieson, ed.) Boca Raton, FL: CRC Press
- Gribbins, K. M., M. Anzalone, M. Collier, G. Granados-González, M. Villagrán-Santa Cruz, and O. Hernández-Gallegos. 2011. "Temporal germ cell development strategy during continuous spermatogenesis within the montane lizard, *Sceloporus bicanthalis* (Squamata; Phrynosomatidae)." *Theriogenology* 76: 1090-1099.
- Harcourt, A. H., P. H. Harvey, S. G. Larson, and R. Short. 1981. "Testis weight, body weight and breeding system in primates." *Nature* 293: 55-57.
- Harmon L. J., J. T. Weir, C. D. Brock, R. E. Glor, and W. Challenger. 2008. "GEIGER: investigating evolutionary radiations." *Bioinformatics* 24: 129-131.
- Higham, J. P., M. Heistermann, and D. Maestripieri. 2011. "The energetics of male–male endurance rivalry in free-ranging rhesus macaques, *Macaca mulatta*." *Animal Behaviour* 81: 1001-1007.

- Holt, W.V., and R.E Lloyd. 2010. "Sperm storage in the vertebrate female reproductive tract: How does it work so well?" *Theriogenology* 73: pp. 713–722.
- Hughes, C. 1998. "Integrating molecular techniques with field methods in studies of social behavior: a revolution results." *Ecology* 79: 383-399.
- Hunt, J., C. J. Breuker, J. A. Sadowski, and A. J. Moore. 2009. "Male–male competition, female mate choice and their interaction: determining total sexual selection." *Journal of Evolutionary Biology* 22: 13-26.
- Immler, S., S. Calhim, and T. R. Birkhead. 2008. "Increased postcopulatory sexual selection reduces the intramale variation in sperm design." *Evolution* 62: 1538-1543.
- Johns, A., H. Gotoh, E.L. McCullough, D.J. Emlen, and L.C. Lavine. 2014. "Heightened condition-dependent growth of sexually selected weapons in the rhinoceros beetle, *Trypoxylus dichotomus* (Coleoptera: Scarabaeidae)." *Integrative and Comparative Biology* 54: 614-621.
- Joseph, P. N., Emberts, Z., Sasson, D. A., & Miller, C. W. 2018. "Males that drop a sexually selected weapon grow larger testes." *Evolution* 72: 113-122.
- Kahrl, A. F., C. L. Cox, and R. M. Cox. 2016. "Correlated evolution between targets of pre-and postcopulatory sexual selection across squamate reptiles." *Ecology and Evolution* 6: 6452-6459.
- Kahrl, A. F. 2017. "Rapid evolution of testis size suggests that postcopulatory sexual selection targets sperm count, not sperm morphology, in *Anolis* lizards." PhD dissertation, University of Virginia.

- Kawano, K. 1995. "Horn and wing allometry and male dimorphism in giant rhinoceros beetles (Coleoptera: Scarabaeidae) of tropical Asia and America." *Annals of the Entomological Society of America* 88: 92-99.
- King, L. M., J. P. Brillard, W. M. Garrett, M. R. Bakst, and A. M. Donoghue. 2002. "Segregation of spermatozoa within sperm storage tubules of fowl and turkey hens." *Reproduction* 123: 79-86.
- Klaczko, J., T. Ingram, and J. Losos. 2015. "Genitals evolve faster than other traits in *Anolis* lizards." *Journal of Zoology* 295: 44-48.
- Kraaijeveld-Smit, F. J. L., S. J. Ward, P. D. Temple-Smith, and D. Paetkau. 2002. "Factors influencing paternity success in *Antechinus agilis*: Last-male sperm precedence, timing of mating and genetic compatibility." *Journal of Evolutionary Biology* 15: 100-107.
- Kupfer, A. 2007. "Chapter 5: Sexual size dimorphism in amphibians: an overview." In: *Sex, Size and Gender Roles: Evolutionary Studies of Sexual Size Dimorphism*: pp. 50-60. (D. J. Fairbairn, W. U. Blanckenhorn, T. Szekely, eds.)
- Kvarnemo, C., and L. W. Simmons. 2013. "Polyandry as a mediator of sexual selection before and after mating." *Philosophical Transactions of the Royal Society B* 368: 20120042.
- Kvarnemo, C., O. Svensson, and W. Manson. 2010. "Investment in testes, sperm-duct glands and lipid reserves differs between male morphs but not between early and late breeding season in *Pomatoschistus minutus*." *Journal of Fish Biology* 76: 1609-1625.
- Liljedal, S, I. Folstad, and F. Skarstein. 1999. "Secondary sex traits, parasites, immunity and ejaculate quality in the Arctic charr." *Proceedings of the Royal Society of London B: Biological Sciences* 266: 1893-1898.

- Lindenfors, P., J. L. Gittleman, and K. E. Jones. 2007. "Chapter 2: Sexual size dimorphism in mammals." In: *Sex, Size and Gender Roles: Evolutionary Studies of Sexual Size Dimorphism*: pp. 16-26. (D. J. Fairbairn, W. U. Blanckenhorn, T. Szekely, eds.).
- Losos, J. B. and C. J. Schneider. 2009. "Anolis lizards." *Current Biology* 19: R316-R318.
- Lovich, J. E., and J. W. Gibbons. 1992. "A review of techniques for quantifying sexual size dimorphism." *Growth Development and Aging* 56: 269-269.
- Lüpold, S., G. M. Linz, J. W. Rivers, D. F. Westneat, and T. R. Birkhead. 2009. "Sperm competition selects beyond relative testes size in birds." *Evolution* 63: 391-402.
- Lüpold, S., J. L. Tomkins, L. W. Simmons, and J. L. Fitzpatrick. 2014. "Female monopolization mediates the relationship between pre-and postcopulatory sexual traits." *Nature Communications* 5: article 3184.
- Lüpold, S, J. Wistuba, O. S. Damm, J. W. Rivers, and T. R. Birkhead. 2011. "Sperm competition leads to functional adaptations in avian testes to maximize sperm quantity and quality." *Reproduction* 141: 595-605.
- Maklakov, A. A., T. Bilde, and Y. Lubin. 2004. "Sexual selection for increased male body size and protandry in a spider." *Animal Behaviour* 68: 1041-1048.
- Marshall, A. J. 1954. "Bower-birds." *Biological Reviews* 29: 1-45.
- McKinney, F., S. R. Derrickson, and P. Mineau. "Forced copulation in waterfowl." *Behaviour* 86.3 (1983): 250-293.
- Millar, J. S., and G. J. Hickling. 1990. "Fasting endurance and the evolution of mammalian body size." *Functional Ecology* 4: 5-12.
- Moller, A. P. 1991. "Sperm competition, sperm depletion, paternal care, and relative testis size in birds." *The American Naturalist* 137: 882-906.

- Nijhout, H. F., and D. J. Emlen. 1998. "Competition among body parts in the development and evolution of insect morphology." *Proceedings of the National Academy of Sciences* 95: 3685-3689.
- Ono, T., M. T. Siva-Jothy, and A. K. I. Kato. 1989. "Removal and subsequent ingestion of rivals' semen during copulation in a tree cricket." *Physiological Entomology* 14: 195-202.
- Paradis, E., J. Claude, and K. Strimmer. 2004. "APE: analyses of phylogenetics and evolution in R language." *Bioinformatics* 20: 289-290.
- Parker, G. A., C. M. Lessells, and L. W. Simmons. 2013. "Sperm competition games: a general model for precopulatory male–male competition." *Evolution* 67: 95-109.
- Perrone, M. 1978. "Mate size and breeding success in a monogamous cichlid fish." *Environmental Biology of Fishes* 3: 193-201.
- Pitnick, S., D. J. Hosken, and T. R. Birkhead. 2009. "Chapter 3: Sperm morphological diversity." In: *Sperm Biology: An Evolutionary Perspective*: pp. 69-149. (Pitnick, S., D. J. Hosken, and T. R. Birkhead, eds.)
- Pitnick, S., K. E. Jones, and G. S. Wilkinson. 2006. "Mating system and brain size in bats." *Proceedings of the Royal Society of London B: Biological Sciences* 273: 719-724.
- Poulin, R., and S. Morand. 2000. "Testes size, body size and male–male competition in acanthocephalan parasites." *Journal of Zoology* 250: 551-558.
- Pyron, R. A., F. T. Burbrink, and J. J. Wiens. 2013. "A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes." *BMC Evolutionary Biology* 13: 93.
- R Core Team (2017). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.

- Racey, P. A. 1979. "The prolonged storage and survival of spermatozoa in Chiroptera." *Journal of Reproduction and Fertility* 56: 391-402.
- Ralls, K., and P. H. Harvey. 1985. "Geographic variation in size and sexual dimorphism of North American weasels." *Biological Journal of the Linnean Society* 25: 119-167.
- Ramm, Steven A., and L. Schärer. 2014. "The evolutionary ecology of testicular function: size isn't everything." *Biological Reviews* 89: 874-888.
- Ridley, M., and D. J. Thompson. 1979. "Size and mating in *Asellus aquaticus* (Crustacea: Isopoda)." *Ethology* 51: 380-397.
- Rönn, J., M. Katvala, and G. Arnqvist. 2007. "Coevolution between harmful male genitalia and female resistance in seed beetles." *Proceedings of the National Academy of Sciences* 104: 10921-10925.
- Rowe, M., J. P. Swaddle, S. Pruett-Jones, and M. S. Webster. 2010. "Plumage coloration, ejaculate quality and reproductive phenotype in the red-backed fairy-wren." *Animal Behaviour* 79: 1239-1246.
- Saino, N., C. R. Primmer, and H. Ellegren. 1997. "An experimental study of paternity and tail ornamentation in the barn swallow (*Hirundo rustica*)." *Evolution* 51: 562-570.
- Schaedelin, F. C., and M. Taborsky. 2010. "Female choice of a non-bodily ornament: an experimental study of cichlid sand craters in *Cyathopharynx furcifer*." *Behavioral ecology and sociobiology* 64: 1437-1447.
- Schärer, L., P. Ladurner, and R. M. Rieger. 2004. "Bigger testes do work more: experimental evidence that testis size reflects testicular cell proliferation activity in the marine invertebrate, the free-living flatworm *Macrostomum* sp." *Behavioral Ecology and Sociobiology* 56: pp. 420-425.

- Schulte-Hostedde, A. I., J. S. Millar, and H. L. Gibbs. 2002. "Female-biased sexual size dimorphism in the yellow-pine chipmunk (*Tamias amoenus*): sex-specific patterns of annual reproductive success and survival." *Evolution* 56: 2519-2529.
- Sears, Richard, and William F. Perrin. 2009. "Blue Whale: *Balaenoptera musculus*." In: *Encyclopedia of Marine Mammals*: 120-124. (W. F. Perrin, B. Wursig, and J. G. M. Thewissen, eds.)
- Serrano-Meneses, M. A., Córdoba-Aguilar, A., Méndez, V., Layen, S. J., and Székely, T. 2007. "Sexual size dimorphism in the American rubyspot: male body size predicts male competition and mating success." *Animal Behaviour* 73: 987-997.
- Sever, D. M., and W. C. Hamlett. 2002. "Female sperm storage in reptiles." *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology* 292: 187-199.
- Sih, A., and J. J. Krupa. 1992. "Predation risk, food deprivation and non-random mating by size in the stream water strider, *Aquarius remigis*." *Behavioral Ecology and Sociobiology* 31: 51-56.
- Simmons, L. W., and D. J. Emlen. 2006. "Evolutionary trade-off between weapons and testes." *Proceedings of the National Academy of Sciences* 103: 16346-16351.
- Suarez, S. S. and A. A. Pacey. 2006. "Sperm transport in the female reproductive tract." *Human Reproduction Update* 12: 23-37.
- Stamps, J. A. 1983. "Sexual selection, sexual dimorphism, and territoriality." In: *Lizard Ecology: Studies of a Model Organism*: 169-204. (R. R. Huey, E. R. Pianka, and T. W. Schoener, eds.)

- Szekely, Tamas, Terje Lislevand, and Jordi Figuerola. 2007. "Chapter 3: Sexual size dimorphism in birds." In: *Sex, Size and Gender Roles: Evolutionary Studies of Sexual Size Dimorphism*: pp. 27-37. (D. J. Fairbairn, W. U. Blanckenhorn, T. Szekely, eds.) Oxford, NY: Oxford University Press.
- Thornhill, R., and J. Alcock. 2014. "Chapter 15: The Evolution of Insect Mating Systems." In: *The Evolution of Insect Mating Systems*. pp. 257-278. Harvard University Press.
- Tilley, S. G. 1968. "Size-fecundity relationships and their evolutionary implications in five desmognathine salamanders." *Evolution* 22: 806-816.
- Williams, G. C. 1966. "Natural selection, the cost of reproduction, and a refinement of Lack's Principle." *The American Naturalist* 100: 687-690
- Zamudio, K. R. 1998. "The evolution of female-biased sexual size dimorphism: A population-level comparative study in horned lizards (*Phrynosoma*)." *Evolution* 52: 1821-1833.
- Zhou, Q., L. Clarke, R. Nie, K. Carnes, L. W. Lai, Y. H. H. Lien, A. Verkman, D. Lubahn, J. S. Fisher, B. S. Katzenellenbogen, and R. A. Hess. 2001. "Estrogen action and male fertility: roles of the sodium/hydrogen exchanger-3 and fluid reabsorption in reproductive tract function." *Proceedings of the National Academy of Sciences* 98: 14132-14137.

Appendix

Appendix. Mean and standard deviation of four cross-sectional area (CSA) measurements (mm²) of *Anolis* testis size and architecture

	Testis CSA	Lumen CSA	Tubule CSA	Epithelium CSA
<i>A.chlorocyanus</i>	10.5±3.12	0.038±0.013	0.094±0.026	0.055±0.014
<i>A.christophei</i>	5.12±0.98	0.044±0.010	0.115±0.023	0.070±0.016
<i>A.cristatellus</i>	7.97±1.35	0.050±0.013	0.133±0.029	0.084±0.020
<i>A.cybotes</i>	13.34±2.12	0.068±0.018	0.172±0.043	0.104±0.028
<i>A.distichus</i>	9.69±1.40	0.053±0.012	0.148±0.0312	0.094±0.021
<i>A.etheridgei</i>	4.72±0.06	0.025±0.008	0.085±0.015	0.061±0.007
<i>A.evermanni</i>	13.22±4.25	0.059±0.012	0.160±0.024	0.101±0.014
<i>A.gundlachi</i>	9.19±1.00	0.053±0.012	0.161±0.033	0.107±0.025
<i>A.insolitus</i>	5.69±0.41	0.041±0.006	0.098±0.004	0.057±0.002
<i>A.krugi</i>	5.13±0.86	0.041±0.011	0.113±0.031	0.072±0.021
<i>A.longitibialis</i>	13.68±2.24	0.071±0.023	0.200±0.044	0.129±0.025
<i>A.marcanoi</i>	11.46±2.33	0.067±0.030	0.185±0.067	0.118±0.038
<i>A.occultus</i>	4.95±1.32	0.041±0.008	0.119±0.022	0.078±0.015
<i>A.olssoni</i>	3.64±0.87	0.050±0.017	0.139±0.032	0.089±0.016
<i>A.poncensis</i>	4.58±0.65	0.043±0.007	0.139±0.021	0.097±0.017
<i>A.pulchellus</i>	4.35±1.44	0.046±0.010	0.139±0.028	0.093±0.020
<i>A.semilineatus</i>	4.68±0.40	0.038±0.007	0.089±0.012	0.051±0.005
<i>A.stratulus</i>	10.32±0.94	0.046±0.012	0.132±0.025	0.086±0.016