The diversity, plasticity, and evolution of sperm morphology in *Anolis* lizards

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A Dissertation presented to the Graduate Faculty
of the University of Virginia in Candidacy for the Degree of
Doctor of Philosophy

Department of Biology

University of Virginia
May, 2017

ABSTRACT

A central goal of evolutionary biology is to understand the sources of phenotypic variation within and among species. Sexually selected traits, those that confer an advantage in both securing a mate and in fertilization, are often highly diverse, especially in males. Though research has historically focused on extravagant traits, such as plumage or horn size, relatively little research has focused on traits that experience selection after mating, or during postcopulatory selection. Sperm is the most diverse of all cell types, ranging four orders of magnitude in size across all animals, and is central to male reproductive success. Because males of all animal species produce sperm, studying this cell provides a unique model for exploring how sexual selection shapes the evolution of traits associated with postcopulatory selection. In this dissertation, I use an integrative approach to compare the processes that generate variation within a species, to the patterns of evolution in sperm morphology among species of *Anolis* lizards. As sexual selection occurs in two episodes (pre- and postcopulatory sexual selection), I first tested for correlated evolution between targets of each episode of selection in snakes and lizards. I found a negative relationship between sexual size dimorphism and residual testis size, suggesting that precopulatory selection constrains the opportunity for postcopulatory selection to occur, and/or that targets of each episode of selection experience an energetic trade-off. Among species of anoles, I then demonstrated that the sperm midpiece length evolves faster than the rest of the sperm cell, but evolves much more slowly than residual testis size. The differences in these rates of evolution indicate that sperm production may be more evolutionarily labile, or more important for reproductive success than sperm

morphology in *Anolis* lizards. In both experimental diet treatments and in wild populations of *Anolis sagrei* I demonstrate that the sperm midpiece length is condition-dependent. In this same study, I showed that fertilization success is condition-dependent, which may be partially mediated by condition-dependent variation in sperm morphology, sperm count, or mating frequency. I also found consistent differences in sperm morphology between native and introduced populations of three species of *Anolis* lizards, suggesting that the environment may influence sperm morphology either through phenotypic plasticity or by genetic adaptation. Finally, I tested for correlations between sperm morphology and sperm velocity in a wild population of brown anoles and found that sperm midpiece length was positively correlated with sperm velocity in this population. These studies reveal high variation in sperm morphology within individuals, between populations, and across species, and demonstrate that this variation is phenotypically plastic, is related to sperm function, and may be associated with male reproductive success. These results also suggest that sperm number and/or copulation rate are more important for fertilization success than sperm morphology in anoles.

ACKNOWLEDGMENTS

The development of a dissertation, and training of a Ph.D. student requires the intellectual, financial, and emotional support of an entire community. I feel exceptionally lucky to have had the support of a wonderful group of people during my dissertation, without whom, this body of work and my development as a researcher would not have occurred. My advisor Bob Cox has been instrumental in this process and has been an amazing mentor during my graduate career. Despite having fairly different research interests, Bob has always been supportive, encouraged my independence, and gave my ideas the critical eye they needed to turn them into interesting and impactful research. Most importantly, Bob has taught me, and shown me by example, how to be an effective communicator, writer, and critic. Bob's ability to build narratives has been invaluable to me as I learn how to share my research and ideas with the broader scientific community. Being his first graduate student has been a privilege, and I know that without his guidance and support I would not be in the position I am in today. He has also taught me a fair amount about beer, which has proven to be a helpful networking skill among fellow herpetologists.

I also would like to thank my committee members for their continued assistance in wrangling methods and topics that are outside most of their areas of expertise. I thank Butch Brodie for his helpful feedback throughout the development of my dissertation, and for constantly pushing me to think broadly and err boldly. He convinced me to come to UVa, and since then has always made time for my questions, both large and small. Laura Galloway's advice and discussion has been immensely helpful to me in committee

meetings and after EEBio seminars. I also appreciate all of the opportunities she has given female graduate students and undergrads to ask questions and have open conversation about being a female scientist. Dave Carr has helped me stay on track by making sure that I had targeted, and question-driven data collection, when my instinct was to grab as much data as I could. Keith Kozminski gave me the initial support I needed to collect data necessary to form my dissertation proposal. I will forever be indebted to him for the use of his microscope, and for his knowledge of cellular biology.

This dissertation would not have taken its completed form without the help of my academic brother and labmate Aaron Reedy. Aaron has taught me to think big, has encouraged me with his endless optimism, and has been a support net in many ways for me throughout my graduate career. I feel lucky to have worked on such a large scale with Aaron because, despite my imminent departure from UVa, we will be able to collaborate for quite a while as we work through all of our data.

I also want to thank the rest of the UVa EEBio community, especially Christian Cox, Robin Costello, Mike Hague, Malcolm Augat, Corlett Wood, Brian Sanderson, Ray Watson, and Vince Formica for their friendship, discussions, help with data collection, and comments on manuscripts. Christian Cox was instrumental in teaching me the basics of phylogenetic comparative methods, and was an excellent mentor, companion, and collaborator in the field. I also have to thank the small army of undergraduate researchers who, by my estimate, have helped me measure close to 10,000 sperm cells, which make up the bulk of my dissertation. From this group of students, I especially want to thank Laura Zemanian, Elizabeth Luebbert, Frank Song, Vida Motamedi, and Matthew Kustra.

It took many hands to collect the number of individuals and species used in Chapter 2 of my dissertation. Michele Johnson, and her dozen or so students from Trinity University, were critical in helping me collect the data needed to pursue this project. Michele has been a wonderful collaborator, friend, and role model to me since we started working together, and I can not thank her enough for including me in trips to Puerto Rico and the Dominican Republic. I also thank all of the Johnson lab undergraduate researchers and technicians who helped me with collections and were great sources of friendship during long hours in the field.

Staying grounded can be a challenge for many people entering into graduate school. I was fortunate that when I moved to Charlottesville I was instantly welcomed into a community of dancers who are some of my closest and dearest friends. Though dance is very different from science, being a part of this organization for six years has made me a better teacher, leader, communicator, and has taught me how to manage larger groups and run events. The people in this group, and especially Franny, Peter, Brian, and Scott, have been inspiring to me in many ways, I feel honored to have celebrated, cried, and learned with them. I want to also thank Kyle Martin who has been an unwavering best friend since my first month in Charlottesville. I feel privileged to have a partner who shares many of my passions, and can inspire me to learn and grow both personally and scientifically.

I would also like to thank my family, and especially my parents, for their constant support and encouragement. Though I have changed career trajectories several times in

my life, they have always been on board and supportive of my choices. I want to thank both of them for the opportunities they have given me throughout my life that allowed me to pursue science as a career and to complete my doctorate.

Finally, I want to thank the National Science Foundation for financial support through a Doctoral Dissertation Improvement Grant, the Herpetologist's League for for their support through an E.E. Williams Research Grant, the American Museum of Natural History for their support through a Theodore Roosevelt Memorial Grant, and support from the UVa Department of Biology.

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INTRODUCTION

The evolution of a sexually selected trait can be complex and difficult to understand without examining the patterns of, and processes that generate, phenotypic variation at multiple levels of organization. Complementing comparative analyses with individual- and species-level studies is critical to understand the evolutionary history of a trait and how it impacts individual fitness. Additionally, assessing the links between morphology and performance gives context for both comparative analyses and experimental studies. Our current understanding of how sexually selected traits evolve is centered on the evolution of traits that experience precopulatory selection. However, in the past 40 years, postcopulatory selection (i.e. selection after mating via sperm competition and cryptic female choice) has been recognized as a strong evolutionary force that can contribute substantially to individual fitness (Pischedda and Rice 2012; Péliissié et al. 2014; Devigili et al. 2015), and is likely responsible for shaping diverse phenotypes among species (Simmons 2001; Birkhead et al. 2009).

Sperm morphology is one of the most diverse traits among animals and experiences postcopulatory selection (Pitnick et al. 2009). Because males of all animal species produce sperm, studying this cell provides a unique opportunity to explore how sexual selection shapes the evolution of a trait critical for fertilization. In this dissertation I aim to determine the evolutionary significance and sources of variation in sperm morphology in *Anolis* lizards. I focus on the causes of individual-level and population-level variation and complement those studies with comparative analyses among species to understand the diversification and evolution of sperm morphology.

Variation on sperm morphology among species

Sperm morphology is a model for studying the evolution of traits associated with postcopulatory selection due to the high diversity in morphology among species (Pitnick et al. 2009; Pizzari and Parker 2009). Although this trait has been studied at multiple levels of organization (from the individual to family-level) there are very few general patterns of selection that emerge among groups (Simmons and Fitzpatrick 2012). The lack of a general pattern is somewhat surprising considering that, for the majority of the species examined, sperm has a similar basic structure with a head, midpiece, and tail. Each of these cellular components plays a role in cell function - the sperm head contains the acrosome and the genetic material, the midpiece contains the mitochondria, and the tail propels the cell and provides some glycolytic activity.

The lack of a consistent pattern of selection among species may be due to the highly species-specific selection that acts on sperm traits. Since sperm competition takes place in the female reproductive tract, it is not surprising that the length of the sperm storage tubule in females is often strongly correlated with sperm morphology (Presgraves et al. 1999; Pitnick et al. 2003; Higginson et al. 2012). Additionally, if sperm competition occurs outside of the female (i.e., in species with external fertilization), a completely different set of traits may be under selection (Snook 2005). Aspects of a species' mating system can also impact how selection acts on sperm traits. For example, in species where mating is frequent, there may be strong selection for increased sperm production, potentially at the cost of sperm quality or size (Snook 2005; Immler et al. 2011).

Depending on the duration of sperm storage, selection may also act differentially on sperm velocity and longevity (which are frequently negatively correlated with one another), potentially resulting in longer or shorter sperm, respectively (Gage et al. 2004; Burness et al. 2004; Helfenstein et al. 2008; 2010). The variation in selective environment, in conjunction with trade-offs among sperm traits, is likely the reason this cell is hyper-diverse. The complex nature of this episode of selection, which is so critical for male reproductive success, has driven research into many of these species-specific trends.

Many studies have tested for associations between the strength of postcopulatory selection and sperm morphology (Simmons and Fitzpatrick 2012). Testis size corrected for body size, or residual testis size, is frequently used as an index of the strength of postcopulatory selection because it is often correlated with the strength of sperm competition across taxa (summarized by Simmons and Fitzpatrick 2012). As larger testes have more sperm-producing tissue (number of seminiferous tubules), males with large testes produce more sperm, and generally, are more successful in sperm competition (Parker 1998; Schärer et al. 2004). Therefore, increases in the strength of sperm competition are predicted to be associated with increases in testis size among species (Parker and Pizzari 2010). This prediction has been bolstered through studies of experimental evolution that document increased residual testis size as a response to increases in sperm competition (Hosken and Ward 2001; Simmons and García-González 2008).

Because selection can be variable among taxa, it is unsurprising that the direction and magnitude of the relationship between sperm morphology and residual testis size is also variable among taxonomic groups (Pizzari and Parker 2009; Simmons and Fitzpatrick 2012). However, if the direction of selection on sperm morphology varies between species (i.e., positive in some, negative in others), it may be difficult to detect a pattern between sperm morphology and residual testis size in a comparative analysis among species even if selection is strong. Estimating the rates of evolution of sperm morphology complements comparative analyses, as rates of evolution can reveal how quickly a trait has diversified over time, regardless of the direction of that change. It can be helpful to pair these rates with tests for evolutionary associations with residual testis size to determine not only how quickly traits have evolved but in what direction they evolved. However, very few studies have implemented both of these tests in their analyses.

Our understanding of the broad patterns of evolution of sperm morphology may be hampered by incomplete taxonomic sampling. Though there are numerous comparative analyses of sperm morphology among species, many of these studies focus on only a few lineages. Though the sperm of birds, mammals, and insects have been sampled across several genera, the breadth of sampling across Metazoa is patchy, and frequently only examines sperm total length rather than parts of the cell (Simmons and Fitzpatrick 2012). Increasing this sampling to include more species, and especially groups that have variable mating systems, will increase our ability to determine how the morphology of this cell has diversified across animals.

Causes for intraspecific and population-level variation in sperm morphology

Sperm is inextricably linked to male fitness, which should generate strong selection and reduce intra-male variation (Immler et al. 2008). Despite this, variation in sperm morphology exists at many levels, within-ejaculate, within-individual, within-population and within-species. Some of this variation can be due to aberrant, irregular morphology or low quality sperm, but much of this variation can also be attributed to differences in testicular structure (Lüpold et al. 2009a), body condition (Rahman et al. 2013; Chapter 4), social environment (Immler et al. 2010; Sasson and Brockmann 2016), geographic patterns (Elgee et al. 2010; Lüpold et al. 2011), and even alternative reproductive tactics (Burness et al. 2004) (summarized in (Pitnick et al. 2009)). However, these causes of variation are typically species-specific and therefore the sources of variation should be characterized within species of a particular lineage to reveal and interpret patterns of sperm morphology among species in that group.

Sexually selected traits often experience strong selection, which can drive them to become energetically expensive and result in a link between trait value and body condition (Andersson 1986; Rowe and Houle 1996). The condition dependence of a trait can generate variation both within and among males (Rowe and Houle 1996; Bonduriansky 2005), and if associated with fitness, may result in condition-dependent reproductive success (Hill 1990; Cotton et al. 2006). Though a single sperm cell may be energetically inexpensive, the production of millions of cells in a single ejaculate can be costly (Dewsbury 1982; Olsson et al. 1997; Perry and Rowe 2010). When resources are

limited, males should invest in the traits that yield the highest fitness benefits, potentially at the cost of other traits (Parker et al. 2013). Limited resources may result in trade-offs within ejaculates, such as, a trade-off between sperm size and sperm number (Immler et al. 2011). Empirically, sperm count (Gasparini et al. 2013; Rahman et al. 2013; Kaldun and Otti 2016; Chapter 4), sperm length (Simmons and Kotiaho 2002; Merrells et al. 2009; Alavi et al. 2009; Rahman et al. 2013; Chapter 4), and within-male variation (Chapter 4) are associated with body condition in a wide range of taxa. However, there are very few studies that have assessed the condition dependence of the individual morphological components of the cell, which could indicate areas of the cell that are energetically expensive, potentially due to sexual selection.

While variation among individuals can lead us to physiological mechanisms and tradeoffs that drive diversity, variation among populations can be used to test if differences in mating systems, selection, or social environment are associated with the divergence of sperm morphology. For example, barn swallow populations with higher rates of extrapair copulation have reduced variation in sperm morphology, likely due to increases in postcopulatory selection (Laskemoen et al. 2013). Several other studies have documented cases of population-level variation in sperm morphology (Elgee et al. 2010; Lüpold et al. 2011; Stewart et al. 2016), but on the whole, we know very little about how and why populations vary. Population-level studies can act to bridge the gap between experimental studies conducted within a species to the broader trends we find among species, and can provide an opportunity to view the evolution and diversification of the sperm cell on a short time scale.

Functional morphology of sperm

Sperm morphology is thought to experience selection primarily because of its link with sperm function. Among species, sperm length is often positively associated with sperm velocity (Gomendio and Roldan 1991; Fitzpatrick et al. 2009; Lüpold et al. 2009b), however, within species these trends are often weak. High within individual-level variation in sperm morphology and velocity may mask the relationship between these two traits (Humphries et al. 2008; Fitzpatrick et al. 2010). One solution to this problem is to measure the morphology and velocity of individual cells. In sea urchins, this method revealed strong positive correlations between sperm morphology and sperm velocity within an ejaculate (Fitzpatrick et al. 2010). Additionally, many studies do not take into account sperm shape when assessing the link between morphology and velocity. The length (and elongation) of the sperm head increases drag, which may make the ratio of sperm head to tail length a better predictor of sperm velocity (Humphries et al. 2008). Incorporating all measures of sperm morphology may help to clarify the association between sperm morphology and velocity among individuals.

Measures of performance, such as longevity and velocity, are predicted to be negatively correlated within species due to cellular energetic trade-offs (Levitan 2000; Firman and Simmons 2010). Occasionally, these trade-offs can result in interactions with sperm morphology, where sperm length may be correlated with both velocity and longevity, but in opposite directions (Gage et al. 2004; Helfenstein et al. 2008; 2010). These trade-offs may select for individuals to maintain variation in their sperm morphology as a way to ensure they have both fast and long-lived sperm represented in

their ejaculate (Helfenstein et al. 2008). Additionally, differences in mating systems among species may cause selection to act more strongly on velocity or longevity, which may cause sperm morphology to be pulled in opposite directions in closely related species or between mating tactics (Burness et al. 2004). Among species, sperm length is often negatively correlated with sperm longevity (Stockley et al. 1997; Firman and Simmons 2008), or the length of sperm storage (Immler et al. 2007). Frequently, sperm length is positively associated with sperm velocity among species, which may indicate that species with short sperm may have experienced selection for sperm longevity, while longer sperm may be the product of selection for sperm velocity.

Comparative analyses among species, and measurements of sperm velocity and morphology for individual sperm cells, show strong correlations between sperm size and velocity (Humphries et al. 2008; Lüpold et al. 2009b; Fitzpatrick et al. 2010). Additionally, sperm velocity has been linked with male reproductive success in many species (Levitan 2000; Malo 2005; Casselman et al. 2006; Gasparini et al. 2010; Boschetto et al. 2011). This link between sperm morphology, function, and fitness makes sperm morphology an ideal trait to study the evolution of traits associated with postcopulatory selection. We might expect that if sperm quality (i.e., performance) is important for reproductive success within species, there may also be an association between relative testis size and sperm morphology among species.

Dissertation specific aims and contributions

In promiscuous species, postcopulatory sexual selection is one of the strongest forces of selection acting on males (Pischedda and Rice 2012; Péliissié et al. 2014; Devigili et al. 2015). Sperm represent one of the few targets of sexual selection with homology across metazoans, and can therefore act as an informative model for understanding how postcopulatory selection generates and influences variation in traits within and among species. Though patterns of diversity in sperm morphology have been characterized across species (Pitnick et al. 2009), the complementary intraspecific analyses required for interpretation of these patterns frequently do not exist. In this dissertation, I examine the evolution of sperm morphology in lizards at multiple levels of organization (i.e. within species, among populations, and among species variation). In these chapters, I assess the link between morphology and performance, test for condition-dependent reproduction mediated by condition-dependent changes in sperm morphology and sperm count, and measure the rates of evolution of the sperm cell relative to residual testis size among species of anoles. Together, these chapters give a broad overview of the potential adaptive significance of sperm morphology and sperm count in *Anolis* lizards.

Anolis lizards are an ideal group to study the evolution of traits associated with postcopulatory selection. Anoles are amenable to both laboratory and field work and have high potential for sperm competition in some species due to high rates of multiple mating and sperm storage (Fox 1963; Licht 1973; Calsbeek et al. 2007; Chapter 4). This group is also highly diverse, and are a classic example of an adaptive radiation that has generated morphological and ecological differences among species (Losos 2009). This radiation

resulted in convergent evolution of ecomorphs that share similar ecological niches, but are not close phylogenetically (Williams 1972; Losos 1998). Anoles also exhibit variation in traits associated with sexual selection (such as sexual size dimorphism) (Butler and Losos 2002; Cox et al. 2007), and many species have a promiscuous mating system. These aspects of anoles imply that they may vary in the strength of postcopulatory selection and in traits associated with postcopulatory selection among species. These lizards are primarily found in Central and South America and the Caribbean, but several species have been introduced into Florida in the last century (Lee 1985; Schwartz and Henderson 1991; Bartlett and Bartlett 1999; Kolbe et al. 2004; 2007; 2012). This creates an opportunity to compare sperm morphology and testis size between populations of several species in their native range versus a sympatric, introduced range.

For intraspecific studies in this dissertation, I study *Anolis sagrei* (the brown anole), a lizard native to the Bahamas that is abundant, and easily collected and kept in a laboratory setting, making it ideal for both experimental and field-based studies. The brown anole has high rates of multiple paternity (Calsbeek et al. 2007), and has extended sperm storage (Chapter 4), making it likely that this species experiences high levels of sperm competition. Additionally, in laboratory breeding designs where two age-matched males were mated to a single female with no male-male competition, large males and males with higher body condition tend to produce more offspring and sons than daughters, suggesting a postcopulatory mechanism that creates these biases (Cox et al. 2011; Chapter 4).

In Chapter 1, I demonstrate a negative relationship between targets of precopulatory selection (as estimated by sexual size dimorphism or SSD) and postcopulatory selection (as estimated by residual testis size) across 151 species of snakes and lizards, where an increase in male-biased sexual size dimorphism is associated with a decrease in residual testis size. I also show that this negative correlation is present in all major lineages of snakes and lizards represented in this dataset. The sequential nature of these two episodes of selection means that when precopulatory selection is strong, female monopolization may be more common, thereby limiting the opportunity for multiple mating and sperm competition. Additionally, the negative correlation between testis size and SSD could represent an energetic trade-off between traits associated with pre- and postcopulatory selection. These two hypotheses are not mutually exclusive, and likely work in concert to drive the negative correlation between testis size and SSD in squamate reptiles.

In Chapter 2, I use measurements of sperm morphology and testis size for 28 species of anoles to test for differences in the rates of evolution in these traits and for evolutionary associations between sperm morphology and residual testis size. Sperm quantity and quality are often associated with male reproductive success within species, but there is very little comparative evidence to demonstrate how these traits evolve relative to one another among species. In this chapter I demonstrate that the sperm midpiece evolves significantly faster than the sperm head or tail, but that residual testis size evolves approximately ten times faster than the sperm midpiece. This difference in the rate of evolution between sperm length and residual testis size indicates that, in *Anolis*

lizards, sperm production may be more tightly linked with male fitness than sperm morphology. In line with this finding, I found no associations between residual testis size (proxy for the strength of postcopulatory selection), and sperm morphology among species. Finally, in this chapter I also test for differences in sperm morphology and testis size among ecomorphs, and found significant differences in sperm head length and residual testis size, suggesting that ecomorphs may experience convergent differences in the strength of postcopulatory selection.

Chapter 3 examines the population-level variation in sperm morphology and testis size in three species of anoles. I sample three species from both their native ranges and from an introduced sympatric range to test for differences in sperm morphology and testis size (though, testis size data were only available for two species). I show that introduced populations of anoles had significantly longer midpieces and shorter tails than native populations. Additionally, anoles in the introduced populations also had significantly smaller testes (corrected for body size) than their native counterparts. These consistent changes indicate that variation in sperm morphology between populations is likely due to differences in the environment that results in either plastic changes, or genetic adaptation in response to postcopulatory selection for long midpieces, short tails and reduced testis size in the introduced population.

To understand how variation in sperm morphology is generated and maintained within a species, in Chapter 4 I test for the condition dependence of sperm morphology, sperm count and fertilization success in the brown anole. To do this, I use both experimental diet treatments, observations of natural variation in condition, as well as

paternity analysis from competitive mating trials between captive males that differed in body condition. I demonstrate that experimental food restriction resulted in males with lower sperm counts, and more variable sperm cells that had larger midpieces and shorter heads. In wild populations of brown anoles, I found a similar significant negative relationship between condition and midpiece length. In re-analysis of paternity results from a previous experiment (Cox et al. 2011) where males were allowed to copulate ad libitum with a female, I found that males in high condition sired more offspring than males in low condition. In competitive matings between males in the experimental diet treatments where males were limited to a single copulation, there was no effect of condition on fertilization success. I also show that sperm head, midpiece length, and sperm count were negatively correlated with fertilization success. These data imply that condition-dependent reproduction in this species may be partially mediated by the condition dependence of sperm traits, but is likely driven by differences in the frequency of copulation between males in high and low condition.

Finally, in Chapter 5 I test for an association between sperm morphology and sperm velocity in a wild population of brown anoles. I measure sperm velocity from over 100 wild male brown anoles and test for associations with sperm morphology (head, midpiece and tail length). I demonstrate a weak positive relationship between the sperm midpiece length and sperm velocity. Detecting a strong association can be very difficult within a species as intra-male variation in both traits can be fairly high.

In this dissertation, I establish that while sperm morphology is highly variable among species and populations of anoles, is weakly associated with both sperm

performance and competitive fertilization success and may not be a strong predictor of male fitness in this group. In fact, the rate of evolution of residual testis size is between 10–30 times faster than any part of the sperm cell, which suggests that selection has acted more strongly on testis size (and sperm production) than on sperm morphology. Adding to this, I found significant differences in condition-dependent fertilization success in Chapter 4, but only when males are allowed unlimited access to females. It may be that for brown anoles, mating frequency (which may be constrained in males who are in low condition due to lower sperm production) may be the most important predictor of reproductive success, rather than sperm morphology or quality. This dissertation also demonstrates a negative evolutionary association between targets of pre- and postcopulatory selection, indicating that species may allocate resources to the traits that yield the highest fitness returns, or that precopulatory selection may constrain the opportunity for postcopulatory selection to occur. In anoles, I found that residual testis size evolves significantly faster than male body size in this group, which indicates that postcopulatory selection for sperm production is likely very strong in anoles, and may be stronger than selection for male body size. This dissertation provides the first comprehensive analysis of lizard sperm morphology that examines both evolutionary patterns among species, and the sources variation in morphology within species. Together, these projects integrate microevolutionary processes with macroevolutionary patterns to better understand the evolution of the male gamete.

References

- Alavi, S. M. H., M. Pšenička, T. Policar, M. Rodina, J. Hamáčková, P. Kozák, and O. Linhart. 2009. Sperm quality in male *Barbus barbus* L. fed different diets during the spawning season. *Fish Physiol Biochem* 35:683–693.
- Andersson, M. 1986. Evolution of condition-dependent sex ornaments and mating preferences: sexual selection based on viability differences. *Evolution* 40:804–816.
- Bartlett, R. D., and P. P. Bartlett. 1999. A field guide to Florida reptiles and amphibians. Gulf Pub. Co., Houston, TX.
- Birkhead, T. R., D. J. Hosken, and S. S. Pitnick. 2009. Sperm biology: an evolutionary perspective. Academic press, USA.
- Bonduriansky, R., and L. Rowe. 2005. Sexual selection, genetic architecture, and the condition dependence of body shape in the sexually dimorphic fly *Prochyliza xanthostoma* (Piophilidae). *Evolution* 59:138–15.
- Boschetto, C., C. Gasparini, and A. Pilastro. 2011. Sperm number and velocity affect sperm competition success in the guppy (*Poecilia reticulata*). *Behav Ecol Sociobiol* 65:813–821. Springer.
- Burness, G., S. J. Casselman, A. I. Schulte-Hostedde, C. D. Moyes, and R. Montgomerie. 2004. Sperm swimming speed and energetics vary with sperm competition risk in bluegill (*Lepomis macrochirus*). *Behav Ecol Sociobiol* 56:65–70.
- Butler, M. A., and J. B. Losos. 2002. Multivariate sexual dimorphism, sexual selection, and adaptation in Greater Antillean *Anolis* lizards. *Ecological Monographs* 72:541–559. Eco Soc America.

- 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. *Evol Ecol Res* 9:495–503.
- Casselman, S. J., A. I. Schulte-Hostedde, and R. Montgomerie. 2006. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). *Can. J. Fish. Aquat. Sci.* 63:2119–2125.
- Cotton, S., J. Small, and A. Pomiankowski. 2006. Sexual selection and condition-dependent mate preferences. *Curr Biol* 16:R755–R765.
- Cox, R. M., M. A. Butler, and H. B. John-Alder. 2007. The evolution of sexual size dimorphism in reptiles. Pp. 38–49 *in* D. J. Fairbairn, W. U. Blanckenhorn, and T. Székely, eds. *Sex, Size and Gender Roles Evolutionary Studies of Sexual Size Dimorphism*. Oxford University Press, Oxford.
- Devigili, A., J. P. Evans, A. Di Nisio, and A. Pilastro. 2015. Multivariate selection drives concordant patterns of pre- and postcopulatory sexual selection in a livebearing fish. *Nat Comm* 6:8291.
- Dewsbury, D. 1982. Ejaculate Cost and Male Choice. *Am Nat* 119:601–610.
- Elgee, K. E., J. P. Evans, I. W. Ramnarine, S. A. Rush, and T. E. Pitcher. 2010. Geographic variation in sperm traits reflects predation risk and natural rates of multiple paternity in the guppy. *J. Evol. Biol.* 23:1331–1338.
- Firman, R. C., and L. W. Simmons. 2008. Polyandry, sperm competition, and reproductive success in mice. *Behav Ecol* 19:695–702.
- Firman, R. C., and L. W. Simmons. 2010. Sperm midpiece length predicts sperm

- swimming velocity in house mice. *Biol Lett* 6:513–516.
- Fitzpatrick, J. L., F. Garcia-Gonzalez, and J. P. Evans. 2010. Linking sperm length and velocity: the importance of intramale variation. *Biol Lett* 6:797–799.
- Fitzpatrick, J. L., R. Montgomerie, J. K. Desjardins, K. A. Stiver, N. Kolm, and S. Balshine. 2009. Female promiscuity promotes the evolution of faster sperm in cichlid fishes. *Proc. Natl. Acad. Sci. U.S.A.* 106:1128–1132.
- Fox, W. 1963. Special tubules for sperm storage in female lizards. *Nature* 198:500–501.
- 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. *Curr Biol* 14:44–47.
- Gasparini, C., A. Devigili, R. Dosselli, and A. Pilastro. 2013. Pattern of inbreeding depression, condition dependence, and additive genetic variance in Trinidadian guppy ejaculate traits. *Ecol Evol* 3:4940–4953.
- Gasparini, C., L. W. Simmons, M. Beveridge, and J. P. Evans. 2010. Sperm swimming velocity predicts competitive fertilization success in the green swordtail *Xiphophorus helleri*. *PLoS ONE* 5:e12146.
- Gomendio, M., and E. R. S. Roldan. 1991. Sperm competition influences sperm size in mammals. *Proc. Biol. Sci. Lond. B.* 243:181–185.
- Helfenstein, F., M. Podelvin, and H. Richner. 2010. Sperm morphology, swimming velocity, and longevity in the house sparrow *Passer domesticus*. *Behav Ecol Sociobiol* 64:557–565.
- Helfenstein, F., T. Szép, Z. Nagy, B. Kempenaers, and R. H. Wagner. 2008. Between-

- male variation in sperm size, velocity and longevity in sand martins *Riparia riparia*.
J Avian Biol 39:647–652.
- Higginson, D. M., K. B. Miller, K. A. Segraves, and S. Pitnick. 2012. Female reproductive tract form drives the evolution of complex sperm morphology. Proc. Natl. Acad. Sci. U.S.A. 109:4538–4543.
- Hill, G. E. 1990. Female house finches prefer colourful males: sexual selection for a condition-dependent trait. Anim Behav 40:563–572.
- Hosken, D. J., and P. I. Ward. 2001. Experimental evidence for testis size evolution via sperm competition. Ecology Letters 4:10–13.
- Humphries, S., J. P. Evans, and L. W. Simmons. 2008. Sperm competition: linking form to function. BMC Evol Biol 8:319.
- Immler, S. S., S. S. Calhim, and T. R. T. Birkhead. 2008. Increased postcopulatory sexual selection reduces the intramale variation in sperm design. Evolution 62:1538–1543.
- Immler, S., M. Saint-Jalme, L. Lesobre, G. Sorci, Y. Roman, and T. R. Birkhead. 2007. The evolution of sperm morphometry in pheasants. J. Evol. Biol. 20:1008–1014.
- Immler, S., S. Pitnick, G. A. Parker, K. L. Durrant, S. Lüpold, S. Calhim, and T. R. Birkhead. 2011. Resolving variation in the reproductive tradeoff between sperm size and number. Proc. Natl. Acad. Sci. U.S.A. 108:5325–5330.
- Immler, S., S. R. Pryke, T. R. Birkhead, and S. C. Griffith. 2010. Pronounced within-individual plasticity in sperm morphometry across social environments. Evolution 64:1634–1643.
- Kahrl, A. F., and R. M. Cox. 2015. Diet affects ejaculate traits in a lizard with condition-

- dependent fertilization success. *Behav Ecol* 26:1502–1511.
- Kaldun, B., and O. Otti. 2016. Condition-dependent ejaculate production affects male mating behavior in the common bedbug *Cimex lectularius*. *Ecol Evol* 6:2548–2558.
- Kolbe, J. J., R. E. Glor, L. Rodríguez Schettino, A. C. Lara, A. Larson, and J. B. Losos. 2004. Genetic variation increases during biological invasion by a Cuban lizard. *Nature* 431:177–181.
- Kolbe, J. J., R. E. Glor, L. R. Schettino, A. C. Lara, A. Larson, and J. B. Losos. 2007. Multiple sources, admixture, and genetic variation in introduced *Anolis* lizard populations. *Conserv Biol* 21:1612–1625.
- Kolbe, J. J., P. S. VanMiddlesworth, N. Losin, N. Dappen, and J. B. Losos. 2012. Climatic niche shift predicts thermal trait response in one but not both introductions of the Puerto Rican lizard *Anolis cristatellus* to Miami, Florida, USA. *Ecol Evol* 2:1503–1516.
- Laskemoen, T., T. Albrecht, A. Bonisoli-Alquati, J. Cepak, F. de Lope, I. G. Hermosell, L. E. Johannessen, O. Kleven, A. Marzal, T. A. Mousseau, A. P. Møller, R. J. Robertson, G. Rudolfson, N. Saino, Y. Vortman, and J. T. Lifjeld. 2013. Variation in sperm morphometry and sperm competition among barn swallow (*Hirundo rustica*) populations. *Behav Ecol Sociobiol* 67:301–309.
- Lee, J. C. 1985. *Anolis sagrei* in Florida: phenetics of a colonizing species I. Meristic characters. *Copeia* 1985:182–194.
- Levitan, D. R. D. 2000. Sperm velocity and longevity trade off each other and influence fertilization in the sea urchin *Lytechinus variegatus*. *Proc. Biol. Sci. Lond. B*.

267:531–534.

- Licht, P. 1973. Influence of temperature and photoperiod on the annual ovarian cycle in the lizard *Anolis carolinensis*. *Copeia* 1973:465–472.
- Losos, J. B. 1998. Contingency and determinism in replicated adaptive radiations of island lizards. *Science* 279:2115–2118.
- Losos, J. B. 2009. Lizards in an evolutionary tree: ecology and adaptive radiation of anoles. University of California Press, Berkeley, CA.
- Lüpold, S., D. F. Westneat, and T. R. Birkhead. 2011. Geographical variation in sperm morphology in the red-winged blackbird (*Agelaius phoeniceus*). *Evol Biol* 25:373–390.
- Lüpold, S., G. M. Linz, J. W. Rivers, D. F. Westneat, and T. R. Birkhead. 2009a. Sperm competition selects beyond relative testes size in birds. *Evolution* 63:391–402.
- Lüpold, S., S. Calhim, S. Immler, and T. R. Birkhead. 2009b. Sperm morphology and sperm velocity in passerine birds. *Proc. R. Soc. Lond. B.* 276:1175–1181.
- Malo, A. F. 2005. Male fertility in natural populations of red deer is determined by sperm velocity and the proportion of normal spermatozoa. *Biology of Reproduction* 72:822–829.
- Merrells, K. J., H. Blewett, J. A. Jamieson, C. G. Taylor, and M. Suh. 2009. Relationship between abnormal sperm morphology induced by dietary zinc deficiency and lipid composition in testes of growing rats. *BJN* 102:226–232.
- Olsson, M., T. Madsen, and R. Shine. 1997. Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. *Proc. Biol. Sci. Lond. B.* 264:455–459.

- Parker, G. A. 1998. Sperm competition and the evolution of ejaculates: towards a theory base. Pp. 3–49 *in* T. R. Birkhead and A. P. Møller, eds. Sperm competition and sexual selection. Academic Press, San Diego, CA.
- Parker, G. A., and T. Pizzari. 2010. Sperm competition and ejaculate economics. *Biological Reviews* 897–934.
- 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.
- Perry, J. C., and L. Rowe. 2010. Condition-dependent ejaculate size and composition in a ladybird beetle. *Proc. R. Soc. Lond. B.* 277:3639–3647.
- Pélissié, B., P. Jarne, V. Sarda, and P. David. 2014. Disentangling precopulatory and postcopulatory sexual selection in polyandrous species. *Evolution* 68:1320–1331.
- Pischedda, A., and W. R. Rice. 2012. Partitioning sexual selection into its mating success and fertilization success components. *Proc. Natl. Acad. Sci. U.S.A.* 109:2049–2053.
- Pitnick, S., D. J. Hosken, and T. R. Birkhead. 2009. Sperm morphological diversity. Pp. 69–149 *in* T. R. Birkhead, D. J. Hosken, and S. Pitnick, eds. *Sperm Biology: An Evolutionary Perspective*. Academic Press, Burlington, MA.
- Pitnick, S., G. T. Miller, K. Schneider, and T. A. Markow. 2003. Ejaculate-female coevolution in *Drosophila mojavensis*. *Proc. Biol. Sci. Lond. B.* 270:1507–1512.
- Pizzari, T., and G. A. Parker. 2009. Sperm competition and sperm phenotype. Pp. 207–245 *in* T. R. Birkhead, D. J. Hosken, and S. S. Pitnick, eds. *Sperm biology: an evolutionary perspective*. Academic press, Burlington, MA.
- Presgraves, D. C., R. H. Baker, and G. S. Wilkinson. 1999. Coevolution of sperm and

- female reproductive tract morphology in stalk-eyed flies. *Proc. Biol. Sci. Lond. B.* 266:1041–1047. The Royal Society.
- Rahman, M. M., J. L. Kelley, and J. P. Evans. 2013. Condition-dependent expression of pre- and postcopulatory sexual traits in guppies. *Ecol Evol* 3:2197–2213.
- Rowe, L., and D. Houle. 1996. The lek paradox and the capture of genetic variance by condition dependent traits. *Proc. R. Soc. Lond. B.* 263:1415–1421.
- Sasson, D. A., and H. J. Brockmann. 2016. Geographic variation in sperm and ejaculate quantity and quality of horseshoe crabs. *Behav Ecol Sociobiol* 70:1715–1725.
- Behavioral Ecology and Sociobiology.
- 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.* *Behav Ecol Sociobiol* 56:420–425. Springer.
- Schwartz, A., and R. W. Henderson. 1991. Amphibians and reptiles of the West Indies: descriptions, distributions, and natural history. University Press of Florida, Gainesville.
- Simmons, L. W. 2001. Sperm competition and its evolutionary consequences in the insects. Princeton University Press, Princeton, NJ.
- Simmons, L. W., and F. García-González. 2008. Evolutionary reduction in testes size and competitive fertilization success in response to the experimental removal of sexual selection in dung beetles. *Evolution* 62:2580–2591.
- Simmons, L. W., and J. L. Fitzpatrick. 2012. Sperm wars and the evolution of male

- fertility. *Reproduction* 144:519–534.
- Simmons, L. W., and J. S. Kotiaho. 2002. Evolution of ejaculates: patterns of phenotypic and genotypic variation and condition dependence in sperm competition traits. *Evolution* 56:1622–1631.
- Snook, R. R. 2005. Sperm in competition: not playing by the numbers. *Ecol. Lett.* 20:46–53.
- Stewart, K. A., R. Wang, and R. Montgomerie. 2016. Extensive variation in sperm morphology in a frog with no sperm competition. *BMC Evol Biol* 16:1–11.
- Stockley, P., M. Gage, G. A. Parker, and A. P. Møller. 1997. Sperm competition in fishes: The evolution of testis size and ejaculate characteristics. *Am Nat* 149:933–954.
- Williams, E. E. 1972. The Origin of Faunas. Evolution of Lizard Congeners in a Complex Island Fauna: A Trial Analysis. *Evol Biol* 6:47–89.

CHAPTER ONE:

Correlated evolution between targets of pre- and postcopulatory sexual selection across
squamate reptiles¹

¹ As published: 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(18): 6452–6459.

Abstract

Sexual selection reflects the joint contributions of precopulatory selection, which arises from variance in mating success, and postcopulatory selection, which arises from variance in fertilization success. The relative importance of each episode of selection is variable among species, and comparative evidence suggests that traits targeted by precopulatory selection often covary in expression with those targeted by postcopulatory selection when assessed across species, though the strength and direction of this association varies considerably among taxa. We tested for correlated evolution between targets of pre- and postcopulatory selection using data on sexual size dimorphism (SSD) and testis size from 151 species of squamate reptiles (120 lizards, 31 snakes). In squamates, male-male competition for mating opportunities often favors large body size, such that the degree of male-biased SSD is associated with the intensity of precopulatory selection. Likewise, competition for fertilization often favors increased sperm production, such that testis size (relative to body size) is associated with the intensity of postcopulatory selection. Using both conventional and phylogenetically based analyses, we show that testis size consistently decreases as the degree of male-biased SSD increases across lizards and snakes. This evolutionary pattern suggests that strong precopulatory selection may often constrain the opportunity for postcopulatory selection, and that the relative importance of each selective episode may determine the optimal resolution of energy allocation tradeoffs between traits subject to each form of sexual selection.

Introduction

Sexual selection can be divided into two episodes that jointly contribute to the net opportunity for selection: precopulatory selection, which arises from variance in mating success, and postcopulatory selection, which arises from variance in fertilization success. When reproductive success is strongly influenced by the acquisition and monopolization of mates, precopulatory selection is the primary determinant of the net opportunity for sexual selection (Pischedda & Rice 2012; Rose et al. 2013; Péliissié et al. 2014). Conversely, when polyandry is high, the opportunity for postcopulatory selection can equal or exceed that for precopulatory selection (Collet et al. 2012; Devigili et al. 2015; Turnell & Shaw 2015). Because these two episodes of sexual selection occur sequentially, the outcome of precopulatory selection has the potential to shape the opportunity for postcopulatory selection (Preston et al. 2003; Hunt et al. 2009; South & Lewis 2012). For example, strong precopulatory selection that results in monopolization of females can reduce the opportunity for sperm competition (Preston et al. 2003; Fitzpatrick et al. 2012; Parker et al. 2013). Consequently, in taxa where precopulatory selection is associated with male-male contest competition and monopolization of females, species are predicted to fall along a continuum ranging from those that invest primarily in traits under precopulatory selection to those that invest primarily in traits under postcopulatory selection (Parker et al. 2013; Lüpold et al. 2014).

Consistent with this prediction, interspecific comparisons across several taxa have revealed that traits subject to precopulatory selection, such as weaponry and body size, often correlate negatively with traits subject to postcopulatory selection, such as testis

size (Heske & Ostfeld 1990; Poulin & Morand 2000; Fitzpatrick et al. 2012; Dines et al. 2015; Dunn et al. 2015). In addition to the potential role of precopulatory selection in limiting the opportunity for postcopulatory selection, such negative interspecific correlations could also arise from (or be strengthened by) energetic tradeoffs between sexually selected traits (Moczek & Nijhout 2004; Simmons & Emlen 2006; Kelly 2008; Parker & Pizzari 2010; Yamane et al. 2010; Somjee et al. 2015). However, analyses in other taxa have found no evidence for negative interspecific correlations between targets of pre- and postcopulatory selection (Ferrandiz-Rovira et al. 2014; Lüpold et al. 2014), and several studies have documented positive correlations (Wedell 1993; Lüpold et al. 2014). The reasons for such discrepancies are still unclear, emphasizing the need for further comparative analyses of this pattern (Lüpold et al. 2014).

Squamate reptiles (lizards and snakes) provide an intriguing group in which to test for correlated evolution between targets of pre- and postcopulatory selection because they often experience strong precopulatory selection on body size and other traits involved in territory defense and mate acquisition (Cox & Kahrl 2014), and because precopulatory selection for large male size is known to influence the direction and magnitude of sexual size dimorphism in this group (Cox et al. 2003; 2007). Postcopulatory selection is also known to favor increased testis size, relative to body size, in squamates (Todd 2008; Uller et al. 2010), as it does in many other taxa (Harcourt et al. 1981; Harvey & Harcourt 1984; Møller 1989; Heske & Ostfeld 1990; Møller 1991; Gage 1994; Møller & Briskie 1995; Stockley et al. 1997; Hosken & Ward 2001; Rowe & Pruett-Jones 2011). Multiple paternity appears to be the rule rather than the exception in squamates, occurring in over

50% of all clutches and in all 23 species examined by Uller and Olsson (2008). Hence, the extent to which males actually monopolize females via mate defense and territoriality is generally uncertain across this lineage. Moreover, some territorial squamates exhibit male-biased SSD in association with high levels of multiple paternity (e.g., *Anolis sagrei*, Calsbeek et al. 2007), whereas other territorial species exhibit female-biased SSD in association with low levels of multiple paternity (e.g., *Sceloporus undulatus*, Haenel et al. 2003). These observations suggest that simple classifications of mating system on the basis of territoriality may be of limited utility for assessing the interplay between pre- and postcopulatory sexual selection, and also raise the question of whether squamates exhibit the same negative association between targets of pre- and postcopulatory selection that characterizes other lineages.

To answer this question, we tested for an interspecific correlation between the phenotypic targets of pre- and postcopulatory selection using a dataset of 151 species of squamate reptiles – the largest comparative dataset to explore this pattern in any lineage. We combined this dataset with a recent phylogeny of Squamata (Pyron et al. 2013) to test for correlated evolution between sexual size dimorphism (SSD), a consequence of precopulatory selection for large male body size, and testis size, a target of postcopulatory selection for sperm production. We predicted that SSD and testis size would be negatively correlated across species for either of two complementary reasons: (1) because strong precopulatory selection may limit the opportunity for postcopulatory selection, and (2) because energetic tradeoffs may limit the extent to which both traits can be simultaneously maximized.

Methods

Comparative dataset

We used estimates of SSD compiled by Cox et al. (2003, 2007) as the basis for our dataset and added measures of testis size from literature searches and unpublished collections (Appendix S1). We recorded mean body size (snout-vent length, SVL) and mean testis size from additional published studies that we obtained by reviewing the literature on testis size and reproductive cycles. We only included species for which we could obtain estimates of testis size during the reproductive season and measures of SVL for adults of each sex. We supplemented this literature review with our own measures of SVL and testis size from 50 wild populations (Appendix S2). In total, our dataset included 151 species (120 lizards, 31 snakes) representing 16 squamate families (Appendix S1). For each species, we calculated an index of SSD as:

$$\text{SSD} = (\text{SVL of larger sex} / \text{SVL of smaller sex}),$$

expressed as a positive value when males are the larger sex and a negative value when females are the larger sex. For analysis, we $\log_{10}$ -transformed this index prior to assigning it a positive or negative value. Testis size was typically reported as either volume (mm^3) or mass (mg), and we converted mass to volume using the density ($1\text{mg}/\text{mm}^3$) given by Licht and Pearson (1969). If only testicular dimensions were reported, we calculated volume using the formula:

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

where a is the radius of the width of the testis and b is the radius of its height. When seasonal patterns of testis size were reported, we used maximum testis size during the breeding season.

Non-phylogenetic analyses

To test the prediction of a negative correlation between traits associated with pre- and postcopulatory selection, we used multiple regression with $\log_{10}$ testis size as the dependent variable and $\log_{10}$ SSD and $\log_{10}$ male SVL (to account for scaling) as independent variables. Because snakes and lizards differ dramatically in body shape and, consequently, in scaling relationships with SVL, we conducted these analyses separately within each group (Losos 1994), despite the fact that snakes comprise a derived clade nested within lizards (Pyron et al. 2013).

Phylogenetic analyses

We used the phylogeny of Pyron et al. (2013), which provides branch lengths and a resolved topology for all species in our dataset, to test for correlated evolutionary changes in SSD and testis size in the R environment (R Development Core Team 2013). First, we removed all species that were not found in our dataset from the phylogeny using APE (Paradis et al. 2004). We used this pruned phylogeny to test for phylogenetic signal in both SSD and testis size using Blomberg's K (Blomberg et al. 2003) and Pagel's λ , implemented in Picante (Kembel et al. 2010) and phytools (Revell 2012), respectively. Blomberg's K is calculated using the variance/covariance matrix of the phylogenetic

relationships among species. It is the ratio of the mean squared error of observed trait values versus the mean squared error of predicted trait values modeled under Brownian motion, with significance ($K > 0$) calculated using simulation tests (Blomberg et al. 2003). If $K < 1$, phenotypic variance is greater within clades than expected under Brownian motion, whereas if $K > 1$, phenotypic variance tends to be relatively greater among clades (Blomberg et al. 2003). Pagel's λ defines the amount of phylogenetic signal, where $\lambda = 0$ corresponds to complete phylogenetic independence and $\lambda = 1$ indicates that traits vary across the phylogeny as predicted by Brownian motion (Pagel 1999). We used log-likelihood ratio tests to determine whether values of Pagel's λ were significantly different from 0 and 1.

We tested for evolutionary associations between SSD and testis size using phylogenetic generalized least squares (PGLS) regressions. As described above, we conducted separate analyses for lizards versus snakes and also tested for relationships between SSD and testis size within any lineage of lizards represented by ≥ 8 species. In all analyses, we used a Brownian motion model of character evolution (Table 1, Table S4). As a complementary method, we also used an Ornstein-Uhlenback model of character evolution, but only had the statistical power to fit the model to lizards due to our limited sampling of snakes and other lineages (Table S3). We conducted these analyses in the R package APE (Harmon et al. 2008) with $\log_{10}$ testis size as the dependent variable and $\log_{10}$ SSD and $\log_{10}$ male SVL (to account for scaling) as independent variables. As a complementary approach, we also tested for a correlation between SSD and testis size using phylogenetically independent contrasts (PIC). In order

to account for a size covariate using PIC, we followed the approach of Garland et al. (1992). First, we calculated contrasts of $\log_{10}$ SSD, $\log_{10}$ testis size, and $\log_{10}$ SVL using the *pic* function in APE (Harmon et al. 2008). Next, we positivized all contrasts with respect to SVL (Garland et al. 1992) and conducted an ordinary least-square regression (forced through the origin) of the contrasts of $\log_{10}$ SSD regressed on the contrasts of $\log_{10}$ SVL, and of the contrasts of $\log_{10}$ testis size regressed on the contrasts of $\log_{10}$ SVL. Finally, we used an ordinary least-square regression (forced through the origin) to test for a negative relationship between the residuals from each of these regressions, which represent phylogenetically independent and size-corrected measures of SSD and testis size (Garland et al. 1992). We conducted these analyses separately for lizards and snakes.

Results

Conventional analyses

We found a significant negative relationship between $\log_{10}$ testis size and $\log_{10}$ SSD in lizards (partial $r = -0.149$, $P = 0.002$) and in snakes (partial $r = -0.449$, $P = 0.012$) with $\log_{10}$ SVL as a covariate (lizards: partial $r = 0.696$, $P < 0.001$; snakes: partial $r = 0.592$, $P < 0.001$). We also found a significant negative relationship between $\log_{10}$ testis size and $\log_{10}$ SSD within the lizard lineages Gekkota (partial $r = -0.835$, $P = 0.001$) and Dactyloidae (partial $r = -0.353$, $P = 0.021$), as well as negative (but non-significant) relationships within Scincidae (partial $r = -0.110$, $P = 0.813$) and Phrynosomatidae (partial $r = -0.282$, $P = 0.161$) (Fig. 1).

Phylogenetic analyses

We detected significant phylogenetic signal in $\log_{10}$ SSD (Blomberg's $K = 0.299$, $P = 0.001$; Pagel's $\lambda = 0.352$, $P < 0.001$, Table 1) and in $\log_{10}$ testis size ($K = 0.291$, $P = 0.001$; $\lambda = 0.167$, $P = 0.002$, Table 1) in lizards. These K and λ values indicate that related species tend to be more similar than expected by chance, but that variance within clades is greater than predicted under Brownian motion (Pagel 1999, Blomberg et al. 2003). We found similar patterns in snakes, though estimates of K and λ were not statistically distinguishable from zero (SSD: $K = 0.294$, $P = 0.305$; $\lambda < 0.001$, $P = 1$; testis size: $K = 0.238$, $P = 0.865$; $\lambda = 0.506$, $P = 0.545$). When accounting for phylogenetic relationships using PGLS regressions, we recovered the same negative relationships between $\log_{10}$ testis size and $\log_{10}$ SSD that we detected using conventional analyses in both lizards and snakes (Fig. 2A-B; Table 1). In lizards, these results are robust to the choice between Brownian motion (Table 1) and Ornstein-Uhlenbeck models of character evolution (Table S3). At a finer taxonomic scale, we consistently found negative (though not always significant) relationships between $\log_{10}$ testis size and $\log_{10}$ SSD within each of the squamate lineages with sufficient representation ($N \geq 8$ species) for separate analyses (Fig. 1; Table S4). We also found that SSD was negatively correlated with testis size after correcting for phylogeny and allometry using phylogenetically independent contrasts in lizards ($r = -0.253$, $P = 0.005$, Fig. 2C) and snakes ($r = -0.438$, $P = 0.015$, Fig. 2D).

Discussion

We found that the evolution of male-biased sexual size dimorphism (SSD) in lizards and snakes is consistently associated with a reduction in relative testis size (Table 1, Figs. 1-2). This negative relationship between the phenotypic targets of pre- and postcopulatory sexual selection is consistent across major squamate lineages (Fig. 1) and is similar to macroevolutionary patterns observed in taxa as disparate as voles (Heske & Ostfeld 1990), primates (Lüpold et al. 2014; Dunn et al. 2015), pinnipeds (Fitzpatrick et al. 2012), cetaceans (Dines et al. 2015) and acanthocephalan worms (Poulin & Morand 2000). Notably, the negative correlations that we observed have regression and correlation coefficients near the high end of those reported for most other lineages (Poulin & Morand, 2000; Fitzpatrick et al. 2012; Lüpold et al. 2014; Dines et al. 2015). These negative interspecific correlations mirror the tradeoff between body size (or weaponry) and relative testis size that is often observed within species (Moczek & Nijhout 2004; Simmons & Emlen 2006; Kelly 2008; Parker & Pizzari 2010; Yamane et al. 2010, Somjee et al. 2015). However, our results stand in contrast to other comparative studies that have documented non-significant or even positive interspecific correlations between the targets of pre- and postcopulatory sexual selection in bushcrickets (Wedell 1993), ungulates (Ferrandiz-Rovira et al. 2014; Lüpold et al. 2014) and a variety of other vertebrate and invertebrate taxa (Lüpold et al. 2014). Below, we consider the mating systems of squamate reptiles in light of current theory on the relationship between pre- and postcopulatory sexual selection (Parker et al. 2013; Lüpold et al. 2014) and discuss

the potential mechanisms structuring the pattern of correlated evolution between SSD and testis size that we have documented in this group.

Recent theory predicts that the interspecific relationship between traits subject to pre- and postcopulatory selection should vary depending on the marginal fitness gains associated with increased investment in either type of sexually selected trait (Parker et al. 2013). In particular, male-male contest competition, and the extent to which it results in reproductive monopolization of females, is predicted to be of primary importance. This theory has been tested across several taxonomic groups, and those with higher rates of male-male contest competition and female monopolization (as defined by the percentage of species within a taxonomic group that exhibit female-defense polygyny) show a stronger negative relationship between SSD or weaponry and testis size (Lüpold et al. 2014). Extrapolating this model to squamate reptiles would imply that strong precopulatory selection for large male body size often results in increased monopolization of females and reduced opportunity for postcopulatory selection. Generally, squamates appear to fit the assumptions of this model, as many snakes and lizards exhibit overt male-male combat, female-defense territorial polygyny, and mate-guarding behavior (Stamps 1983; Carothers 1984; Shine 1994). However, multiple paternity occurs in all squamate species that have been studied to date (Uller and Olsson 2008), so the extent to which postcopulatory selection is actually reduced by mate guarding and territory defense is generally uncertain in this lineage. For example, some territorial species exhibit high levels of multiple paternity (e.g., *Anolis sagrei*, Calsbeek et al. 2007), whereas others exhibit low levels of multiple paternity (e.g., *Sceloporus*

undulatus, Haenel et al. 2003). Although both species follow the expected pattern ranging from male-biased SSD and small testis size (*A. sagrei*) to female-biased SSD and large testis size (*S. undulatus*), this pattern occurs in the absence of categorical variation in territoriality and opposite what would be predicted from the apparent opportunity for postcopulatory sexual selection based on paternity analyses. As such, it is difficult to assess whether the negative correlations that we have documented between SSD and testis size occur for the reasons envisioned by recent theory (Parker et al. 2013; Lüpold et al. 2014). Future studies that partition the opportunities for pre- and postcopulatory sexual selection to compare them between related species with contrasting patterns of SSD and testis size could be extremely informative in this regard, and squamates offer a variety of lineages in which mating systems or traits such as sexual size dimorphism vary considerably (e.g., Scincidae, Phrynosomatidae).

Several proximate and ultimate factors may interact to produce the negative correlations that we observed between targets of pre- and postcopulatory sexual selection. Ultimately, the sequential nature of these two episodes of selection means that, when precopulatory selection is strong and results in reproductive monopolization of females, the opportunity for postcopulatory selection and sperm competition should be reduced (Preston et al. 2003; Hunt et al. 2009; South & Lewis 2012; Fitzpatrick et al. 2012; Parker et al. 2013). Conversely, when precopulatory selection on males is relaxed, females may re-mate more frequently, allowing postcopulatory selection to occur (Kvarnemo & Simmons 2013). This does not imply that postcopulatory selection will always be high when precopulatory selection is low, nor does it mean that strong

precopulatory selection will necessarily preclude postcopulatory selection, particularly when females can re-mate frequently despite male-male contest competition. As such, the tendency for SSD and testis size to covary negatively despite the presumably imperfect relationship between the strength of pre- and postcopulatory selection suggests that proximate energetic constraints on the expression of these traits may further help to structure the negative relationships that we observed. When precopulatory selection is strong, males typically allocate more resources to body size and weaponry, whereas in species where postcopulatory selection is strong, males often invest primarily in traits that improve their success in sperm competition (Moczek & Nijhout 2004; Simmons & Emlen 2006; Kelly 2008; Parker & Pizzari 2010; Yamane et al. 2010; Somjee et al. 2015). Evidence for the latter is limited in squamates, though sperm production is energetically costly in snakes and lizards (Olsson et al. 1997; Chapter 4). Thus, energy allocation tradeoffs among sexually selected traits are likely to act in concert with an inherent covariance in the opportunity for pre- and postcopulatory selection to shape the correlated evolution of traits such as body size, weaponry, and testis size.

Acknowledgments

We thank M. Johnson, B. Kircher, M. Oberndorf, J. Strecula, J. Murray, B. Andre, M. Jaramillo, M. Webber, A. Zed, F. Deckard, A. Hanninen, J. Striecher, and M. Landestoy for assistance collecting data for several species. We thank L. J. Vitt for providing unpublished data and M. Augat, R. Costello, H. Edgington, M. Hague, A. Reedy, B. Sanderson, and C. Wood for comments during manuscript preparation. We

thank S. Lüpold for statistical advice. This work was supported by startup funding from The University of Virginia (to RMC), an E.E. Williams Research Grant from the Herpetologist's League (to AFK), a Theodore Roosevelt Memorial Grant from the American Museum of Natural History (to AFK), and a Doctoral Dissertation Improvement Grant from the National Science Foundation (DEB-1501680 to RMC and AFK). The authors declare that they have no conflict of interest.

References

- Blomberg, S.P., Garland, T. & Ives, A.R. 2003. Testing for phylogenetic signal in comparative data: behavioral traits are more labile. *Evolution* **57**:717–745.
- Calsbeek, R., Bonneaud, C., Prabhu, S., Manoukis, N. & Smith, T.B. 2007. Multiple paternity and sperm storage lead to increased genetic diversity in *Anolis* lizards. *Evol. Ecol.* **9**: 495-503.
- Carothers, J.H. 1984. Sexual selection and sexual dimorphism in some herbivorous lizards. *Am. Nat.* **124**:244-254.
- Collet, J., Richardson, D.S., Worley, K., & Pizzari, T. 2012. Sexual selection and the differential effect of polyandry. *Proc. Nat. Acad. Sci.* **109**: 8641–8645.
- Cox, R.M., Skelley, S.L. & John-Alder, H.B. 2003. A comparative test of adaptive hypotheses for sexual size dimorphism in lizards. *Evolution* **57**:1653-1669.
- Cox, R.M., Butler, M.A. & John-Alder, H.B. 2007. The evolution of sexual size dimorphism in reptiles. In: *Sex, Size and Gender Roles: Evolutionary Studies of Sexual Size Dimorphism*. (D.J. Fairbairn, W.U. Blanckenhorn, & T. Székely, eds.), pp. 38-49. Oxford University Press, Oxford, UK.
- Cox, R.M., & A.F. Kahrl. 2014. Sexual selection and sexual dimorphism. In: *Reproductive Biology and Phylogeny of Lizards and Tuatara*. (J.L. Rheubert, D.S. Siegel, S.E. Trauth & B. G. Jamieson, eds.), pp. 78-108. CRC Press, Boca Raton, U.S.A.

- Devigili, A., Evans, J.P., Di Nisio, A. & Pilastro, A. 2015. Multivariate selection drives concordant patterns of pre- and postcopulatory sexual selection in a livebearing fish. *Nat. Commun.* **6**:1-9.
- Dines, J.P., Mesnick, S.L., Ralls, K., May-Collado, L., Agnarsson, I. & Dean, M.D. 2015. A trade-off between precopulatory and postcopulatory trait investment in male cetaceans. *Evolution* **69**:1560-1572.
- Dunn, J.C., Halenar, L.B., Davies, T.G., Cristobal-Azkarate, J., Reby, D., Sykes, D., Dengg, S., Fitch, W.T. & Knapp, L.A. 2015. Evolutionary trade-off between vocal tract and testes dimensions in howler monkeys. *Curr. Biol.* **25**:2839-2844.
- Ferrandiz-Rovira, M., Lemaître, J.F., Lardy, S., López, B.C. & Cohas, A. 2014. Do pre- and post-copulatory sexually selected traits covary in large herbivores? *BMC Evol. Biol.* **14**:79.
- Fitzpatrick, J.L., Almbro, M., Gonzalez-Voyer, A., Kolm, N. & Simmons, L.W. 2012. Male contest competition and the coevolution of weaponry and testes in pinnipeds. *Evolution* **66**:3595–3604.
- Gage, M. 1994. Associations between body size, mating pattern, testis size, and sperm lengths across butterflies. *Proc. R. Soc. Lond. B.* **258**:247–254.
- Garland, T., Harvey, P.H. & Ives, A.R. 1992. Procedures for the analysis of comparative data using phylogenetically independent contrasts. *Syst. Biol.* **41**:18–32.
- Haenel, G.J., Smith, L.C. & John-Alder, H.B. 2003. Home-range analysis in *Sceloporus undulatus* II: A test of spatial relationships and reproductive success. *Copeia* **2003**:113-123.

- Harcourt, A.H., Harvey, P.H., Larson, S.G. & Short, R.V. 1981. Testis weight, body weight and breeding system in primates. *Nature* **293**:55–57.
- Harmon, L.J., Weir, J.T., Brock, C.D., Glor, R.E. & Challenger, W. 2008. GEIGER: investigating evolutionary radiations. *Bioinformatics* **24**:129–131.
- Harvey, P.H. & Harcourt, A.H. 1984. Sperm competition, testes size, and breeding systems in primates. In: *Sperm competition and the evolution of animal mating systems*. (R. Smith ed.), pp. 589–599. Academic Press, London, U.K.
- Heske, E.J. & Ostfeld, R.S. 1990. Sexual dimorphism in size, relative size of testes, and mating systems in North American voles. *J. Mammal.* **71**:510–519.
- Hosken, D.J. & Ward, P.I. 2001. Experimental evidence for testis size evolution via sperm competition. *Ecol. Lett.* **4**:10–13.
- Hunt, J., Breuker, C.J., Sadowski, J.A. & Moore, A.J. 2009. Male-male competition, female mate choice and their interaction: determining total sexual selection. *J. Evol. Biol.* **22**:13–26.
- Kahrl, A.F. & Cox, R.M. 2015. Diet affects ejaculate traits in a lizard with condition-dependent fertilization success. *Behav. Ecol.* **26**: 1502–1511.
- Kelly, C.D. 2008. Sperm investment in relation to weapon size in a male trimorphic insect? *Behav. Ecol.* **19**:1018–1024.
- Kembel, S.W., Cowan, P.D., Helmus, M.R., Cornwell, W.K., Morlon, H., Ackerly, D.D., Blomberg, S.P. & Webb, C.O. 2010. Picante: R tools for integrating phylogenies and ecology. *Bioinformatics* **26**:1463–1464.

- Kvarnemo, C. & Simmons, L.W. 2013. Polyandry as a mediator of sexual selection before and after mating. *Phil. Trans. R. Soc. B.* **368**:20120042.
- Licht, P. & Pearson, A.K. 1969. Effects of adenohipophysectomy on testicular function in the lizard *Anolis carolinensis*. *Biol. Reprod.* **1**:107-119.
- Losos, J.B. 1994. Historical contingency and lizard community ecology. In: *Lizard ecology: historical and experimental perspectives*. (L.J. Vitt & E.R. Pianka, eds.), pp. 319-333. Princeton University Press, Princeton, NJ.
- Lüpold, S., Tomkins, J.L., Simmons, L.W. & Fitzpatrick, J.L. 2014. Female monopolization mediates the relationship between pre- and postcopulatory sexual traits. *Nat. Commun.* **5**:3184-3191.
- Moczek, A.P. & Nijhout, H.F. 2004. Trade-offs during the development of primary and secondary sexual traits in a horned beetle. *Evolution* **163**:184-191.
- Møller, A.P. 1989. Ejaculate quality, testes size and sperm production in mammals. *Funct. Ecol.* **3**:91-96.
- Møller, A.P. 1991. Sperm competition, sperm depletion, paternal care, and relative testis size in birds. *Am. Nat.* **137**:882-906.
- Møller, A.P. & Briskie, J.V. 1995. Extra-pair paternity, sperm competition and the evolution of testis size in birds. *Behav. Ecol. Sociobiol.* **36**:357-365.
- Olsson, M., Madsen, T. and Shine, R. 1997. Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. *Proc. R. Soc. Lond. B.* **264**:455-459.
- Pagel, M. 1999. Inferring the historical patterns of biological evolution. *Nature* **401**:877-884.

- Paradis, E., Claude, J. & Strimmer, K. 2004. APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* **20**:289-290.
- Parker, G.A. & Pizzari, T. 2010. Sperm competition and ejaculate economics. *Biol. Rev.* **85**:897-934.
- Parker, G.A., Lessells, C.M. & Simmons, L.W. 2013. Sperm competition games: a general model for pre-copulatory male-male competition. *Evolution* **67**:95-109.
- Pélissié, B., Jarne, P., Sarda, V. & David, P. 2014. Disentangling precopulatory and postcopulatory sexual selection in polyandrous species. *Evolution* **68**:1320-1331.
- Pischedda, A. & Rice, W.R. 2012. Partitioning sexual selection into its mating success and fertilization success components. *Proc. Nat. Acad. Sci.* **109**: 2049-2053.
- Poulin, R. & Morand, S. 2000. Testes size, body size and male–male competition in acanthocephalan parasites. *J. Zool.* **250**:551-558.
- Preston, B.T., Stevenson, I.R., Pemberton, J.M., Coltman, D.W. & Wilson, K. 2003. Overt and covert competition in a promiscuous mammal: the importance of weaponry and testes size to male reproductive success. *Proc. R. Soc. Lond. B.* **270**: 633-640.
- Pyron, R.A., Burbrink, F.T. & Wiens, J.J. 2013. A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. *BMC Evol. Biol.* **13**:93.
- R Development Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org>.

- Revell, L.J. 2012. phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.* **3**:217-223.
- Rose, E., Paczolt, K.A. & Jones, A.G. 2013. The contributions of premating and postmating selection episodes to total selection in sex-role-reversed Gulf Pipefish. *Am. Nat.* **182**:410-420.
- Rowe, M. & Pruett-Jones, S. 2011. Sperm competition selects for sperm quantity and quality in the Australian Maluridae. *PLoS One* **6**(1):e15720-e15720.
- Shine, R. 1994. Sexual size dimorphism in snakes revisited. *Copeia*. **1994**:326-346.
- Simmons, L.W. & Emlen, D.J. 2006. Evolutionary trade-off between weapons and testes. *Proc. Nat. Acad. Sci.* **103**:16346-16351.
- Somjee, U., Allen, P.E. & Miller, C.W. 2015. Different environments lead to a reversal in the expression of weapons and testes in the heliconia bug, *Leptoscelis tricolor* (Hemiptera: Coreidae). *Biol. J. Linn. Soc.* **115**:802-809.
- South, A. & Lewis, S.M. 2012. Determinants of reproductive success across sequential episodes of sexual selection in a firefly. *Proc. R. Soc. B.* **279**:3201-3208.
- Stamps, J.A. 1983. Sexual selection, sexual dimorphism and territoriality. In: *Lizard ecology: historical and experimental perspectives*. (L.J. Vitt & E.R. Pianka, eds.), pp. 169-204. Princeton Univ. Press, Princeton, NJ.
- Stockley, P., Gage, M., Parker, G.A. & Møller, A.P. 1997. Sperm competition in fishes: the evolution of testis size and ejaculate characteristics. *Am. Nat.* **45**:933-954.
- Todd, A.C. 2008. Using testis size to predict the mating systems of New Zealand geckos. *New Zeal. J. Zool.* **35**:103-114.

- Turnell, B.R. & Shaw, K.L. 2015. High opportunity for postcopulatory sexual selection under field conditions. *Evolution* **69**:2094-2104.
- Uller, T. & Olsson, M. 2008. Multiple paternity in reptiles: patterns and processes. *Mol. Ecol.* **17**:2566-2580.
- Uller, T., Stuart-Fox, D. & Olsson, M. 2010. Evolution of primary sexual characters in reptiles. In: *The evolution of primary sexual characteristics in animals*. (J.L. Leonard & A. Córdoba-Aguilari, eds.), pp. 426-429 Oxford University Press, Oxford, UK.
- Wedell, N. 1993. Spermatophore size in bushcrickets: comparative evidence for nuptial gifts as a sperm protection device. *Evolution* **47**:1203-1212.
- Yamane, T., Okada, K., Nakayama, S. & Miyatake, T. 2010. Dispersal and ejaculatory strategies associated with exaggeration of weapon in an armed beetle. *Proc. R. Soc. Lond. B.* **277**: 1705-1710.

Tables and Figures

Figure 1. Partial slopes ($\pm$ standard errors) from phylogenetic generalized least squares (PGLS, black) and ordinary least squares (OLS, white) regressions of $\log_{10}$ testis size on $\log_{10}$ sexual size dimorphism (SSD) with $\log_{10}$ snout-vent length (SVL) as a covariate. We conducted separate regressions for all lineages represented by ≥ 8 species, with sample sizes for each lineage shown at the tips of the phylogeny.

Figure 2. (A-B) Relationships between phylogenetically adjusted and size-corrected measures of testis size and sexual size dimorphism (SSD) across (A) 120 lizard species, and (B) 31 snake species. Residuals were obtained from phylogenetic generalized least squares (PGLS) regression of $\log_{10}$ testis size on $\log_{10}$ SVL (residual testis size), and PGLS regression of $\log_{10}$ SSD on $\log_{10}$ SVL (residual SSD). These residuals were used to visualize the covariance between testis size and SSD after accounting for phylogeny and allometry, but actual inferences (and reported partial r and P values) are based on multiple regressions using PGLS with a size covariate. (C-D) Relationships between size-

corrected residuals obtained from regression (through the origin) of phylogenetically independent contrasts for $\log_{10}$ SSD regressed on independent contrasts for $\log_{10}$ SVL (residual contrasts of SSD), and of independent contrasts for $\log_{10}$ testis size regressed on independent contrasts for $\log_{10}$ SVL (residual contrasts of testis size). As above, relationships are shown separately for (C) lizards ($N = 119$ contrasts) and (D) snakes, ($N = 30$ contrasts).

Table 1. Summary of results from phylogenetic generalized least squares (PGLS) regression of $\log_{10}$ testis size on $\log_{10}$ sexual size dimorphism (SSD) with $\log_{10}$ mean male body size (snout-vent length, SVL) as a covariate, using a Brownian Motion model of character evolution. Analyses were conducted separately for lizards (N = 120 species) and snakes (N = 31 species) due to differences in shape and in allometry of testis size and SSD between groups. Pagel's λ is given with superscripts corresponding to P -values testing significance using log-likelihood ratio tests against models of $\lambda = 0$ and $\lambda = 1$, respectively.

Taxon	Trait	Partial slope $\pm$ s.e.	df	t	P	Partial r (95% CI)	Pagel's λ
Lizards	Log ₁₀ SSD	-0.764 $\pm$ 0.226	117	-3.369	< 0.001	-0.293 (-5.367, -1.356)	0.352 ^{<0.001, <0.001}
	Log ₁₀ SVL	2.221 $\pm$ 0.279	117	7.941	< 0.001	0.586 (5.725, 10.129)	0.167 ^{0.067, 1}
Snakes	Log ₁₀ SSD	-0.534 $\pm$ 2.423	28	-2.204	0.035	-0.692 (-7.677, -2.946)	<0.001 ^{1, <0.001}
	Log ₁₀ SVL	3.277 $\pm$ 0.915	28	3.578	0.001	0.507 (1.132, 5.376)	0.506 ^{0.545, 1}

Appendix 1. All species of snakes and lizards entered into analysis. Mean sexual size dimorphism (SSD) was calculated as the mean SVL of the larger sex divided by the mean SVL of the smaller sex. The $\log_{10}$ values used in analysis were taken from these values, and were given a negative value for female larger species, and a positive value for male larger species.

Details of data collected for this manuscript (listed in source), can be found in Appendix 2.

Species	Mean SSD	Mean SVL (M)	Mean SVL (F)	Mean testis size (mm ³)	Number (M,F)	Family	Source
Lizards							
<i>Ameiva ameiva</i>	1.022	117.8	115.3	221.6	18, 11	Tetridae	Vit unpublished
<i>Amphibolurus muricatus</i>	1.013	91.7	90.5	47	87, 69	Agamidae	Harlow and Taylor 2000
<i>Anolis alinger</i>	1.169	51.72	44.25	40.44	11, 8	Dactyloidae	This manuscript, Appendix 2
<i>Anolis angusticeps</i>	1.190	53.57	45	23.08	4, 3	Dactyloidae	This manuscript, Appendix 2
<i>Anolis auratus</i>	1.019	43.6	42.8	21.57	110, 122	Dactyloidae	Sexton et al. 1971
<i>Anolis bahucoensis</i>	1.149	44.76	38.94	6.048	20, 18	Dactyloidae	This manuscript, Appendix 2
<i>Anolis barahonae</i>	-1.053	142.4	150	236.45	5, 1	Dactyloidae	This manuscript, Appendix 2
<i>Anolis brevirostris</i>	1.119	49.1	43.87	12.20	19, 16	Dactyloidae	This manuscript, Appendix 2
<i>Anolis carolinensis</i>	1.192	62.8	52.7	32.5	69, 60	Dactyloidae	Dessauer 1955, Ruby 1984, Stamps et al. 1997
<i>Anolis christophei</i>	1.102	47.5	43.1	13.66	20, 20	Dactyloidae	This manuscript, Appendix 2
<i>Anolis coelestinus</i>	1.270	68.7	54.1	24.8	20, 10	Dactyloidae	This manuscript, Appendix 2
<i>Anolis conspersus</i>	1.534	63.5	41.4	18	-	Dactyloidae	Licht & Gorman 1970
<i>Anolis chlorocyanus</i>	1.254	73.89	58.9	48.18	19, 11	Dactyloidae	This manuscript, Appendix 2
<i>Anolis cristatellus</i>	1.365	66.35	48.6	30.95	20, 41	Dactyloidae	This manuscript, Appendix 2
<i>Anolis cupreus</i>	1.068	42.7	40	18	-	Dactyloidae	Fleming & Hooker 1975, Stamps et al. 1997

<i>Anolis cybotes</i>	1.170	64.2	54.88	45.01	20, 20	Dactyloidae	This manuscript, Appendix 2
<i>Anolis distichus</i>	1.140	51.63	45.3	15.15	29, 25	Dactyloidae	This manuscript, Appendix 2
<i>Anolis equestris</i>	1.128	151.77	134.5	310.2	9, 6	Dactyloidae	This manuscript, Appendix 2
<i>Anolis etheridgei</i>	1.216	40.75	33.5	11.34	12, 10	Dactyloidae	This manuscript, Appendix 2
<i>Anolis evermanni</i>	1.277	60.29	47.21	42.27	20, 14	Dactyloidae	This manuscript, Appendix 2
<i>Anolis fuscoauratus</i>	-1.109	41.1	45.6	24.49	8, 78	Dactyloidae	Vitt unpublished
<i>Anolis grahami</i>	1.389	63.9	46	42	-	Dactyloidae	Licht & Gorman 1970
<i>Anolis gundlachi</i>	1.443	62.05	43	24.16	20, 5	Dactyloidae	This manuscript, Appendix 2
<i>Anolis insolitus</i>	-1.026	39	40	11.55	6, 2	Dactyloidae	This manuscript, Appendix 2
<i>Anolis longitibialis</i>	1.353	65.88	48.69	43.99	17, 20	Dactyloidae	This manuscript, Appendix 2
<i>Anolis krugi</i>	1.302	50.05	38.44	10.53	20, 18	Dactyloidae	This manuscript, Appendix 2
<i>Anolis limifrons</i>	1.012	43.6	43.1	15.7	244, 369	Dactyloidae	Sexton et al. 1971
<i>Anolis lineatopus</i>	1.368	62.5	45.7	41	-	Dactyloidae	Licht & Gorman 1970
<i>Anolis marcanoi</i>	1.085	52.08	48	35.71	12, 11	Dactyloidae	This manuscript, Appendix 2
<i>Anolis ocellus</i>	-1.036	38.4	39.8	12.78	5, 10	Dactyloidae	This manuscript, Appendix 2
<i>Anolis olssoni</i>	1.053	43.85	41.66	6.11	20, 15	Dactyloidae	This manuscript, Appendix 2
<i>Anolis opalinus</i>	1.196	48.33	40.42	28.8	101, 148	Dactyloidae	Floyd & Jenssen 1983, Jenssen 1994
<i>Anolis ortonii</i>	1.045	46.8	44.8	26.84	9, 8	Dactyloidae	Fitch 1976, Vitt unpublished
<i>Anolis poncensis</i>	1.186	45.68	38.5	9.42	20, 8	Dactyloidae	This manuscript, Appendix 2
<i>Anolis porcatus</i>	1.521	66.63	43.81	23.83	20, 20	Dactyloidae	This manuscript, Appendix 2
<i>Anolis pulchellus</i>	1.195	44.96	37.63	9.29	20, 16	Dactyloidae	This manuscript, Appendix 2
<i>Anolis punctatus</i>	1.050	77.8	74.1	31.09	10, 11	Dactyloidae	Vitt unpublished
<i>Anolis richardii</i>	1.226	93.2	76	95	-	Dactyloidae	Licht & Gorman 1970
<i>Anolis sagrei</i>	1.286	56.57	44	30.3	25, 25	Dactyloidae	This manuscript, Appendix 2
<i>Anolis semilineatus</i>	1.000	44	44	8.62	5, 2	Dactyloidae	This manuscript, Appendix 2
<i>Anolis smaragdinus</i>	1.235	60.5	49	33.91	25, 25	Dactyloidae	This manuscript, Appendix 2
<i>Anolis stratulus</i>	1.218	47	38.6	31.06	20, 20	Dactyloidae	This manuscript, Appendix 2
<i>Anolis trachyderma</i>	-1.158	46.9	54.3	19.84	38, 26	Dactyloidae	Vitt unpublished

<i>Anolis trinitatis</i>	1.373	63.3	46.1	22	-	Dactyloidae	Licht & Gorman 1970
<i>Anolis tropidogaster</i>	-1.002	44.9	45	24.5	83, 29	Dactyloidae	Sexton et al. 1971
<i>Aspidoscelis tigris</i>	-1.008	77.1	77.7	63	44, 75	Teiidae	Vitt and Ohmart 1977
<i>Barisia imbricata</i>	1.000	111.8	111.8	67	18, 36	Anguidae	Guillette & Casas-Andreu 1987
<i>Basiliscus vittatus</i>	1.197	116.5	97.31	62.93	15, 15	Coryphanidae	Hirth 1963, This manuscript, Appendix 2
<i>Callisaurus draconoides</i>	1.201	88.375	73.6	65.62	16, 10	Phrynosomatidae	This manuscript, Appendix 2
<i>Cnemidophorus lemniscatus</i>	1.081	65.6	54.7	62.93	67, 21	Teiidae	Vitt & de Carvalho 1995, Vitt et al. 1997, This manuscript, Appendix 2 This manuscript, Appendix 2
<i>Cophosaurus texanus</i>	1.199	64.14	59.32	42.63	16, 31	Phrynosomatidae	Vitt et al. 1997, This manuscript, Appendix 2 This manuscript, Appendix 2
<i>Crotaphytus collaris</i>	1.056	96.9	91.8	365	422, 518	Iguanidae	Sexton et al. 1992, Trauth 1979
<i>Ctenophorus pictus</i>	1.000	54	54	150	69, 45	Agamidae	Niejalke 2006
<i>Ctenotus brooksi</i>	-1.045	44.4	46.4	16	105, 98	Scincidae	James 1991a,b,c
<i>Ctenotus leonhardtii</i>	-1.006	65.1	65.5	21.4	106, 110	Scincidae	James 1991a,b,c
<i>Ctenotus pantherinus</i>	-1.088	79.8	86.8	86.4	61, 75	Scincidae	James 1991a,b,c
<i>Ctenotus plankai</i>	-1.048	50.4	52.8	14.5	81, 40	Scincidae	James 1991a,b,c
<i>Ctenotus</i>	-1.038	60.4	62.7	10.3	309, 247	Scincidae	James 1991a,b,c
<i>quattuordecimlineatus</i>							
<i>Ctenotus schomburgkii</i>	-1.076	42.1	45.3	10.1	115, 124	Scincidae	James 1991a,b,c
<i>Dipsosaurus dorsalis</i>	1.058	127	120	250	377, 200	Iguanidae	Mayhew 1971, Licht 1973
<i>Gambelia sila</i>	1.088	106.1	97.5	48.67	-	Crotaphytidae	Tollestrup 1982
<i>Gambelia wislizenii</i>	-1.142	104.6	119.5	39.79	-	Crotaphytidae	Tollestrup 1982
<i>Gekko gekko</i>	1.087	148.61	136.76	59	68, 39	Gekkonidae	Aowphol et al. 2006, This manuscript, Appendix 2
<i>Gonatodes concinnatus</i>	1.010	42.4	42	11.25	8, 12	Sphaerodactylidae	Fitch 1981, Duellman unpublished data, Appendix 2
<i>Gonatodes humeralis</i>	1.014	36.2	35.7	2.64	19, 28	Sphaerodactylidae	Vitt unpublished data
<i>Hemidactylus mabouia</i>	1.003	63.6	63.4	35.83	12, 14	Gekkonidae	Vitt unpublished
<i>Hemidactylus palachinus</i>	-1.004	54.8	55	38.11	13, 18	Gekkonidae	Vitt unpublished
<i>Kentropyx calcarata</i>	-1.012	79.8	80.76	231.4	-	Teiidae	Vitt unpublished; Vitt 1991
<i>Laudakia stellio</i>	1.113	118	106	350	66, 77	Agamidae	Childress 1970

<i>Leiocephalus carinatus</i>	1.283	106	82.62	36.96	3, 8	Leiocephalidae	This manuscript, Appendix 2
<i>Leiocephalus barahonensis</i>	1.240	77.5	62.5	47.11	2, 6	Leiocephalidae	This manuscript, Appendix 2
<i>Lepidophyma sylvaticum</i>	-1.013	75	76	50	44, 57	Xantusiidae	Ramírez-Bautista et al. 2008
<i>Mabuya nigropunctata</i>	1.068	87.8	82.2	30.28	15, 12	Scincidae	Vitt unpublished, Vitt and Blackburn 1991
<i>Ouriborus cataphractus</i>	1.009	107	106	45	70, 52	Cordylidae	Flemming and Mouton 2002
<i>Phrynosoma cornutum</i>	-1.069	69.6	74.4	466.49	4, 2	Phrynosomatidae	This manuscript, Appendix 2, Fitch 1981
<i>Phrynosoma modestum</i>	-1.125	55.8	62.8	158.93	4, 2	Phrynosomatidae	This manuscript, Appendix 2, Fitch 1981
<i>Phrynosoma solare</i>	-1.133	90	102	598	23, 23	Phrynosomatidae	Parker 1971, Sherbrooke unpublished
<i>Phyllodactylus lanei</i>	1.040	68.1	65.5	78.9	199, 144	Phyllodactylus	Ramírez-Sandoval, Ramírez-Bautista, and Vitt 2006
<i>Plestiodon copei</i>	-1.075	58.62	63	47	100, 100	Scincidae	Ramírez-Bautista et al. 1996
<i>Plestiodon laticeps</i>	1.154	109.4	94.8	240	164, 97	Scincidae	Vitt & Cooper 1985a, 1985b
<i>Plica plica</i>	1.085	118.2	108.9	163.69	5, 10	Tropiduridae	Vitt 1991, Vitt unpublished
<i>Plica umbra</i>	-1.053	84.5	89	82.29	14, 7	Tropiduridae	Vitt et al. 1997, Vitt unpublished
<i>Podarcis siculus</i>	1.118	62.6	56	55	31, 42	Lacertidae	Fitch 1981, Nevo et al. 1972
<i>Pseudocordylus melanotus</i>	1.103	118.3	107.3	248	83, 89	Cordylidae	Flemming 1993a, 1993b
<i>Sceloporus clarkii</i>	1.079	84.9	78.66	273.42	6, 3	Phrynosomatidae	This manuscript, Appendix 2
<i>Sceloporus graciosus</i>	-1.035	52.1	53.9	315	85, 76	Phrynosomatidae	Jameson 1974, Fitch 1978
<i>Sceloporus grammicus</i>	1.040	51.26	49.28	92	23, 32	Phrynosomatidae	Fitch 1978, Guillelte and Bearce 1986
<i>Sceloporus horridus</i>	-1.099	83.2	91.4	10.39	65, 37	Phrynosomatidae	Valdez-Gonzalez and Ramirez-Bautista 2002
<i>Sceloporus jarrovi</i>	1.068	88.2	82.58	129.07	6, 5	Phrynosomatidae	Cox and John-Alder 2007, This manuscript, Appendix 2
<i>Sceloporus magister</i>	1.146	102.5	89.41	108.61	17, 24	Phrynosomatidae	This manuscript, Appendix 2
<i>Sceloporus malachiticus</i>	1.048	79.12	75.49	259	146, 208	Phrynosomatidae	Marion & Sexton 1971, Fitch 1978
<i>Sceloporus occidentalis</i>	1.036	75.36	72.77	231	97, 46	Phrynosomatidae	Fitch 1978, Wilhoft and Quay 1961
<i>Sceloporus olivaceus</i>	-1.135	93.16	105.72	183.55	11, 11	Phrynosomatidae	This manuscript, Appendix 2
<i>Sceloporus orcutti</i>	1.109	102	92	225	117, 77	Phrynosomatidae	Mayhew 1963
<i>Sceloporus scalaris</i>	-1.077	47.59	51.25	60	45, 203	Phrynosomatidae	Newlin 1976

<i>Sceloporus spinosus</i>	1.039	90.4	87	14.5	73, 23	Phrynosomatidae	Valdéz-González and Ramírez-Bautista 2002
<i>Sceloporus undulatus</i>	-1.054	62.07	65.43	247	85, 79	Phrynosomatidae	Cooper & Vitt 1989, Marion 1982
<i>Sceloporus uniformis</i>	1.079	64.44	59.7	15.6	9, 10	Phrynosomatidae	Fitch 1978, Ramírez-Bautista, and Gutiérrez-Mayén 2003
<i>Sceloporus virgatus</i>	-1.108	56.4	62.5	114.66	12, 11	Phrynosomatidae	Rose 1981, This manuscript, Appendix 2
<i>Sceloporus woodi</i>	-1.061	47.6	50.5	55.69	400	Phrynosomatidae	Jackson & Telford 1974
<i>Strophurus ciliaris</i>	-1.083	67.4	73	150	64, 82	Diplodactylidae	How et al. 1986
<i>Strophurus elderi</i>	-1.059	40.6	43	37	72, 81	Diplodactylidae	How et al. 1986
<i>Strophurus intermedius</i>	-1.092	58.7	64.1	140	33, 43	Diplodactylidae	How et al. 1986
<i>Strophurus rankini</i>	-1.053	53.1	55.9	60	20, 23	Diplodactylidae	How et al. 1986
<i>Strophurus spinigerus</i>	-1.077	56.1	60.4	134	77, 101	Diplodactylidae	How et al. 1986
<i>Strophurus strophurus</i>	-1.127	58.1	65.5	97	53, 73	Diplodactylidae	How et al. 1986
<i>Takydromus</i>	-1.012	60.5	61.2	42	78, 105	Lacertidae	Huang 1998
<i>hsuehshanensis</i>							
<i>Takydromus sylvaticus</i>	-1.053	53	55.8	19.05	5, 4	Lacertidae	Tang 2007
<i>Trachylepis capensis</i>	-1.223	75.47	92.31	105	90, 71	Scincidae	Flemming 1994
<i>Tropidurus hispidus</i>	1.290	93.5	72.5	58.6	39, 42	Tropiduridae	Vitt unpublished, Vitt 1993
<i>Tropidurus itambere</i>	1.128	75.9	67.3	56.23	66, 60	Tropiduridae	Van Sluys 1993a,b, 1998, 2000
<i>Tropidurus torquatus</i>	1.168	96.1	82.3	200	224, 233	Tropiduridae	Vitt & Goldberg 1983
<i>Uma inornata</i>	1.259	102	81	190	191, 213	Phrynosomatidae	Mayhew 1965
<i>Uma notata</i>	1.263	96	76	123.3	214,270	Phrynosomatidae	Mayhew 1966a
<i>Uma scoparia</i>	1.169	97	83	210	248,236	Phrynosomatidae	Mayhew 1966b
<i>Uranoscodon</i>	-1.127	101.2	114.1	289.5	22,15	Tropiduridae	Vitt unpublished
<i>superciliatus</i>							
<i>Urosaurus graciosus</i>	1.034	54.1	52.3	25	227, 195	Phrynosomatidae	Vitt et al. 1978
<i>Urosaurus ornatus</i>	1.122	55.65	49.6	34.28	17,25	Phrynosomatidae	This manuscript, Appendix 2
<i>Uta stansburiana</i>	1.100	54.47	49.5	19.57	17,14	Phrynosomatidae	This manuscript, Appendix 2
<i>Xantusia vigilis</i>	-1.079	38	41	16	-	Xantusiidae	Miller 1951, Licht 1973

Snakes

<i>Acrochordus arafurae</i>	-1.286	1050	1350	5654	217, 331	Acrochordidae	Shine 1986
<i>Acrochordus granulatus</i>	-1.077	648	698	600	168, 147	Acrochordidae	Gorman et al. 1981
<i>Agkistrodon piscivorus</i>	1.100	767	697	470	93, 49	Viperidae	Scott et al. 1995
<i>Austrelaps superbis</i>	1.085	766	706	200	110, 27	Elapidae	Shine 1977a,b, Shine 1978
<i>Boa constrictor</i>	-1.139	1800	2050	15840	56, 50	Boidae	Bertona and Chiaraviglio 2003
<i>Boiga irregularis</i>	1.213	1308	1078	735	455, 327	Colubridae	Savidge et al. 2007
<i>Bothrops asper</i>	-1.126	1263	1422	3000	64, 83	Viperidae	Solorzano and Cerdas 1989
<i>Cerastes vipera</i>	-1.102	235	259	80	165, 165	Viperidae	Sivan et al. 2012
<i>Cerberus rynchops</i>	-1.041	511	532	720	203, 179	Colubridae	Gorman et al. 1981
<i>Crotalus adamanteus</i>	1.003	1107	1104	474	32, 31	Viperidae	Steen et al. 2007, Hoss et al. 2011
<i>Crotalus oreganus</i>	1.332	986	740	890	39, 19	Viperidae	Lind et al. 2010
<i>Cylindrophis ruffus</i>	-1.042	686	715	508	48, 88	Cylindrophidae	Brooks et al. 2009
<i>Enhydrina schistosa</i>	-1.083	782	847	25000	38, 13	Elapidae	Voris and Jayne 1979
<i>Enhydris bocourti</i>	-1.077	532	573	787.6	68, 68	Colubridae	Brooks et al. 2009
<i>Enhydris enhydris</i>	-1.063	475	505	203.75	2341, 1754	Colubridae	Brooks et al. 2009, Karns et al. 2005
<i>Enhydris longicauda</i>	-1.106	442	489	195.56	720, 906	Colubridae	Brooks et al. 2009
<i>Erypeton tentaculatum</i>	-1.092	459	501	114.5	443, 424	Colubridae	Brooks et al. 2009
<i>Hemiaspis signata</i>	-1.005	431	433	170	53, 15	Elapidae	Shine 1977a,b, Shine 1978
<i>Homalopsis buccata</i>	1.000	740	740	1074.14	707, 874	Colubridae	Brooks et al. 2009
<i>Latiauda colubrina</i>	-1.419	884.5	1255	950	158, 73	Elapidae	Gorman et al. 1981
<i>Micruurus fulvius</i>	-1.329	547	727	2240	51, 27	Elapidae	Jackson and Franz 1981
<i>Nerodia taxispilota</i>	-1.214	608	738	225000	70, 54	Colubridae	White et al. 1982
<i>Notechis scutatus</i>	1.002	810	808	500	174, 32	Elapidae	Shine 1977a,b, Shine 1978
<i>Pseudechis porphyriacus</i>	1.052	1116	1061	360	225, 55	Elapidae	Shine 1977a,b, Shine 1978
<i>Pseudonaja textilis</i>	1.068	1250	1170	320	-	Elapidae	Shine 1977a,b, Whittaker and Shine 2003
<i>Sibynomorphus mikantii</i>	-1.241	352	437	117.6	92, 52	Colubridae	Rojas et al. 2013, Pizzatto et al. 2008
<i>Thamnophis elegans</i>	-1.081	405	438	451.5	22, 20	Colubridae	Fox 1954

<i>Thamnophis melanogaster</i>	1.021	546	535	103.5	15, 28	Colubridae	Garstka and Crews 1982
<i>Thamnophis sirtalis</i>	-1.012	417	422	451.5	68, 92	Colubridae	White and Kolb 1974
<i>Tropidoclonion lineatum</i>	-1.565	200	313	141.2	65, 100	Colubridae	Krohmer and Aldridge 1985a, 1985b
<i>Xenochrophis piscator</i>	-1.202	504	606	628.9	97, 135	Colubridae	Brooks et al. 2009

Appendix 2

Several species in this dataset were collected from wild populations between May 2013 - June 2015. All species were collected during the peak of their breeding season (May-June) to ensure the testes were at their maximum size. We collected lizards using nooses, and measured them for mass and snout-vent length. We checked each male for sexual maturity by collecting a sperm sample, and any male who did not produce sperm after one day of rest was eliminated from the dataset. Testis size for each species was measured either through surgical laparotomy or by dissection. For our surgical laparotomies, each lizard received a 2-4ul injection of 0.25% bupivacain (Auromedics, Dayton, NJ), a local anesthetic, to their abdomen. We then cooled the lizards at -20°F for 5-10 minutes (depending on the size of the species) and placed them on an ice pack during their surgery. We made a small ventral incision and exposed the left testis to measure length and width. The testis was then placed back in the abdomen, and the incision was closed using Nexaband surgical glue (Veterinary Products Laboratories, Phoenix, AZ). We held each individual in separate container overnight to allow them to recover, and then released them to their capture locations. Some species were brought back to the lab, sacrificed and dissected. Dr. Michele Johnson generously provided us with testis length and width from these species.

Below we list the details of our permits that specify which method was used for collecting testis size.

Species collected from Arizona were collected under license number: SP673841 issued by the State of Arizona's Game and Fish Department. Species collected from Puerto Rico were collected under permit numbers: 2014-IC-045 and 2014-IC-029 (issued to Dr. Michele Johnson) issued by el Departamento de Recursos Naturales y Ambientales. Species collected in the Dominican Republic were collected under with permission issued by el Ministerio de Medio Ambiente y Recursos Naturales (issued to Dr. Michele Johnson). Species collected from Texas were collected under permit number: SPR 0814-

159 issued by Texas Parks and Wildlife Department to Corey E. Roelke. Finally, species collected in Dade county Florida were collected under permit number: 213, issued by Miami-Dade County Parks and Recreation to Ariel F. Kahrl.

Species	Testes measurements	Date	GPS
<i>Anolis aliniger</i>	Dissection	June 2015	N 19° 01' 57.52" W 70° 32' 35.50"
<i>Anolis angusticeps</i>	Dissection	June 2013	N 23° 30' 23.17" W 75° 45' 57.53"
<i>Anolis bahorucoensis</i>	Dissection	June 2015	N 18° 07' 36.66" W 71° 16' 06.67"
<i>Anolis barahonae</i>	Dissection	June 2015	N 18° 05' 59.92" W 71° 15' 14.42"
<i>Anolis brevirostris</i>	Dissection	June 2015	N 18° 03' 29.24" W 71° 06' 46.51"
<i>Anolis christophei</i>	Dissection	June 2015	N 19° 01' 57.52" W 70° 32' 35.50"
<i>Anolis coelestinus</i>	Dissection	June 2015	N 18° 03' 29.24" W 71° 06' 46.51"
<i>Anolis chlorocyanus</i>	Dissection	June 2015	N 18° 31' 38.92" W 70° 30' 30.43"
<i>Anolis cristatellus</i>	Dissection	June 2014	N 18° 20' 32.28" W 65° 49' 33.72"
<i>Anolis cybotes</i>	Dissection	June 2015	N 18° 03' 29.24" W 71° 06' 46.51"
<i>Anolis distichus</i>	Dissection	June 2013	N 23° 30' 23.17" W 75° 45' 57.53"
<i>Anolis equestris</i>	Dissection	May 2014	N 25° 36' 56.36" W 80° 18' 24.19"
<i>Anolis etheridgei</i>	Dissection	June 2015	N 19° 01' 57.52" W 70° 32' 35.50"
<i>Anolis evermanni</i>	Dissection	June 2014	N 18° 20' 32.28" W 65° 49' 33.72"
<i>Anolis gundlachi</i>	Dissection	June 2014	N 18° 20' 32.28" W 65° 49' 33.72"
<i>Anolis insolitus</i>	Dissection	June 2015	N 19° 02' 26.71" W 70° 31' 17.34"
<i>Anolis longitibialis</i>	Dissection	June 2015	N 17° 50' 10.36" W 71° 27' 00.09"
<i>Anolis krugi</i>	Dissection	June 2014	N 18° 20' 32.28" W 65° 49' 33.72"
<i>Anolis marcanoii</i>	Dissection	June 2015	N 18° 24' 21.22" W 70° 25' 02.36"
<i>Anolis occultus</i>	Dissection	June 2014	N 18° 27' 09.00" W 66° 35' 49.56"
<i>Anolis olssoni</i>	Dissection	June 2015	N 18° 13' 50.80" W 70° 20' 44.15"
<i>Anolis poncensis</i>	Dissection	June 2014	N 17° 56' 56.94" W 66° 52' 32.64"
<i>Anolis porcatus</i>	Dissection	May 2014	N 25° 42' 28.51" W 80° 09' 27.75"
<i>Anolis pulchellus</i>	Dissection	June 2014	N 18° 19' 52.56" W 65° 49' 26.58"
<i>Anolis sagrei</i>	Dissection	June 2013	N 23° 30' 23.17" W 75° 45' 57.53"
<i>Anolis semilineatus</i>	Dissection	June 2015	N 18° 51' 25.33" W 70° 41' 45.48"
<i>Anolis smaragdinus</i>	Dissection	June 2013	N 23° 29' 49.14" W 75° 45' 54.59"
<i>Anolis stratulus</i>	Dissection	June 2014	N 18° 20' 32.28" W 65° 49' 33.72"
<i>Basiliscus vittatus</i>	Dissection	May 2014	N 25° 40' 42.31" W 80° 16' 25.60"
<i>Callisaurus draconoides</i>	Laparotomy	May 2014	N 32° 16' 50.33" W 110° 55' 58.28"
<i>Cnemidophorus lemniscatus</i>	Dissection	May 2014	N 25° 47' 34.96" W 80° 12' 48.18"
<i>Cophosaurus texanus</i>	Dissection	May 2014	N 32° 20' 22.36" W 110° 54' 34.43"
<i>Gekko gecko</i>	Dissection	May 2014	N 25° 36' 55.16" W 80° 18' 23.42"
<i>Leiocephalus carinatus</i>	Dissection	May 2014	N 25° 47' 34.96" W 80° 12' 48.18"
<i>Leiocephalus barahonensis</i>	Dissection	May 2014	N 17° 50' 10.36" W 71° 27' 00.09"
<i>Phrynosoma cornutum</i>	Laparotomy	May 2014	N 31 °54' 50.32" W 109° 08' 29.32"
<i>Phrynosoma modestum</i>	Laparotomy	May 2014	N 31 °54' 50.32" W 109° 08' 29.32"

<i>Sceloporus clarkii</i>	Laparotomy	May 2014	N 32° 19' 48.41" W 110° 51' 21.71"
<i>Sceloporus jarrovi</i>	Laparotomy	May 2014	N 31° 52' 10.95" W 109° 11' 10.20"
<i>Sceloporus magister</i>	Laparotomy	May 2014	N 32° 16' 50.33" W 110° 55' 58.28"
<i>Sceloporus olivaceus</i>	Dissection	July 2014	N 32° 45' 01.44" W 97° 07' 54.52"
<i>Sceloporus virgatus</i>	Laparotomy	May 2014	N 31° 54' 11.36" W 109° 14' 35.39"
<i>Urosaurus ornatus</i>	Laparotomy	May 2014	N 32° 16' 50.33" W 110° 55' 58.28"
<i>Uta stansburiana</i>	Laparotomy	May 2014	N 32° 16' 50.33" W 110° 55' 58.28"

Appendix 3

- Aowphol, A., Thirakhupt, K., Nabhitabhata, J. & Voris, H.K. 2006. Foraging ecology of the Tokay gecko, *Gekko gecko* in a residential area in Thailand. *Amphibia-Reptilia* **27**:491-503.
- Bertona, M. & Chiaraviglio, M. 2003. Reproductive biology, mating aggregations, and sexual dimorphism of the Argentine Boa Constrictor (*Boa constrictor occidentalis*). *J. Herpetol.* **37**:510-516.
- Brooks, S.E., Allison, E.H., Gill, J.A. & Reynolds, J.D. 2009. Reproductive and trophic ecology of an assemblage of aquatic and semi-aquatic snakes in Tonle Sap, Cambodia. *Copeia* **2009**:7-20.
- Childress, J.R. 1970. Observations on the reproductive cycle of *Agama stellio picea*. *Herpetologica*. **26**:149-155.
- Cooper, W.E. & Vitt, L.J. 1989. Sexual dimorphism of head and body size in an iguanid lizard: paradoxical results. *Am. Nat.* **133**:729-735.
- Cox, R.M. & John-Alder, H.B. 2007. Growing apart together: the development of contrasting sexual size dimorphisms in sympatric *Sceloporus* lizards. *Herpetologica*. **63**:245-257.
- Dessauer, H.C. 1955. Seasonal changes in the gross organ composition of the lizard, *Anolis carolinensis*. *J. Exp. Zool.* **128**:1-12.
- Fitch, H.S. 1976. Sexual size differences in the mainland anoles. *Occas. Pap. Mus. Nat. Hist. Univ. Kans.* 50:1-21.
- Fitch, H.S. 1978. Sexual size differences in the genus *Sceloporus*. *Uni. Kansas. Sci. Bull.*

51:441-461.

Fitch, H.S. 1981. Sexual size differences in reptiles. *Mis. Publ. Mus. Nat. Hist. Uni. Kans.*

70:1-72.

Fleming, T.H. & Hooker, R.S. 1975. *Anolis cupreus*: the response of a lizard to tropical seasonality. *Ecology*. 56:1243-1261.

Flemming, A.F. 1993a. The male reproductive cycle of the lizard *Pseudocordylus m. melanotus* (Sauria: Cordylidae). *J. Herpetol.* 27:473-478.

Flemming, A.F. 1993b. The female reproductive cycle of the lizard *Pseudocordylus m. melanotus* (Sauria: Cordylidae). *J. Herpetol.* 27:103-107.

Flemming, A.F. 1994. Male and female reproductive cycles of the viviparous lizard, *Mabuya capensis* (Sauria: Scincidae) from South Africa. *J. Herpetol.* 28:334-341.

Flemming, A.F. & Mouton, P.L.F.N. 2002. Reproduction in a group-living lizard, *Cordylus cataphractus* (Cordylidae), from South Africa. *J. Herpetol.* 36:691-696.

Floyd, H.B. & Jenssen, T.A. 1983. Food habits of the Jamaican lizard *Anolis opalinus*: resource partitioning and seasonal effects examined. *Copeia* 1983:319-331.

Fox, W. 1954. Genetic and environmental variation in the timing of the reproductive cycles of male garter snakes. *J. Morphol.* 95: 415–450.

Garstka, W.R. & Crews, D. 1982. Female control of male reproductive function in a Mexican snake. *Science* 217:1159-1160.

Gorman, G.C., Licht, P. & McCollum, F. 1981. Annual reproductive patterns in three species of marine snakes from the central Philippines. *J. Herpetol.* 15:335-354.

- Guillette Jr., L.J. & Bearce, D.A. 1986. The reproductive and fat body cycles of the lizard, *Sceloporus grammicus disparilis*. *Trans. Kans. Acad. Sci.* **89**:31-39.
- Guillette Jr., L.J. & Casas-Andreu, G. 1987. The reproductive biology of the high elevation Mexican lizard *Barisia imbricata*. *Herpetologica* **43**:29-38.
- Harlow, P.S. & Taylor, J.E. 2000. Reproductive ecology of the jacky dragon (*Amphibolurus muricatus*): an agamid lizard with temperature-dependent sex determination. *Austral Ecol.* **25**:640-652.
- Hirth, H.F. 1963. The ecology of two lizards on a tropical beach. *Ecol. Monogr.* **33**:83-112.
- How, R.A., Dell, J. & Wellington, B.D. 1986. Comparative biology of eight species of *Diplodactylus* gecko in Western Australia. *Herpetologica* **42**:471-482.
- Hoss, S.K., Schuett, G.W., Earley, R.L. & Smith, L.L. 2011. Reproduction in male *Crotalus adamanteus* Beauvois (Eastern diamond-backed rattlesnake): relationship of plasma testosterone to testis and kidney dimensions and the mating season. *Southeast. Nat.* **10**:95-108.
- Huang, W.S. 1998. Reproductive cycles of the grass lizard, *Takydromus hsuehshanensis*, with comments on reproductive patterns of lizards from the central high elevation area of Taiwan. *Copeia* **1998**:866-873.
- Jackson, D.R. & Franz, R. 1981. Ecology of the eastern coral snake (*Micrurus fulvius*) in northern peninsular Florida. *Herpetologica* **37**:213-228.
- Jackson, J.F. & Telford Jr., S.R. 1974. Reproductive ecology of the Florida scrub lizard, *Sceloporus woodi*. *Copeia* **1974**:689-694.

- James, C.D. 1991. Population dynamics, demography, and life history of sympatric scincid lizards (*Ctenotus*) in central Australia. *Herpetologica* **47**:194-210.
- James, C.D. 1991. Annual variation in reproductive cycles of scincid lizards (*Ctenotus*) in central Australia. *Copeia* **1991**:744-760.
- James, C.D. 1991. Growth rates and ages at maturity of sympatric scincid lizards (*Ctenotus*) in central Australia. *J. Herpetol.* **25**:284-295.
- Jameson Jr, E.W. 1974. Fat and breeding cycles in a montane population of *Sceloporus graciosus*. *J. Herpetol.* **8**:311-322.
- Jenssen, T.A. & Nunez, S.C. 1994. Male and female reproductive cycles of the Jamaican lizard, *Anolis opalinus*. *Copeia* **1994**:767-780.
- Karns, D.R., Murphy, J.C., Voris, H.K. & Suddeth, J.S. 2005. Comparison of semi-aquatic snake communities associated with the Khorat Basin, Thailand. *The Natural History Journal of Chulalongkorn University* **5**:73-90.
- Krohmer, R.W. & Aldridge, R.D. 1985a. Male reproductive cycle of the lined snake (*Tropidoclonion lineatum*). *Herpetologica* **41**:33-38.
- Krohmer, R.W. & Aldridge, R.D. 1985b. Female reproductive cycle of the lined snake (*Tropidoclonion lineatum*). *Herpetologica* **41**:39-44.
- Licht, P. & Gorman, G.C. 1970. Reproductive and fat cycles in Caribbean *Anolis* lizards (Vol. 95). USA: University of California Press.
- Licht, P. 1973. Environmental influences on the testis cycles of the lizards *Dipsosaurus dorsalis* and *Xantusia vigilis*. *Comp. Biochem. Phys. A.* **45**:7-20.

- Lind, C.M., Husak, J.F., Eikenaar, C., Moore, I.T. & Taylor, E.N. 2010. The relationship between plasma steroid hormone concentrations and the reproductive cycle in the Northern Pacific Rattlesnake, *Crotalus oreganus*. *Gen. Comp. Endocr.* **166**:590-599.
- Marion, K.R. & Sexton, O.J. 1971. The reproductive cycle of the lizard *Sceloporus malachiticus* in Costa Rica. *Copeia* **1971**:517-526.
- Marion, K.R. 1982. Reproductive cues for gonadal development in temperate reptiles: temperature and photoperiod effects on the testicular cycle of the lizard *Sceloporus undulatus*. *Herpetologica* **38**:26-39.
- Mayhew, W.W. 1963. Reproduction in the granite spiny lizard, *Sceloporus orcutti*. *Copeia* **1963**:144-152.
- Mayhew, W.W. 1965. Reproduction in the sand-dwelling lizard *Uma inornata*. *Herpetologica* **21**:39-55.
- Mayhew, W.W. 1966a. Reproduction in the arenicolous lizard *Uma notata*. *Ecology* **47**:9-18.
- Mayhew, W.W. 1966b. Reproduction in the psammophilous lizard *Uma scoparia*. *Copeia* **1966**:114-122.
- Mayhew, W.W. 1971. Reproduction in the desert lizard, *Dipsosaurus dorsalis*. *Herpetologica* **27**:57-77.
- Nevo, E., Gorman, G.C., Soulé, M., Yang, S.Y., Clover, R. & Jovanović, V. 1972. Competitive exclusion between insular *Lacerta* species (Sauria, Lacertidae). *Oecologia* **10**:183-190.

- Miller, M.R. 1951. Some aspects of the life history of the yucca night lizard, *Xantusia vigilis*. *Copeia* **1951**:114-120.
- Newlin, M.E. 1976. Reproduction in the bunch grass lizard, *Sceloporus scalaris*. *Herpetologica* **32**:171-184.
- Niejalke, D.P. 2006. Reproduction by a small agamid lizard, *Ctenophorus pictus*, during contrasting seasons. *Herpetologica* **62**:409-420.
- Parker, W.S. 1971. Ecological observations on the regal horned lizard (*Phrynosoma solare*) in Arizona. *Herpetologica* **27**:333-338.
- Pizzatto, L., Cantor, M., De Oliveira, J.L., Marques, O.A., Capovilla, V. & Martins, M. 2008. Reproductive ecology of dipsadine snakes, with emphasis on South American species. *Herpetologica* **64**:168-179.
- Ramirez-Bautista, A., Guillette Jr., L.J., Gutierrez-Mayen, G. & Uribe-Peña, Z. 1996. Reproductive Biology of the Lizard "*Eumeces Copei*" (Lacertilia: Scincidae) from the Eje Neovolcanico, Mexico. *Southwest. Nat.* **41**:103-110.
- Ramírez-Bautista, A. & Gutiérrez-Mayén, G. 2003. Reproductive ecology of *Sceloporus utiformis* (Sauria: Phrynosomatidae) from a tropical dry forest of Mexico. *J. Herpetol.* **37**:1-10.
- Ramírez-Bautista, A., Vitt, L.J., Ramírez-Hernández, A., Quijano, F.M. & Smith, G.R. 2008. Reproduction and sexual dimorphism of *Lepidophyma sylvaticum* (Squamata: Xantusiidae), a tropical night lizard from Tlanchinol, Hidalgo, Mexico. *Amphibia-Reptilia* **29**:207-216.

- Ramírez-Sandoval, E., Ramírez-Bautista, A. & Vitt, L.J. 2006. Reproduction in the lizard *Phyllodactylus lanei* (Squamata: Gekkonidae) from the Pacific Coast of Mexico. *Copeia* **2006**:1-9.
- Rojas, C.A., Barros, V.A. & Almeida-Santos, S.M. 2013. The reproductive cycle of the male sleep snake *Sibynomorphus mikanii* (Schlegel, 1837) from southeastern Brazil. *J. Morphol.* **274**:215-228.
- Rose, B. 1981. Factors affecting activity in *Sceloporus virgatus*. *Ecology* **62**:706-716.
- Ruby, D.E. 1984. Male breeding success and differential access to females in *Anolis carolinensis*. *Herpetologica* **40**:272-280.
- Savidge, J.A., Qualls, F.J. & Rodda, G.H. 2007. Reproductive biology of the brown tree snake, *Boiga irregularis* (Reptilia: Colubridae), during colonization of Guam and comparison with that in their native range 1. *Pac. Sci.* **61**:191-199.
- Scott, D.E., Fischer, R.U., Congdon, J.D. & Busa, S.A. 1995. Whole body lipid dynamics and reproduction in the eastern cottonmouth, *Agkistrodon piscivorus*. *Herpetologica* **51**:472-487.
- Sexton, O.J., Ortleb, E.P., Hathaway, L.M., Ballinger, R.E. & Licht, P. 1971. Reproductive cycles of three species of anoline lizards from the Isthmus of Panama. *Ecology* **52**:202-215.
- Sexton, O.J., Andrews, R.M. & Bramble, J.E. 1992. Size and growth rate characteristics of a peripheral population of *Crotaphytus collaris* (Sauria: Crotaphytidae). *Copeia* **1992**:968-980.

- Shine, R. 1986. Sexual differences in morphology and niche utilization in an aquatic snake, *Acrochordus arafurae*. *Oecologia* **69**:260-267.
- Shine, R. 1977a. Reproduction in Australian elapid snakes I. Testicular cycles and mating seasons. *Aust. J. Zool.* **25**:647-653.
- Shine, R. 1977b. Reproduction in Australian elapid snakes II. Female reproductive cycles. *Aust. J. Zool.* **25**:655-666.
- Shine, R. 1978. Growth rates and sexual maturation in six species of Australian elapid snakes. *Herpetologica* **34**:73-79.
- Sivan, J., Kam, M., Hadad, S., Degen, A.A., Rozenboim, I. & Rosenstrauch, A. 2012. Reproductive cycle of free-living male Saharan sand vipers, *Cerastes vipera* (Viperidae) in the Negev desert, Israel. *Gen. Comp. Endocr.* **179**:241-247.
- Solórzano, A. & Cerdas, L. 1989. Reproductive biology and distribution of the terciopelo, *Bothrops asper* Garman (Serpentes: Viperidae), in Costa Rica. *Herpetologica* **45**:444-450.
- Stamps, J.A., Losos, J.B. & Andrews, R.M. 1997. A comparative study of population density and sexual size dimorphism in lizards. *Am. Nat.* **149**:64-90.
- Steen, D.A., Smith, L.L., Conner, L., Brock, J.C. & Hoss, S.K. 2007. Habitat use of sympatric rattlesnake species within the Gulf Coastal Plain. *J. Wildlife Manage.* **71**:759-764.
- Tang, X.S., Lu, S.Q. & Chou, W.H. 2007. Description of male *Takydromus sylvaticus* (Squamata: Lacertidae) from China, with notes on sexual dimorphism and a revision of the morphological diagnosis of the species. *Zool. Sci.* **24**:496-503.

- Tollestrup, K. 1982. Growth and reproduction in two closely related species of leopard lizards, *Gambelia silus* and *Gambelia wislizenii*. *Am. Midl. Nat.* **108**:1-20.
- Trauth, S.E. 1979. Testicular cycle and timing of reproduction in the collared lizard (*Crotaphytus collaris*) in Arkansas. *Herpetologica* **35**:184-192.
- Valdéz-González, M.A. & Ramírez-Bautista, A. 2002. Reproductive characteristics of the spiny lizards, *Sceloporus horridus* and *Sceloporus spinosus* (Squamata: Phrynosomatidae) from México. *J. Herpetol.* **36**:36-43.
- Van Sluys, M. 1993a. Food habits of the lizard *Tropidurus itambere* (Tropiduridae) in southeastern Brazil. *J. Herpetol.* **27**:347-351.
- Van Sluys, M. 1993b. The reproductive cycle of *Tropidurus itambere* (Sauria: Tropiduridae) in southeastern Brazil. *J. Herpetol.* **27**: 28-32.
- Van Sluys, M. 1998. Growth and body condition of the saxicolous lizard *Tropidurus itambere* in southeastern Brazil. *J. Herpetol.* **32**:359-365.
- Van Sluys, M. 2000. Population dynamics of the saxicolous lizard *Tropidurus itambere* (Tropiduridae) in a seasonal habitat of southeastern Brazil. *Herpetologica* **56**:55-62.
- Vitt, L.J. 1991. Ecology and life history of the wide-foraging lizard *Kentropyx calcarata* (Teiidae) in Amazonian Brazil. *Can. J. Zoolog.* **69**:2791-2799.
- Vitt, L.J. 1993. Ecology of isolated open-formation *Tropidurus* (Reptilia: Tropiduridae) in Amazonian lowland rain forest. *Can. J. Zoolog.* **71**:2370-2390.
- Vitt, L.J. & Blackburn, D.G. 1991. Ecology and life history of the viviparous lizard *Mabuya bistrata* (Scincidae) in the Brazilian Amazon. *Copeia* **1991**:916-927.

- Vitt, L.J. & de Carvalho, C.M. 1995. Niche partitioning in a tropical wet season: lizards in the lavrado area of northern Brazil. *Copeia* **1995**:305-329.
- Vitt, L.J. & Cooper Jr., W.E. 1985a. The evolution of sexual dimorphism in the skink *Eumeces laticeps*: an example of sexual selection. *Can. J. Zoolog.* **63**:995-1002.
- Vitt, L.J. & Cooper Jr., W.E. 1985b. The relationship between reproduction and lipid cycling in the skink *Eumeces laticeps* with comments on brooding ecology. *Herpetologica* **41**:419-432.
- Vitt, L.J. & Goldberg, S.R. 1983. Reproductive ecology of two tropical iguanid lizards: *Tropidurus torquatus* and *Platynotus semitaeniatus*. *Copeia* **1983**:131-141.
- Vitt, L.J. & Ohmart, R.D. 1977. Ecology and reproduction of Lower Colorado River lizards: II. *Cnemidophorus tigris* (Teiidae), with comparisons. *Herpetologica* **33**:223-234.
- Vitt, L.J., Sels, R.C.V.L. & Ohmart., R.D. 1978. Lizard reproduction: annual variation and environmental correlates in the iguanid lizard *Urosaurus graciosus*. *Herpetologica* **34**:241-253.
- Vitt, L.J., Zani, P.A., Caldwell, J.P., De Araujo, M.C. & Magnusson, W.E. 1997. Ecology of whiptail lizards (*Cnemidophorus*) in the Amazon region of Brazil. *Copeia* **1997**:745-757.
- Voris, H.K. & Jayne, B.C. 1979. Growth, reproduction and population structure of a marine snake, *Enhydrina schistosa* (Hydrophiidae). *Copeia* **1979**:307-318.
- White, M. & Kolb, J.A. 1974. A preliminary study of *Thamnophis* near Sagehen Creek, California. *Copeia* **1974**:126-136.

- White, D.R., Mitchell, J.C. & Woolcott, W.S. 1982. Reproductive cycle and embryonic development of *Nerodia taxispilota* (Serpentes: Colubridae) at the northeastern edge of its range. *Copeia* **1982**:646-652.
- Wilhoft, D.C. & Quay, W.B. 1961. Testicular histology and seasonal changes in the lizard, *Sceloporus occidentalis*. *J. Morphol.* **108**:95-106.
- Whitaker, P.B. & Shine, R. 2003. A radiotelemetric study of movements and shelter-site selection by free-ranging brownsnakes (*Pseudonaja textilis*, Elapidae). *Herpetol. Monogr.* **17**:130-144.

CHAPTER 2:

Rapid evolution of testis size suggests that postcopulatory sexual selection targets sperm count, not sperm morphology, in *Anolis* lizards

Abstract

Postcopulatory selection acts on many ejaculate traits associated with sperm quality or quantity. To determine how the sperm cell evolves relative to body size-corrected testis size, we measured sperm morphology (head, midpiece, and tail length) and testis size from 28 species of *Anolis* lizards. We first tested for differences in the Brownian rates of evolution among residual testis size, body size, and the lengths of the sperm head, midpiece and tail. We then tested for correlated evolution between residual testis size and sperm morphology to determine if sperm morphology is associated with the strength of postcopulatory selection among species. Finally, we tested for differences in these traits among *Anolis* ecomorphs, which are groups composed of species that have independently evolved convergent morphology, ecology, and behavior. We found that midpiece length has evolved 2–3 times faster than other parts of the sperm cell, but that testis size evolves between 10–30 times faster than any aspect of sperm morphology. The midpiece may experience stronger selection or be more evolutionarily labile than the rest of the sperm cell, however, the rapid rate of evolution of residual testis size suggests that sperm production may be more important than sperm morphology for reproductive success in *Anolis* lizards. In line with this, we found no significant correlations between sperm morphology and testis size, suggesting further that selection on sperm morphology in anoles is weak or inconsistent in direction. However, anole ecomorphs differ significantly in their testis size and sperm head length, which suggests that the strength of postcopulatory selection may be variable among ecomorph groups.

Introduction

Sexually selected traits are predicted to evolve quickly due to strong selection arising from male-male competition and female choice (West-Eberhard 1983; Andersson 1994; Gonzalez-Voyer and Kolm 2011). In fact, recent work has demonstrated that traits associated with precopulatory sexual selection (or selection before mating), such as male weapons or ornaments have accelerated rates of evolution relative to other morphological traits (Fitzpatrick et al. 2012; Seddon et al. 2013; Friedman and Remeš 2015; Simmons and Fitzpatrick 2016). When females mate with more than one male, sperm from several males may be in the reproductive tract of a female simultaneously, which can result in sperm competition and selection for a variety of ejaculate traits that enhance fertilization success (Birkhead et al. 2009). Sperm is the most morphologically diverse cell among animals, which indicates that selection for sperm size and shape may be strong (Pitnick et al. 2009). The direction and magnitude of selection on these traits can be variable even among closely related species (Simmons and Fitzpatrick 2012). However, measuring the rates of diversification and examining evolutionary patterns of sperm morphology among species can broadly demonstrate how postcopulatory selection has shaped these traits over time.

Though sperm size and number are important for reproductive success in many groups (Radwan 1996; García-González and Simmons 2007; Laskemoen et al. 2010; Boschetto et al. 2011; Bennison et al. 2014), their relative importance for male fitness, and how these traits have evolved in respect to one another, is unclear. Because sperm production is often correlated with testis size (Schärer et al. 2004) and sperm count is

frequently linked with male reproductive success (Parker 1998; Schärer et al. 2004), body-size corrected testis size is often used as an indicator for the strength of postcopulatory selection among species (Simmons and Fitzpatrick 2012). Residual testis size is viewed as both a trait shaped by sperm competition, as well as a rough estimate of the strength of postcopulatory selection among species. Interspecific studies, therefore, frequently test for associations between sperm morphology and residual testis size to understand how postcopulatory selection has shaped sperm morphology (reviewed in Simmons and Fitzpatrick 2012).

Anolis lizards are a model group for studies of sexual selection, as species in this genus exhibit diversity in traits thought to be targeted by sexual selection such as hemipene morphology (Klaczko et al. 2015), body size (Butler et al. 2000; Butler and Losos 2002), and dewlap size and color (Ng et al. 2012). Further, on the islands of the Greater Antilles, species have convergently evolved morphological, ecological, and behavioral specializations to particular microhabitats, resulting in groups of phenotypically similar (but distantly related) species called ecomorphs (Williams 1983; Losos 1994; Losos et al. 1998; Butler et al. 2007; Johnson and Wade 2010). These ecomorph groups also differ in traits such as sexual size dimorphism, which may signal differences in the strength of precopulatory sexual selection they experience (Butler and Losos 2002; Cox et al. 2007). It is unclear, however, whether these ecomorphs also differ in the strength of postcopulatory selection they experience. Many of the species that have high sexual size dimorphism also occur at high densities (Stamps et al. 1997), can store sperm for several months (Licht 1973; Birkhead 1993; Chapter 4), and have high rates of multiple mating

(Calsbeek et al. 2007), likely resulting in strong postcopulatory selection. Previous work among species of snakes and lizards has demonstrated a negative correlation between the strengths of pre- and postcopulatory selection (Chapter 1). We predict that ecomorphs will exhibit a similar pattern, where groups that experience strong precopulatory selection will experience weak postcopulatory selection (Chapter 1). If ecomorphs differ in the strength of postcopulatory selection, this may result in convergent evolution of traits associated with postcopulatory selection (e.g., sperm morphology, testis size, hemipene morphology) among these groups.

Our goal in this study was to understand the evolution and diversification of sperm morphology and testis size among species of *Anolis* lizards. We examined three parts of the sperm cell: the head of the cell, which contains the nuclear material and the acrosome (containing proteolytic enzymes necessary for egg penetration); the midpiece, which contains the mitochondria of the cell; and the tail, which, aids in propulsion and provides some glycolytic activity. We also collected measures of testis size, which when adjusted for variation in body size among species, provides an index of the strength of postcopulatory sexual selection (Birkhead and Møller 1998; Simmons 2001; Simmons and Fitzpatrick 2012). Using these data, we first tested for differences in the rate of evolution of sperm morphology, residual testis size, and male SVL (snout-vent length, a standard measure of body size in lizards). Second, as residual testis size can act as an estimate of the strength of postcopulatory selection among species, we also tested the hypothesis that sperm morphology is correlated with evolutionary shifts in the intensity of postcopulatory sexual selection. By estimating the rates of evolution of each trait, and

testing for evolutionary correlations between sperm morphology and testis size, we can demonstrate how rapidly, and in what direction, these traits have diversified. Finally, we tested the hypothesis that anole ecomorphs differ in traits associated with postcopulatory selection, potentially indicating convergent differences in the strength of postcopulatory selection.

Methods

Specimen and Sperm Collection

We collected sperm samples and testis measurements from 432 males of 28 different *Anolis* species (mean = 15.4 males per species, Figure 1, Table S1) from the Bahamas, the Dominican Republic, Puerto Rico, and Miami, Florida during the peak of their reproductive seasons (May 15 – June 30 in 2013 – 2015). Some species occur at low densities or are rare and protected, which limited our sample size (especially for twig and crown-giant anoles). Because anoles have testicular recrudescence, we collected all individuals when testis size was at its maximum and only sampled individuals who were adults (Gorman 1970). For each species, we collected between 5–20 adult males (mean = 15.4 individuals/species) and recorded snout-vent length (SVL, to the nearest 1mm) and body mass (to the nearest 0.1g) for all males (see Table S1 for phenotypic data as well as locality and collection details). We then collected a sperm sample from each male, which was suspended in 500 µl of phosphate buffered saline with 4% PFA to fix the cells. These fixed cells were transferred to a microscope slide, allowed to dry and were stained with Sperm BlueTM (Microptic SL, Barcelona, Spain). We imaged these cells using an

Olympus Magnafire camera (Olympus America, Melville, NY) at $\times 100$ magnification and measured the length of the sperm head, midpiece, and tail for 15 cells per male using ImageJ (NIH, Bethesda, MD). From these measurements, we calculated individual means, which were then used to calculate species-level means for each sperm trait.

We also measured testis size for these individuals either through surgical laparotomy or by dissection following euthanasia. For surgical laparotomy, each lizard received a 4 μ l subcutaneous injection of 0.25% bupivacain (Auromedics, Dayton, NJ) as a local anesthetic and analgesic at the site of incision. We then cold-immobilized each animal by placing it at -20°F for 5–10 minutes (depending on the size of the species) and placed them on a slightly thawed ice pack during surgery. To measure the testis, we made a five mm ventral incision in the abdomen lateral to the midline, located and exposed the right testis using forceps. We then measured the length and width of the testis using calipers (nearest 0.1 mm) while the testis was still in the body cavity. We closed the incision using Nexaband surgical glue (Veterinary Products Laboratories, Phoenix, AZ). All lizards were held overnight for observation, and were returned to the wild at their location of capture on the following day. We converted testis length and width into volume using the formula for the volume of an ellipsoid:

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

where a is the radius of the width of the testis and b is the radius of its length. We used the residuals from a linear regression of natural log-transformed mean testis volume and mean male SVL to calculate residual testis size as a single value for each species. In order to calculate a species-level error in residual testis size, we also calculated residual

testis size for each individual in the same way, and then used these values to calculate species-level error and covariances between residual testis size and all other traits for each species.

Phylogenetic signal

All of our statistical testing and modeling was conducted in the R environment v.3.3.2 (R Development Core Team 2016). We used the Dactyloidae clade of the squamate phylogeny from Pyron and Burbrink (2014), which provides a fully resolved topology and branch lengths for all *Anolis* species in our dataset. We first trimmed the original phylogeny to include only species in our dataset in *APE* (Paradis et al. 2004). We then used this trimmed phylogeny and dataset to test for phylogenetic signal in residual testis size, SVL, and sperm head, midpiece, and tail length. We calculated Pagel's λ in *phytools* (Revell 2011), and Blomberg's K in *Picante* (Kembel et al. 2010), which function as complementary methods of assessing phylogenetic signal. Pagel's λ generally ranges from 0–1 (upper end unbounded), with 0 corresponding to complete phylogenetic independence and 1 corresponding to trait evolution under a Brownian motion model of character evolution (Pagel 1999). Values of $\lambda > 1$ can indicate that sister species are more similar than predicted under Brownian motion, or that the tips of the tree exhibit less variation than expected given the variation at the roots of the tree. Blomberg's K is calculated by comparing the observed trait variance to the expected trait variance calculated under Brownian motion (Blomberg et al. 2003), with P -values indicating significant phylogenetic signal (i.e., $K > 0$). If $K < 1$, species are less phenotypically

similar than expected under Brownian motion, given their phylogenetic relationships, and if $K > 1$, species are more similar than expected under a Brownian motion model of evolution.

Models of character evolution

We first compared the fit of three different models of character evolution to our traits using the *fitContinuous* function in the package *geiger* (Harmon et al. 2008). We fit models of Brownian motion, Ornstein-Uhlenbeck, and Early-burst character evolution to each of the traits in our analysis, and found that a Brownian motion model of evolution was the best fit for all of our traits using AICc model comparison (Table 1). For all of our traits, the Early-burst model of character evolution did not converge using the default bounds, so we specified the maximum bounds to be set to 0 in the function *fitContinuous* to allow model convergence. For most traits, Ornstein-Uhlenbeck and Early-burst models did not have a significantly better fit models using Brownian motion ($\Delta\text{AICc} > 2$). However, the Akaike weights were always higher for models under Brownian motion, so we used this model of character evolution for all subsequent analyses.

Rates of evolution

To compare the rates of evolution of traits in this study, we used a likelihood approach developed by Adams (2013) that allows the Brownian rate parameter (σ^2) to be estimated for several traits simultaneously in either a model where traits have independent evolution or in a model where the evolution of traits covaries. These models

also allow for the incorporation of within-species trait errors and covariances. One of the assumptions of the method is that traits evolve under a model of Brownian motion, which we confirmed before running these tests. This method determines the rates of each trait independently, and compares these rates to a common model where all traits are constrained to have a common rate of evolution. Likelihood ratio tests are used to test for significance differences between the fit of the observed-rate and common-rate models. Because dimensionality can significantly impact trait variances, we natural log-transformed all length traits (and calculated error and covariances from these transformed values) prior to analyses. We then estimated the rates of evolution of residual testis size, male SVL, sperm head, midpiece, and tail length. All models that did not include evolutionary covariance (i.e., the rates of evolution of the covariance between traits was not estimated in both common and observed matrices) converged under the L-BFGS-B optimization function provided in Adams' (2013) code. We ran models both with and without estimates of within-species error and covariance, and though the rate estimates did not vary from model to model, we found the best fit and model convergence occurred when error and covariance were specified for each species. We then did post hoc tests where we followed this same model-fitting procedure, but only used two traits at a time to fit all pairwise combinations of the traits.

Tests for Correlated Evolution

To test for associations between sperm morphology and residual testis size (which may reflect a history of strong postcopulatory selection), we used phylogenetic

generalized least squares (PGLS) regression under a Brownian motion model of character evolution in *APE* (Paradis et al. 2004), where testis size was the response variable and sperm morphology and male SVL (covariate to account for body size) were independent variables. We also tested for evolutionary correlations between all pairwise combinations of sperm traits using PGLS modeled under Brownian motion to determine the most biologically relevant evolutionary rate models (i.e., models including evolutionary covariance) when testing for differences in evolutionary rates between different parts of the cell. Finally, we conducted a phylogenetic ANOVA to test for differences in residual testis size and sperm morphology among ecomorphs (i.e. trunk, trunk-crown, trunk-ground, crown-giant, grass-bush, and twig anoles) using the function *aov.phylo* in *geiger* (Harmon et al. 2008) and *phylANOVA* (Revell 2011) to run post hoc comparisons across groups.

Results

Phylogenetic Signal

All traits in our study exhibited strong phylogenetic signal, as assessed by Blomberg's K and Pagel's λ , with the exception of male SVL (Table 2). Several traits exhibited $\lambda > 1$, likely because several closely related taxa have smaller differences in their trait values than expected under Brownian motion (Figure S1).

Rates of evolution

Using likelihood ratio tests, we found significant differences in the evolutionary rates among all of the traits we examined when using a model that assumes independent trait evolution, and specifies intraspecific trait covariance and measurement error (full model: $LRT = 68.71$, $P < 0.0001$, $AIC_{\text{observed}} = -72.08$, $AIC_{\text{common}} = -11.37$, Figure 2) (post hoc tests: Table S2). This result was robust across all of the models tested.

Although the three measures of sperm morphology covaried, only models that excluded evolutionary covariance converged properly, likely because of low covariance among the entire set of traits in the model. These models show that residual testis size had the highest rates of evolution, followed by SVL, sperm midpiece, sperm tail length, and then sperm head length (Figure 2, Table S2).

Tests for Correlated Evolution

We also tested for correlations between each of our traits. We found no significant relationships between residual testis size and any measure of sperm morphology (i.e., head, midpiece or tail) using PGLS modeled under a Brownian motion model of character evolution (Figure 3, Table 3). We found nearly identical results when we used an Ornstein-Uhlenbeck model of character evolution (Table S3). Within sperm morphology, we found significant positive correlations between sperm head and tail length ($r = 0.45$, $t = 2.67$, $P = 0.013$) and head and midpiece length ($r = 0.64$, $t = 4.472$, $P = 0.0001$), but not between sperm tail and midpiece length ($r = -0.02$, $t = -0.121$, $P = 0.905$).

We also found significant differences among ecomorphs in residual testis size ($F_{6,21} = 9.58$, $P = 0.001$) and marginal differences in sperm head length ($F_{6,21} = 3.514$, $P = 0.051$), but not in sperm midpiece length ($F_{6,21} = 2.25$, $P = 0.153$) or tail length ($F_{6,21} = 2.102$, $P = 0.183$). In particular, we found that three ecomorphs: trunk-crown, trunk-ground and twig ecomorphs have significantly larger residual testis size than grass-bush and trunk anoles (Figure 4).

Discussion

In a group of 28 *Anolis* lizard species, we found that the sperm midpiece evolves more quickly than the sperm head or tail, and that testis size evolves more than ten times faster than sperm morphology in this group. These rates of evolution demonstrate that testis size may be more evolutionarily labile than sperm morphology and/or that postcopulatory selection may be targeting sperm number rather than sperm size. Reinforcing this idea, the variation in testis size among species (acting as an estimate of the strength of postcopulatory selection) was unrelated to variation in sperm morphology, suggesting postcopulatory selection on sperm morphology may be weak. We also showed that *Anolis* ecomorphs differ significantly in testis size and marginally sperm head length, which indicating that the strength of postcopulatory selection may vary among ecomorphs.

Rates of evolution of sperm morphology

Theory and empirical data show that sexually-selected traits tend have higher rates of evolution than other morphological traits, in part due to persistently strong selection (Arnqvist 1998; Gonzalez-Voyer and Kolm 2011; Seddon et al. 2013). The sperm midpiece contains mitochondria, and is associated with body condition (Bonanno and Schulte-Hostedde 2009; Chapter 4), sperm velocity (Firman and Simmons 2010; Blengini et al. 2014), and sperm motility (Ruiz-Pesini et al. 1998; Anderson and Dixon 2002), which are frequently associated with male reproductive success (Simmons and Fitzpatrick 2012). For these reasons, it is perhaps not surprising that the midpiece has the highest rate of evolution among sperm morphological traits. Only recently have researchers quantified the rates of evolution of sperm morphology. These studies in passerine birds show that the sperm midpiece evolves at a faster rate than the sperm tail and head (Rowe et al. 2015; Supriya et al. 2016). In fact, both studies found an almost identical pattern of evolutionary rates reported in this study. The consistency in these rates of evolution suggests that some parts of the cell – in this case, the sperm midpiece – may be more evolutionarily labile than others, and/or may experience stronger selection, which would allow for faster diversification of this trait within clades.

In contrast to the quickly evolving midpiece, the sperm head, which contains both the acrosome and the nuclear material, exhibited the slowest rate of evolution, which may be due to functional constraints. For example, longer filiform heads increase drag on the sperm cell, which then requires a larger flagellum to propel them at an equal speed (Humphries et al. 2008), a prediction that has been supported by tests in humans, birds, and fish (Lüpold et al. 2009; Gillies et al. 2009; Helfenstein et al. 2010; Simpson et al.

2013). Moreover, genome size may prevent the sperm head from attaining a smaller size, though few have tested this hypothesis, and results are somewhat mixed (Alvarez-Fuster et al. 1991; Gage 1998; Gallardo et al. 2002). While we do not know the relationship between sperm morphology and function in anoles, the patterns of evolution and sperm function exhibited in birds lead us to hypothesize that sperm head size may be constrained because of functional limitations resulting in stabilizing selection and therefore a lower rate of evolution (Rowe et al. 2015).

We also found significant differences in the rate of evolution among sperm morphology, residual testis size and body size. Our estimates of the rates of evolution of these traits show that residual testis size evolves between 10–30 times faster than the components of the sperm cell, suggesting that sperm count may be a better predictor of fertilization success in this group than sperm morphology. We also found that residual testis size evolves significantly faster than body size. Body size is used in this study as a point of comparison rather than a test of a hypothesis, as it experiences a variety of constraints and selective pressures (both natural and sexual). Tests for differences in the rates of evolution between testis size and body size have only been made in a handful of groups, but in all cases, the opposite pattern was found. In *Onthophagus* beetles, which have strong male-male competition, body size and horn length evolve faster than testis size and total sperm length (Simmons and Fitzpatrick 2016). In pinnipeds, body size evolves significantly faster in harem forming species than non-harem forming species while testis size exhibited no significant difference between these groups, suggesting that male-male competition increases the rate of evolution of body size but not testis size

(Fitzpatrick et al. 2012). The difference in the rates of evolution of body size and testis size between these groups may be reflective of variation in the mating system between dung beetles, pinnipeds and anoles (as well as differences in natural selection). Recent models and empirical studies demonstrate that species which are unable to monopolize females should invest in traits associated with precopulatory selection in order to acquire mates (Schantz et al. 1994; Badyaev et al. 1998; Jacob et al. 2009; Cotton et al. 2009; Parker et al. 2013; Lüpold et al. 2014; Simmons and Fitzpatrick 2016). This could potentially lead to higher rates of evolution of traits associated with precopulatory selection. These models also predicted (Parker et al. 2013), and empirical studies demonstrated (Lüpold et al. 2014), that as the rate of female monopolization within a group increases, there is an increasing evolutionary trade-off between traits associated with pre- and postcopulatory selection, suggesting that species will allocate to both pre- and postcopulatory sexual traits. Anoles are territorial, but the rates of monopolization among species are unknown. The difference in the rate of evolution between residual testis size and body size may indicate that the rates of monopolization among anoles is highly variable, creating higher variance in the strength of postcopulatory selection and consequently, higher rates of evolution of relative testis size. Indeed, another study has demonstrated that anole hemipenis morphology has an accelerated rate of evolution relative to traits that are associated with visual signaling or ecology (dewlap and limb length), implying that postcopulatory selection for genital morphology is strong and can lead to high diversification (Klaczko et al. 2015).

We have interpreted the differences in the rate of evolution between traits in this study as differences in the strength of selection, however, there are several other causes that should be considered. First, difference in trait heritabilities could cause the rates of evolution to vary (Reznick 1997), so that traits with low heritabilities would have lower rates of evolution than traits with high heritabilities. Most estimates for the heritability of sperm length and testis size among species are fairly high (Simmons and Moore 2008), however, there are no existing estimates of heritability of these traits in reptiles. Second, the type of selection acting on each trait could dramatically change the variance in trait value over a phylogeny. We predict that testis size should experience strong directional selection for increased size in species with strong postcopulatory selection, however, the pattern is less clear for sperm morphology. We mentioned above that sperm head size may be constrained in size, and experience stabilizing selection. This may be true, to some extent, for the rest of the cell. As sperm experiences selection in the female reproductive tract, the reproductive tract itself may create stabilizing selection for a specific sperm size. There is evidence for this in birds where species with strong postcopulatory selection have reduced variation in sperm morphology (Calhim et al. 2007; Immler et al. 2008). Both the heritability and the type of selection could change the evolutionary lability of each trait we examine, and we can not exclude the possibility that the differences in the rates of evolution we estimated are due to differences in heritability and/or the type of selection.

Selection on sperm among taxonomic groups

Comparative analyses testing for an evolutionary association between residual testis size and sperm morphology have spanned all major orders of animals. However, there are still gaps in our knowledge about how the sperm cell evolves among species, particularly in cases where closely related lineages have inconsistent patterns in the evolution of sperm morphology (Immler and Birkhead 2007; Simmons and Fitzpatrick 2012). These cases offer an opportunity to examine the differences in the mating systems between groups that may lead to the disparities in sperm evolution. First, selection on sperm morphology may depend on the length of sperm storage (Immler et al. 2007) or coevolution with female storage organs (Gomendio and Roldan 1993; Presgraves et al. 1999; Pitnick et al. 2003; Beese et al. 2006; Snook et al. 2010; Higginson et al. 2012). Second, some species may experience an energetic trade-off between sperm size and number, and investment into one or the other may result in different patterns of evolution of sperm morphology (Immler and Birkhead 2007; Parker et al. 2010; Immler et al. 2011; Simmons and Fitzpatrick 2012; Lüpold and Fitzpatrick 2015).

There is relatively little known about sperm morphology in reptiles, and snakes are the only other major lineage that has been examined in a comparative context. Though snakes are phylogenetically nested within lizards, the evolution of sperm morphology in this group is fairly different than within anoles. Among species of snakes, sperm head, midpiece and tail length are all positively correlated with residual testis size (phylogenetically controlled R^2 between 0.17 - 0.20) (Tourmente et al. 2009). Snakes and *Anolis* lizards differ significantly in their mating systems and their reproductive biology, which may affect how postcopulatory selection acts in these groups. While snakes have

both oviparous and viviparous species, tend to breed during an abbreviated reproductive season, and have single clutches with many eggs, anoles reproduce continuously from the late spring through the early summer with females laying multiple clutches of a single egg. These differences in their reproduction may cause variation in how selection acts on ejaculates. Several species of snakes can store sperm for greater than five years [*Acrochordus javanicus* (7 years), *Letodeira polysticta* (6 years), *Agkistrodon piscivorus* (5 years) (Birkhead 1993)], and may experience selection to produce high-quality sperm cells that can survive in the female reproductive tract for several reproductive seasons (Friesen et al. 2014a,b). Anoles, on the other hand, reproduce and copulate frequently over an extended breeding season, which may result in selection that acts more strongly on sperm production than sperm morphology or quality.

The differences in the mating systems between snakes and anoles may lead to variation in the length of sperm storage, which may result in differences in selection for sperm morphology. The significant differences in the mating systems between these groups implies that while anoles may experience selection for higher sperm numbers, snakes may rely on sperm storage and sperm longevity to a much higher degree. These different selective pressures may explain the difference in midpiece length between these groups. While total sperm length is similar between snakes and anoles (snakes range: 85.75 – 159.45 μm , lizards 84.65 – 111.01 μm), the midpiece length in snakes is on average 59.71% of total sperm length (range: 35.88 – 111.53 μm , Tourmente et al. 2009), and is on average 3.34% of total sperm length (range: 2.46 – 4.59 μm) in anoles. The midpiece contains the mitochondria, as well as the beginning of the axoneme, outer dense

fibers and fibrous sheath, which contribute to the cells metabolism through glycolysis and ATP production (Narisawa et al. 2002; Miki et al. 2004; Turner 2005; Tourmente et al. 2009). Sperm length is correlated with sperm longevity in birds (Immler et al. 2007; Helfenstein et al. 2008; 2010), and it may be that this significant increase in midpiece size enables sperm to be stored in the female reproductive tracts of snakes longer than in anoles.

Sexual selection in Anolis ecomorphs

The *Anolis* ecomorph groups have many differences in their ecological (e.g., density, habitat specialization), morphological (e.g., body size, tail length, limb length, sexual size dimorphism, lamellae), and behavioral traits (e.g., movement rate, display) (summarized in Losos 2009), some of which can be attributed to the type and magnitude of sexual selection they experience. Across species of lizards (and within only anoles) there is a negative correlation between traits associated with precopulatory selection (sexual size dimorphism, SSD) and postcopulatory selection (residual testis size) (Chapter 2), which may suggest that ecomorphs with high SSD would have low residual testis size. Though there are significant differences in both SSD (Butler et al. 2000; Butler and Losos 2002) and residual testis size in this group, there does not seem to be a predictable pattern between these traits. The ecomorphs with the highest SSD do not have the lowest residual testis size, and vice versa. Ecomorphs may experience convergence in traits associated with pre- and postcopulatory selection, but how those differences arise is unknown. It may be that variation in their mating systems or density alter the relative

importance of each episode of selection, which may lead to the differences in SSD, testis size and sperm head length we see between groups. Our study has demonstrated how sperm traits evolve in *Anolis* lizards, but it is still unclear how these traits impact male reproductive success. Measurement of multivariate selection in a wild population of anoles will be essential to describe the relative importance of these postcopulatory traits for male reproductive success.

Acknowledgments

We thank B. Kircher, M. Oberndorf, J. Stercula, J. Murray, B. Ivanov, M. Jaramillo, M. Webber, A. Zeb, F. Deckard, A. Hanninen, C. Gilman and M. Landestoy for assistance with field collection, and V. Motamedi, L. Zemanian, and E. Thompson for assistance measuring sperm cells. We also thank John Fitzpatrick, Dean Adams, and Matt Pennell for assistance with data analysis and implementing R packages. This study was conducted under permits from Estado Libre Asociado de Puerto Rico, Bahamas Ministry of Agriculture, Bahamas Environment Science and Technology Commission, Ministerio de Medio Ambiente y Recursos Naturales of the Dominican Republic, the Miami-Dade County Parks and Recreation, and approved under the University of Virginia IACUC protocol 3896. This work was supported by startup funding from UVA (to RMC), the National Science Foundation (IOS-1257021 to MAJ), a Doctoral Dissertation Improvement Grant from the National Science Foundation (DEB-1501680 to AFK and RMC), an E.E. Williams Research Grant from the Herpetologist's League (to AFK), and

a Theodore Roosevelt Memorial Grant from the American Museum of Natural History
(to AFK).

References

- Adams, D. C. 2013. Comparing evolutionary rates for different phenotypic traits on a phylogeny using likelihood. *Syst. Biol.* 62:181–192.
- Alvarez-Fuster, A., C. Juan, and E. Petitpierre. 1991. Genome size in *Tribolium* flour-beetles: inter-and intraspecific variation. *Genet. Res.* 58:1–5. Cambridge Univ. Press, Cambridge, UK.
- Anderson, M. J., and A. F. Dixson. 2002. Sperm competition: motility and the midpiece in primates. *Nature* 416:496.
- Andersson, M. B. 1994. Sexual selection. Princeton Univ. Press, Princeton, NJ.
- Arnqvist, G. 1998. Comparative evidence for the evolution of genitalia by sexual selection. *Nature* 393:784–785.
- Badyaev, A. V., W. J. Etges, J. D. Faust, and T. E. Martin. 1998. Fitness correlates of spur length and spur asymmetry in male wild turkeys. *J. Anim. Ecol.* 67:845–852.
- Beese, K., K. Beier, and B. Baur. 2006. Coevolution of male and female reproductive traits in a simultaneously hermaphroditic land snail. *J. Evol. Biol.* 19:410–418.
- Bennison, C., N. Hemmings, J. Slate, and T. Birkhead. 2014. Long sperm fertilize more eggs in a bird. *Proc. Biol. Sci. Lond. B.* 282:20141897.
- Birkhead, T. R. 1993. Sexual selection and the temporal separation of reproductive events: sperm storage data from reptiles, birds and mammals. *Biol. J. Linn. Soc.* 50:295–311.
- Birkhead, T. R., and A. P. Møller. 1998. Sperm competition and sexual selection. Academic Press, San Diego, CA.

- Birkhead, T. R., D. J. Hosken, and S. S. Pitnick. 2009. *Sperm biology: an evolutionary perspective*. Academic Press, San Diego, CA.
- Blengini, C. S., N. Sergio, C. Gabriela, L. C. Giojalas, and C. Margarita. 2014. Variability in sperm form and function in the context of sperm competition risk in two *Tupinambis* lizards. *Ecol. Evol.* 4:4080–4092.
- Blomberg, S. P., T. Garland, and A. R. Ives. 2003. Testing for phylogenetic signal in comparative data: behavioral traits are more labile. *Evolution* 57:717.
- Bonanno, V. L., and A. I. Schulte-Hostedde. 2009. Sperm competition and ejaculate investment in red squirrels (*Tamiasciurus hudsonicus*). *Behav. Ecol. Sociobiol.* 63:835–846.
- Boschetto, C., C. Gasparini, and A. Pilastro. 2011. Sperm number and velocity affect sperm competition success in the guppy (*Poecilia reticulata*). *Behav. Ecol. Sociobiol.* 65:813–821. Springer.
- Butler, M. A., T. W. Schoener, and J. B. Losos. 2000. The relationship between sexual size dimorphism and habitat use in Greater Antillean *Anolis* lizards. *Evolution* 54:259–272.
- Butler, M. A., and J. B. Losos. 2002. Multivariate sexual dimorphism, sexual selection, and adaptation in Greater Antillean *Anolis* lizards. *Ecol. Monogr.* 72:541–559.
- Butler, M. A., S. A. Sawyer, and J. B. Losos. 2007. Sexual dimorphism and adaptive radiation in *Anolis* lizards. *Nature* 447:202–205.
- Calhim, S., S. Immler, and T. R. Birkhead. 2007. Postcopulatory sexual selection is associated with reduced variation in sperm morphology. *PLoS ONE* 2:e413.

- 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. *Evol. Ecol. Res.* 9:495–503.
- Cotton, S., J. Small, R. Hashim, and A. Pomiankowski. 2009. Eyespan reflects reproductive quality in wild stalk-eyed flies. *Evol. Biol.* 24:83–95.
- Cox, R. M., M. A. Butler, and H. B. John-Alder. 2007. The evolution of sexual size dimorphism in reptiles. Pp. 38–49 *in* D. J. Fairbairn, W. U. Blanckenhorn, and T. Székely, eds. *Sex, Size and Gender Roles Evolutionary Studies of Sexual Size Dimorphism*. Oxford Univ. Press, Oxford, UK.
- Firman, R. C., and L. W. Simmons. 2010. Sperm midpiece length predicts sperm swimming velocity in house mice. *Biol Lett* 6:513–516.
- 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.
- Friedman, N. R., and V. Remeš. 2015. Rapid evolution of elaborate male coloration is driven by visual system in Australian fairy-wrens (Maluridae). *J. Evol. Biol.* 28:2125–2135.
- Friesen, C. R., A. R. Kerns, and R. T. Mason. 2014a. Factors influencing paternity in multiply mated female red-sided garter snakes and the persistent use of sperm stored over winter. *Behav. Ecol. Sociobiol.* 68:1419–1430.
- Friesen, C. R., R. T. Mason, S. J. Arnold, and S. Estes. 2014b. Patterns of sperm use in two populations of Red-sided Garter Snake (*Thamnophis sirtalis parietalis*) with

- long-term female sperm storage. *Can. J. Zool.* 92:33–40.
- Gage, M. J. 1998. Mammalian sperm morphometry. *Proc. Biol. Sci. Lond. B.* 265:97–103.
- Gallardo, M. H., F. C. Mondaca, and R. A. Ojeda. 2002. Morphological diversity in the sperms of caviomorph rodents. *J. Neotrop. Mammal* 9:159–170.
- García-González, F., and L. W. Simmons. 2007. Shorter sperm confer higher competitive fertilization success. *Evolution* 61:816–824.
- Gillies, E. A., R. M. Cannon, R. B. Green, and A. A. Pacey. 2009. Hydrodynamic propulsion of human sperm. *J. Fluid Mech.* 625:445.
- Gomendio, M., and E. R. Roldan. 1993. Coevolution between male ejaculates and female reproductive biology in eutherian mammals. *Proc. Biol. Sci. Lond. B.* 252:7–12.
- Gonzalez-Voyer, A., and N. Kolm. 2011. Rates of phenotypic evolution of ecological characters and sexual traits during the Tanganyikan cichlid adaptive radiation. *J. Evol. Biol.* 24:2378–2388.
- Harmon, L. J., J. T. Weir, C. D. Brock, R. E. Glor, and W. Challenger. 2008. GEIGER: investigating evolutionary radiations. *Bioinformatics* 24:129–131.
- Helfenstein, F., M. Podelvin, and H. Richner. 2010. Sperm morphology, swimming velocity, and longevity in the house sparrow *Passer domesticus*. *Behav. Ecol. Sociobiol.* 64:557–565.
- Helfenstein, F., T. Szép, Z. Nagy, B. Kempenaers, and R. H. Wagner. 2008. Between-male variation in sperm size, velocity and longevity in sand martins *Riparia riparia*. *J. Avian Biol.* 39:647–652.
- Higginson, D. M., K. B. Miller, K. A. Segraves, and S. Pitnick. 2012. Female

- reproductive tract form drives the evolution of complex sperm morphology. *Proc. Natl. Acad. Sci. U.S.A.* 109:4538–4543.
- Immler, S. S., S. S. Calhim, and T. R. T. Birkhead. 2008. Increased postcopulatory sexual selection reduces the intramale variation in sperm design. *Evolution* 62:1538–1543.
- Immler, S., and T. R. Birkhead. 2007. Sperm competition and sperm midpiece size: no consistent pattern in passerine birds. *Proc. R. Soc. Lond. B.* 274:561–568.
- Immler, S., M. Saint-Jalme, L. Lesobre, G. Sorci, Y. Roman, and T. R. Birkhead. 2007. The evolution of sperm morphometry in pheasants. *J. Evol. Biol.* 20:1008–1014.
- Immler, S., S. Pitnick, G. A. Parker, K. L. Durrant, S. Lüpold, S. Calhim, and T. R. Birkhead. 2011. Resolving variation in the reproductive tradeoff between sperm size and number. *Proc. Natl. Acad. Sci. U.S.A.* 108:5325–5330.
- Jacob, A., G. Evanno, E. Renai, R. Sermier, and C. Wedekind. 2009. Male body size and breeding tubercles are both linked to intrasexual dominance and reproductive success in the minnow. *Anim. Behav.* 77:823–829.
- Johnson, M. A., and J. Wade. 2010. Behavioural display systems across nine *Anolis* lizard species: sexual dimorphisms in structure and function. *Proc. Biol. Sci. Lond. B.* 277:1711–1719.
- Kahrl, A. F., and R. M. Cox. 2015. Diet affects ejaculate traits in a lizard with condition-dependent fertilization success. *Behav. Ecol.* 26:1502–1511.
- Kahrl, A. F., C. L. Cox, and R. M. Cox. 2016. Correlated evolution between targets of pre- and postcopulatory sexual selection across squamate reptiles. *Ecol. Evol.* 6:6452–6459.

- Kembel, S. W., P. D. Cowan, M. R. Helmus, W. K. Cornwell, H. Morlon, D. D. Ackerly, S. P. Blomberg, and C. O. Webb. 2010. Picante: R tools for integrating phylogenies and ecology. *Bioinformatics* 26:1463–1464.
- Klaczko, J., T. Ingram, and J. Losos. 2015. Genitals evolve faster than other traits in *Anolis* lizards. *J. Zool.* 295:44–48.
- Laskemoen, T., O. Kleven, F. Fossøy, R. J. Robertson, G. Rudolfson, and J. T. Lifjeld. 2010. Sperm quantity and quality effects on fertilization success in a highly promiscuous passerine, the tree swallow *Tachycineta bicolor*. *Behav. Ecol. Sociobiol.* 64:1473–1483.
- Licht, P., and G. C. Gorman. 1970. Reproductive and fat cycles in Caribbean *Anolis* lizards. Univ. California Press, Berkely, CA.
- Licht P. 1973. Influence of temperature and photoperiod on the annual ovarian cycle in the lizard, *Anolis carolinensis*. *Copeia* 1973: 465-472.
- Losos, J. B. 1994. Integrative approaches to evolutionary ecology: *Anolis* lizards as model systems. *Annu. Rev. Ecol. Syst.* 25:467–493.
- Lüpold, S., and J. L. Fitzpatrick. 2015. Sperm number trumps sperm size in mammalian ejaculate evolution. *Proc. Biol. Sci. Lond. B.* 282:20152122.
- Lüpold, S., J. L. Tomkins, L. W. Simmons, and J. L. Fitzpatrick. 2014. Female monopolization mediates the relationship between pre- and postcopulatory sexual traits. *Nat. Comm.* 5:3184.
- Lüpold, S., S. Calhim, S. Immler, and T. R. Birkhead. 2009. Sperm morphology and sperm velocity in passerine birds. *Proc. R. Soc. Lond. B.* 276:1175–1181.

- Miki, K., W. Qu, E. H. Goulding, W. D. Willis, D. O. Bunch, L. F. Strader, S. D. Perreault, E. M. Eddy, and D. A. O'Brien. 2004. Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proc. Natl. Acad. Sci. U.S.A.* 101:16501–16506.
- Narisawa, S., N. B. Hecht, E. Goldberg, K. M. Boatright, J. C. Reed, and J. L. Millán. 2002. Testis-specific cytochrome c-null mice produce functional sperm but undergo early testicular atrophy. *Mol. Cell. Biol.* 22:5554–5562.
- Ng, J., E. L. Landeen, R. M. Logsdon, and R. E. Glor. 2012. Correlation between *Anolis* lizard dewlap phenotype and environmental variation indicates adaptive divergence of a signal important to sexual selection and species recognition. *Evolution* 67:573–582.
- Pagel, M. 1999. Inferring the historical patterns of biological evolution. *Nature* 401:877–884.
- Paradis, E., J. Claude, and K. Strimmer. 2004. APE: Analyses of Phylogenetics and Evolution in R language. *Bioinformatics* 20:289–290.
- Parker, G. A. 1998. Sperm competition and the evolution of ejaculates: towards a theory base. Pp. 3–49 in T. R. Birkhead and A. P. Møller, eds. *Sperm competition and sexual selection*. Academic Press, San Diego, CA.
- 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.
- Parker, G. A., S. Immler, S. Pitnick, and T. R. Birkhead. 2010. Sperm competition games: Sperm size (mass) and number under raffle and displacement, and the

- evolution of P2. *J. Theor. Biol.* 264:1003–1023.
- Pitnick, S., D. J. Hosken, and T. R. Birkhead. 2009. Sperm morphological diversity. Pp. 69–149 *in* T. R. Birkhead, D. J. Hosken, and S. Pitnick, eds. *Sperm Biology: An Evolutionary Perspective*. Academic Press, San Diego, CA.
- Pitnick, S., G. T. Miller, K. Schneider, and T. A. Markow. 2003. Ejaculate-female coevolution in *Drosophila mojavensis*. *Proc. Biol. Sci. Lond. B.* 270:1507–1512.
- Presgraves, D. C., R. H. Baker, and G. S. Wilkinson. 1999. Coevolution of sperm and female reproductive tract morphology in stalk-eyed flies. *Proc. Biol. Sci. Lond. B.* 266:1041–1047.
- Pyron, R. A., and F. T. Burbrink. 2014. Early origin of viviparity and multiple reversions to oviparity in squamate reptiles. *Ecol. Lett.* 17:13–21.
- Radwan, J. 1996. Intraspecific variation in sperm competition success in the bulb mite: a role for sperm size. *Proc. Biol. Sci. Lond. B.* 263:855–859. The Royal Society.
- Revell, L. J. 2011. phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.* 3:217–223.
- Reznick, D. N. 1997. Evaluation of the rate of evolution in natural populations of guppies (*Poecilia reticulata*). *Science* 275:1934–1937.
- Ruiz-Pesini, E., C. Diez, A. C. Lapeña, A. Pérez-Martos, J. Montoya, E. Alvarez, J. Arenas, and M. J. López-Pérez. 1998. Correlation of sperm motility with mitochondrial enzymatic activities. *Clin. Chem.* 44:1616–1620.
- Schantz, von, T., M. Grahn, and G. Goransson. 1994. Intersexual selection and reproductive success in the pheasant *Phasianus colchicus*. *Am. Nat.* 144:510–527.

- 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.* Behav. Ecol. Sociobiol. 56:420–425.
- Seddon, N., C. A. Botero, J. A. Tobias, P. O. Dunn, H. E. A. MacGregor, D. R. Rubenstein, J. A. C. Uy, J. T. Weir, L. A. Whittingham, and R. J. Safran. 2013. Sexual selection accelerates signal evolution during speciation in birds. Proc. Biol. Sci. Lond. B. 280:20131065–20131065.
- Simmons, L. W. 2001. Sperm competition and its evolutionary consequences in the insects. Princeton Univ. Press, Princeton, NJ.
- Simmons, L. W., and A. J. Moore. 2008. Evolutionary quantitative genetics of sperm. Pp. 405–434 in T. Birkhead, D. J. Hosken, and S. Pitnick, eds. Sperm biology: an evolutionary perspective. Academic press, San Diego, CA.
- Simmons, L. W., and J. L. Fitzpatrick. 2016. Sperm competition and the coevolution of pre- and postcopulatory traits: Weapons evolve faster than testes among onthophagine dung beetles. Evolution 70:998–1008.
- Simmons, L. W., and J. L. Fitzpatrick. 2012. Sperm wars and the evolution of male fertility. Reproduction 144:519–534.
- Simpson, J. L., S. Humphries, J. P. Evans, L. W. Simmons, and J. L. Fitzpatrick. 2013. Relationships between sperm length and speed differ among three internally and three externally fertilizing species. Evolution 68:92–104.
- Snook, R. R., L. D. Bacigalupe, and A. J. Moore. 2010. The quantitative genetics and

- coevolution of male and female reproductive traits. *Evolution* 64:1926–1934.
- Stamps, J. A., J. B. Losos, and R. M. Andrews. 1997. A comparative study of population density and sexual size dimorphism in lizards. *Am. Nat.* 149:64–90.
- Tourmente, M., M. Gomendio, E. R. S. Roldan, L. C. Giojalas, and M. Chiaraviglio. 2009. Sperm competition and reproductive mode influence sperm dimensions and structure among snakes. *Evolution* 63:2513–2524.
- Turner, R. M. 2005. Moving to the beat: a review of mammalian sperm motility regulation. *Reprod. Fertil. Dev.* 18:25–38.
- West-Eberhard, M. J. 1983. Sexual selection, social competition, and speciation. *Q. Rev. Biol.* 58:155–183.

Figures and Tables

Table 1. Summary of model fit and parameters for Brownian motion, Ornstein-Uhlenbeck, and Early-burst models of character evolution for each trait presented in this study. Values of σ^2 (Brownian rate parameter), α (selection strength parameter), and a (rate of evolutionary change parameter) are presented for Brownian motion, Ornstein-Uhlenbeck, and Early-burst models, respectively. AICc, Δ AICc and ω_i (Akaike weights) indicate that Brownian motion is the preferred model of evolution for each trait.

	Brownian motion					Ornstein-Uhlenbeck					Early-burst				
	σ^2	lnL	AICc	Δ_i	ω_i	α	lnL	AICc	Δ_i	ω_i	a	lnL	AICc	Δ_i	ω_i
Body size (SVL)	0.0029	-7.61	19.69	0	0.60	0.016	-7.47	21.94	2.24	0.19	0.032	-6.73	21.94	2.24	0.19
Residual testis size	0.0059	-17.34	39.17	0	0.51	0.027	-16.82	40.65	1.47	0.24	0.053	-16.82	40.65	1.47	0.24
Sperm head length	1.71 e-4	32.42	-6.04	0	0.66	~0	32.42	-5.78	2.52	0.18	0.006	32.31	-57.43	2.93	0.15
Sperm midpiece length	4.45 e-4	19.04	-33.59	0	0.65	~0	19.04	-31.07	2.52	0.18	0.006	18.92	-30.85	2.74	0.16
Sperm tail length	2.55 e-4	26.81	-49.15	0	0.44	0.023	27.59	-48.19	0.95	0.27	0.046	27.59	-48.19	0.95	0.27

Table 2. Phylogenetic signal (Blomberg's K and Pagel's λ) for each trait examined (all ln-transformed). Significance testing for Blomberg's K indicates a difference from $K = 0$, which indicates significant phylogenetic signal. For Pagel's λ , the maximum likelihood estimate of λ was tested against models where $\lambda = 0$ using log likelihood ratio tests.

Trait	Blomberg's K	P	Pagel's λ	P
Residual testis size	0.885	0.036	1.030	0.167
Body size (SVL)	0.829	0.125	0.670	0.651
Sperm head length	1.178	0.003	1.315	<0.001
Sperm midpiece length	1.041	0.005	1.346	<0.001
Sperm tail length	0.880	0.039	0.613	0.072

Table 3. Summary of results from phylogenetic generalized least squares regressions of sperm morphology on testis size with a male SVL covariate. All traits were ln-transformed prior to analyses, and all analyses used a Brownian motion model of character evolution.

Sperm Morphology	Predictor	Partial <i>r</i> (95% CI)	<i>t</i>	<i>P</i>
Head Length	Testis Size	0.23 (-0.18 – 0.57)	1.25	0.222
	SVL	-0.14 (-0.5 – 0.26)	-0.77	0.443
Midpiece Length	Testis Size	0.31 (-0.49 – 1.11)	0.82	0.422
	SVL	-0.02 (-0.41 – 0.37)	-0.11	0.911
Tail Length	Testis Size	0.12 (-0.28 – 0.48)	0.63	0.528
	SVL	-0.08 (-0.46 – 0.32)	-0.45	0.654

Figure 1. Pruned phylogeny of *Anolis* from Pyron et al. (2014) that includes the 28 species used in this study and their ecomorphs (A), alongside the mean and standard deviation of sperm head, represented in blue (B), midpiece, represented in orange (C) and tail length, represented in green (D) for each species, with an image of an anole sperm on the right. GB = grass-bush, T = trunk, CG = crown-giant, TG = trunk-ground, TW = twig, TC = trunk-crown.

Figure 2. Brownian rates of evolution (with 95% confidence intervals) of the sperm head, midpiece and tail, and residual testis size and male SVL for 28 species of *Anolis* lizards. These rates were calculated using ln-transformed values, and post hoc tests were conducted to determine significant differences between traits (Table S2).

Figure 3. Relationship between species' residual testis size and sperm head (A), midpiece (B), and tail length (C) for 28 species of *Anolis* lizards.

Figure 4. Least square means and SEM extracted from a phylogenetic ANOVA of residual testis size across ecomorphs with significance determined by post hoc tests using the function *phylANOVA* in the package *phytools* (Revell 2011). The number of species included for each group are listed below the graph.

Supplementary Table S1. Species averages ($\pm$ SD) of male body size (SVL), testis size, and sperm head, midpiece and tail length.

Species	N (M)	SVL (M) (mm)	Mean testis size (mg)	Head length (μ m)	Midpiece length (μ m)	Tail length (μ m)	Collection location
<i>Anolis aliniger</i>	10	51.7 $\pm$ 4.9	40.4 $\pm$ 10.1	16.30 $\pm$ 0.11	3.49 $\pm$ 0.11	77.46 $\pm$ 0.76	Ebano Verde, The Dominican Rep.
<i>Anolis angusticeps</i>	4	53.8 $\pm$ 2.2	23.1 $\pm$ 5.1	14.49 $\pm$ 0.06	2.69 $\pm$ 0.06	79.08 $\pm$ 2.27	Georgetown, The Bahamas
<i>Anolis bahorucoensis</i>	20	44.8 $\pm$ 2.8	6.0 $\pm$ 2.7	15.66 $\pm$ 0.10	3.60 $\pm$ 0.10	76.43 $\pm$ 1.40	Polo, The Dominican Rep.
<i>Anolis barahonae</i>	5	142.4 $\pm$ 23.2	236.5 $\pm$ 94.1	15.03 $\pm$ 0.02	3.53 $\pm$ 0.02	84.63 $\pm$ 2.68	Polo, The Dominican Rep.
<i>Anolis brevirostris</i>	19	49.1 $\pm$ 2.8	12.25 $\pm$ 5.0	17.38 $\pm$ 0.18	3.38 $\pm$ 0.18	90.25 $\pm$ 1.33	La Cienaga, The Dominican Rep.
<i>Anolis carolinensis</i>	13	54.8 $\pm$ 3.4	32.5 $\pm$ 2.2	16.98 $\pm$ 0.13	4.60 $\pm$ 0.14	63.08 $\pm$ 1.10	Florida, The United States
<i>Anolis christophei</i>	20	47.5 $\pm$ 4.2	13.7 $\pm$ 11.2	13.00 $\pm$ 0.09	2.84 $\pm$ 0.09	90.33 $\pm$ 1.35	Ebano Verde, The Dominican Rep.
<i>Anolis chlorocyanus</i>	18	73.9 $\pm$ 5.9	48.2 $\pm$ 20.3	16.31 $\pm$ 0.08	3.25 $\pm$ 0.09	78.31 $\pm$ 0.37	Bani, The Dominican Rep.
<i>Anolis coelestinus</i>	20	68.7 $\pm$ 4.1	24.8 $\pm$ 9.6	16.48 $\pm$ 0.12	3.40 $\pm$ 0.13	77.47 $\pm$ 0.58	San Jose de Ocoa, The Dominican Rep.
<i>Anolis cristatellus</i>	19	66.4 $\pm$ 5.9	31.0 $\pm$ 11.2	15.09 $\pm$ 0.08	3.25 $\pm$ 0.08	82.37 $\pm$ 1.8	El Verde, Puerto Rico
<i>Anolis cybotes</i>	20	64.2 $\pm$ 5.6	45.0 $\pm$ 20.3	15.14 $\pm$ 0.12	3.08 $\pm$ 0.13	84.60 $\pm$ 1.32	La Cienaga, The Dominican Rep.
<i>Anolis distichus</i>	19	51.7 $\pm$ 1.6	15.2 $\pm$ 2.9	16.55 $\pm$ 0.14	3.40 $\pm$ 0.14	95.52 $\pm$ 2.03	Georgetown, The Bahamas
<i>Anolis equestris</i>	9	151.8 $\pm$ 20.8	310.2 $\pm$ 178.0	15.09 $\pm$ 0.17	3.92 $\pm$ 0.17	77.78 $\pm$ 1.16	Florida, United States
<i>Anolis etheridgei</i>	12	40.8 $\pm$ 2.3	11.3 $\pm$ 3.6	13.99 $\pm$ 0.07	2.98 $\pm$ 0.07	72.99 $\pm$ 0.82	Ebano Verde, The Dominican Rep.
<i>Anolis evermanni</i>	20	58.6 $\pm$ 5.1	52.3 $\pm$ 17.1	16.86 $\pm$ 0.09	3.66 $\pm$ 0.09	85.50 $\pm$ 1.60	El Verde, Puerto Rico
<i>Anolis gundlachi</i>	20	63.3 $\pm$ 2.9	26.9 $\pm$ 6.2	16.24 $\pm$ 0.16	3.52 $\pm$ 0.16	96.62 $\pm$ 2.11	El Verde, Puerto Rico
<i>Anolis insolitus</i>	5	39.0 $\pm$ 6.7	11.6 $\pm$ 6.6	14.05 $\pm$ 0.03	3.18 $\pm$ 0.03	70.20 $\pm$ 0.38	Ebano Verde, The Dominican Rep.
<i>Anolis krugi</i>	20	49.5 $\pm$ 3.1	7.2 $\pm$ 5.1	16.40 $\pm$ 0.10	3.63 $\pm$ 0.10	86.90 $\pm$ 1.09	El Verde, Puerto Rico
<i>Anolis longitibialis</i>	17	65.9 $\pm$ 6.2	44.0 $\pm$ 10.1	16.39 $\pm$ 0.12	3.00 $\pm$ 0.12	83.60 $\pm$ 0.79	Oviedo, The Dominican Rep.
<i>Anolis marcanoi</i>	12	52.1 $\pm$ 8.5	35.7 $\pm$ 12.6	15.00 $\pm$ 0.04	3.06 $\pm$ 0.04	89.50 $\pm$ 1.37	Bani, The Dominican Rep.
<i>Anolis occultus</i>	5	38.4 $\pm$ 2.1	13.5 $\pm$ 1.7	18.21 $\pm$ 0.18	3.86 $\pm$ 0.18	92.35 $\pm$ 0.67	Arecibo, Puerto Rico
<i>Anolis olsoni</i>	20	43.9 $\pm$ 2.9	6.1 $\pm$ 2.1	13.28 $\pm$ 0.03	2.65 $\pm$ 0.03	79.02 $\pm$ 1.02	El Verde, Puerto Rico
<i>Anolis poncensis</i>	20	45.7 $\pm$ 1.7	7.1 $\pm$ 2.1	15.01 $\pm$ 0.36	3.55 $\pm$ 0.36	74.19 $\pm$ 1.06	Ponce, Puerto Rico
<i>Anolis pulchellus</i>	20	45.0 $\pm$ 3.1	9.1 $\pm$ 3.0	16.51 $\pm$ 0.12	3.50 $\pm$ 0.12	86.16 $\pm$ 1.50	El Verde, Puerto Rico
<i>Anolis sagrei</i>	20	56.1 $\pm$ 5.6	29.3 $\pm$ 8.4	14.13 $\pm$ 0.11	2.47 $\pm$ 0.11	80.86 $\pm$ 2.33	Georgetown, The Bahamas
<i>Anolis semilineatus</i>	5	44.0 $\pm$ 5.6	8.6 $\pm$ 6.7	13.44 $\pm$ 0.08	2.78 $\pm$ 0.08	76.56 $\pm$ 0.18	Constanza, The Dominican Rep.
<i>Anolis smaragdinus</i>	20	61.2 $\pm$ 3.2	32.0 $\pm$ 10.4	17.80 $\pm$ 0.18	4.07 $\pm$ 0.18	76.13 $\pm$ 1.41	Georgetown, The Bahamas
<i>Anolis stratulus</i>	20	47.0 $\pm$ 4.6	41.0 $\pm$ 9.7	17.24 $\pm$ 0.09	3.63 $\pm$ 0.09	88.56 $\pm$ 1.29	El Verde, Puerto Rico

Supplementary Table S2. Pairwise testing for differences in rates of evolution between all traits in this study. Each model was run under the L-BFGS-B optimization function in Adams' (2013) model. All traits reported here are from the best fit model, which modeled independent evolution among all traits (with the exception of the comparison of residual testis size (RTS) and SVL, and comparisons between sperm traits) while specifying intraspecific covariance but excluding intraspecific error.

Pairwise Comparison	Log(L)_{obs}	Log(L)_{com.}	LTR	P	AIC_{obs}	AIC_{com.}
RTS ~ SVL	-24.436	-69.119	89.366	<0.0001	56.870	144.238
RTS ~ Head	15.193	-15.841	62.071	<0.0001	-22.387	37.683
RTS ~ Midpiece	1.180	-17.070	37.762	<0.0001	4.379	40.141
RTS ~ Tail	19.589	-16.225	51.630	<0.0001	-11.179	38.451
SVL ~ Head	26.834	4.417	44.833	<0.0001	-45.669	-2.835
SVL ~ Midpiece	10.268	1.229	18.077	<0.0001	-12.536	3.541
SVL ~ Tail	16.690	-16.434	66.248	<0.0001	-25.380	38.868
Head ~ Midpiece	59.441	-122.009	362.913	<0.0001	-110.895	250.018
Head ~ Tail	59.981	-122.002	363.966	<0.0001	-111.962	250.004
Midpiece ~ Tail	46.651	-122.013	337.333	<0.0001	-85.308	250.024

Supplementary Table S3. Summary of results from phylogenetic generalized least squares regression of sperm morphology on testis size with a male SVL covariate. All traits were ln-transformed prior to analyses, and all analyses used an Ornstein-Uhlenbeck motion model of character evolution.

Sperm Morphology	Predictor	Partial r (95% CI)	t	P
Head Length	Testis Size	0.24 (-0.17 – 0.57)	1.28	0.212
	SVL	-0.18 (-0.53 – 0.23)	-0.97	0.339
Midpiece Length	Testis Size	0.10 (-0.30 – 0.47)	0.56	0.580
	SVL	0.01 (-0.38 – 0.40)	0.05	0.956
Tail Length	Testis Size	0.03 (-0.36 – 0.42)	0.17	0.868
	SVL	-0.02 (-0.40 – 0.37)	-0.09	0.928

CHAPTER 3:

Consistent differences in sperm morphology and testis size between native and introduced populations of three *Anolis* lizard species³

³ Accepted: Kahrl, A. F. and R. M. Cox. 2017. Consistent differences in sperm morphology and testis size between native and introduced populations of three *Anolis* lizard species. *Journal of Herpetology*.

Abstract

Sperm morphology can be highly variable among individuals and across species, but less is known about its variation among populations. Within the past 20–80 years, several species of *Anolis* lizards have been introduced to southern Florida from different source islands in the Caribbean, thereby permitting comparisons of sperm morphology between native and introduced populations of multiple species. We collected sperm samples from native populations of *A. sagrei* (Bahamas), *A. distichus* (Dominican Republic), and *A. cristatellus* (Puerto Rico) and compared them to samples from introduced populations of each species that are now sympatric in Miami, Florida. In each of these three species, lizards from introduced populations had sperm with shorter tails and larger midpieces relative to lizards from native populations. We also measured testis size in *A. distichus* and *A. cristatellus* and found that introduced populations of each species had smaller testes for a given body size, relative to their native counterparts. The consistency of these differences across species argues against random genetic drift as an explanation, suggesting instead that sperm morphology and testis size may exhibit predictable phenotypic plasticity or genetic adaptation in response to the process of introduction and/or the shared local environment in Florida. Though these population differences in male reproductive physiology and morphology may be repeatable, their underlying causes require further study.

Keywords: *Anolis cristatellus*; *Anolis distichus*; *Anolis sagrei*; intraspecific variation; postcopulatory sexual selection; testis size

Introduction

Sperm is the most morphologically diverse cell type among animals, showing high variation both within and among species and ranging several orders of magnitude in size (Pitnick et al., 2009). Much of the variation among species can be attributed to differences in the strength of postcopulatory sexual selection due to cryptic female choice and sperm competition (Immler et al., 2008; Tourmente et al., 2009; Higginson et al., 2012). Variation in sperm morphology within species can be influenced by an individual's genes (Simmons and Moore, 2008), social environment (Immler et al., 2010; Johnson et al., 2012), and diet or condition (Merrells et al., 2009; Rahman et al., 2013; Chapter 4; Kaldun and Otti, 2016). Collectively, these studies indicate that variation in sperm morphology arises from a combination of genetic divergence, which is most evident at the interspecific level, and phenotypic plasticity, which is most evident at the individual level within species.

Studies that have compared sperm morphology among populations of a species have found that variation in sperm morphology can be influenced by genetic drift (Stewart et al., 2016) and the strength of selection (Pitnick et al., 2003; Manier and Palumbi, 2008; Elgee, et al. 2010; Laskemoen et al., 2013). Sperm morphology can evolve relatively quickly, resulting in divergence between populations after only a few generations of selection (Landry et al., 2003; Pitnick et al., 2009; Hogner et al., 2013). Though several studies have documented population-level variation in sperm morphology (Pitnick et al., 2003; Hettyey and Roberts, 2005; Elgee, et al., 2010; Lüpold et al., 2011; Stewart et al., 2016), none have explored how the process of introduction into a novel

environment may structure this variation. Introduction into a novel environment can drive rapid phenotypic changes via adaptive evolution (Novak, 2007), phenotypic plasticity in response to novel environmental conditions (Davidson et al., 2011), or random divergence due to population bottlenecks (i.e. founder effects and/or genetic drift) (Prentis et al., 2008). One way to discern between these possibilities is to test whether differences in sperm morphology between native and introduced populations are consistent across species, which is predicted in the case of adaptive genetic change or phenotypic plasticity, but not in the case of genetic drift or founder effects. Genetic drift or founder effects may cause shifts in sperm morphology between populations, but it is unlikely that drift or founder effects would cause these shifts be the same direction across several species.

To address this question, we sampled native and introduced populations of three species of anoles (*Anolis sagrei*, *A. distichus*, and *A. cristatellus*) to test whether sperm morphology and testis size differ consistently between geographically disparate populations in the native range of each species, versus introduced populations that are now sympatric in southern Florida, USA (Fig. 1). These species are native to different islands in the Caribbean and were separately introduced to Miami, Florida, USA within the last 20–80 years (Lee, 1985; Schwartz and Henderson, 1991; Bartlett and Bartlett, 1999). Sperm morphology and testis size are highly variable both within (Chapter 4), and among species of lizards (Uller et al., 2010), but the extent of variation among populations is unknown. We predicted that, if either the process of introduction or the local environment in Miami favors similar reproductive phenotypes, then any differences

between native and introduced populations within each species would be broadly consistent across species. We also tested for the condition-dependence of sperm morphology in these populations as a signal of phenotypic plasticity.

Methods

We collected a total of 111 male lizards of *Anolis sagrei* (= *Norops sagrei*) (Duméril and Bibron, 1837), *Anolis cristatellus* (= *Ctenonotus cristatellus*) (Duméril and Bibron, 1837), and *Anolis distichus* (= *Ctenonotus distichus*) (Cope, 1861) from their native range in the Bahamas, Puerto Rico, the Dominican Republic, respectively, as well as introduced populations of all three species from southern Florida, USA (Fig. 1). Collections occurred during the middle of their breeding seasons (between May 15 and June 30) in 2013–2015 (collection times and localities in Table 1). We captured individual lizards using nooses or by hand and measured their SVL (snout-vent length, to the nearest mm) and mass (to the nearest 0.01 g). We sampled sperm from each male by applying pressure to the abdomen and collecting the ejaculate into a microcapillary tube inserted partially into the cloaca, then transferred this sample to 500 µl of phosphate-buffered saline (PBS) with 4% paraformaldehyde (PFA) to fix the cells. We centrifuged the sample and re-suspended it in water, then dried the cells on a microscope slide and stained them with SpermBlue™ (Microptic SL, Barcelona, Spain). We imaged the cells with an Olympus Magnafire Camera (Olympus America, Melville, NY) at ×100 magnification using differential interference contrast microscopy. We then measured the length of the sperm head, midpiece, and tail of 15 cells per male using ImageJ (NIH,

Bethesda, MD) and calculated the mean length of each part of the cell for each individual. We measured the length and width (to the nearest 0.1 mm) of the right testis from all individuals of *A. cristatellus* and *A. distichus* by dissection after euthanasia, but did not collect these data for *A. sagrei*. We calculated the volume of the testis using the equation for an ellipsoid, $(4/3\pi a^2 b)$, where a is the radius of the width of the testis and b is the radius of its length.

We tested for differences in sperm morphology using a generalized linear model (GLM) with the mean sperm phenotype for each individual as the dependent variable and effects of species, population (native or introduced), and their interaction. We conducted post hoc comparisons using Tukey's honestly significant difference (HSD) test to determine which populations were significantly different. We then pooled data across species and populations and calculated restricted maximum likelihood (REML) variance component estimates to partition the total variance in sperm morphology into species-level, population-level, and residual (individual-level) variation.

Previous work indicated correlations between body condition and sperm morphology within a native population of *A. sagrei* (Chapter 4). To test for condition-dependence of sperm morphology within native and introduced populations of each species in this study, we calculated body condition for each male by using the residuals from a regression of $\log_{10}$ mass on $\log_{10}$ SVL, which was done separately for each species. $\log_{10}$ transformation was used to remove dimensionality of mass and SVL in order to linearize the regression between these two variables. We then tested for correlations between these residual measures of condition and the mean measures of sperm

morphology for each male using ordinary least-squares regression for each species. We also tested for differences in condition between native and introduced populations of each species using ANCOVA with $\log_{10}$ mass as the dependent variable, population (native or introduced) as the independent variable, and $\log_{10}$ SVL as a covariate, after confirming homogeneity of slopes. We then tested for a correlation between body condition and residual testis size (calculated from a regression of $\log_{10}$ testis volume on $\log_{10}$ SVL) for each species. We also tested for differences in testis size between native and introduced populations of *A. cristatellus* and *A. distichus* using separate ANCOVAs with SVL as a covariate, after first confirming homogeneity of slopes between populations within each species. Finally, we used *t*-tests to test for differences in SVL and mass between native and introduced populations of each species.

Results

Across three *Anolis* species, we found significant overall effects of species and population (native versus introduced) on sperm morphology (head, midpiece, and tail length) (Table 2). We also found a significant interaction between species and population for each aspect of sperm morphology, indicating that the extent to which native and introduced populations differed in sperm morphology was variable across species, though the direction of change from native to introduced was nearly always consistent across species (Fig. 2, Table 2). Tukey's HSD post hoc test revealed significant differences between populations of *A. sagrei* (Fig. 2D) versus nonsignificant differences between populations of *A. cristatellus* (Fig. 2B) and *A. distichus* (Fig. 2C). The significant

interaction for midpiece length was driven by the combination of significant differences between populations of *A. distichus* (Fig. 2F) and *A. sagrei* (Fig. 2G) versus nonsignificant differences between populations of *A. cristatellus* (Fig. 2E). Finally, the interaction for tail length was driven by variation in the magnitude of significant differences in tail length between populations of all three species: *A. cristatellus* (Fig. 2H), *A. distichus* (Fig. 2I), and *A. sagrei* (Fig. 2J). In general, introduced populations had sperm with shorter tails and longer midpieces, relative to native populations (Fig 2). When we partitioned the total variation in sperm morphology, we found that majority of the total phenotypic variance occurred among species (mean = 87% across the three sperm traits), whereas a moderate amount of variation occurred between populations of a species (6.5%) and among individuals within a population (6.5%, Table 3).

Based on ANCOVA with $\log_{10}$ body mass as the dependent variable and $\log_{10}$ SVL as a covariate, we found no differences in body condition between native and introduced populations of *A. cristatellus* (Population: $F_{2,36} = 0.01$ $P = 0.934$, SVL: $F_{2,36} = 192.73$ $P < 0.001$) or *A. distichus* (Population: $F_{2,25} = 3.39$ $P = 0.078$, SVL: $F_{2,25} = 43.04$ $P = < 0.001$), but the introduced population of *A. sagrei* had higher body condition than the native population ($F_{2,38} = 12.60$ $P = 0.001$, SVL: $F_{2,38} = 229.81$ $P = < 0.001$). However, we did not find a correlation between body condition (residuals from the regression of $\log_{10}$ mass on $\log_{10}$ SVL) and sperm morphology in any population (all $P > 0.5$) except the native population of *A. sagrei*, which had a weak negative relationship between condition and midpiece size ($r = -0.454$, $t = -2.18$, $P = 0.043$). We also found no relationship between condition and relative testis size in either *A. cristatellus* ($t = -0.12$, P

= 0.907) or *A. distichus* ($t = -0.86$, $P = 0.401$), the two species in which we measured testis size.

We found significant differences in SVL and body mass between native and invasive populations of *A. sagrei* (SVL: $t = -3.33$, $P = 0.002$, mass: $t = -4.85$, $P < 0.001$) and *A. cristatellus* (SVL: $t = -2.64$, $P = 0.012$, mass: $t = -2.26$, $P = 0.030$), where individuals from the introduced populations were significantly longer and more massive. By contrast, we found no difference in SVL or mass between the native and invasive populations of *A. distichus* (SVL: $t = -0.64$, $P = 0.525$, mass: $t = 0.23$, $P = 0.820$). Individuals from native populations also had larger testes, relative to their SVL, than individuals from introduced populations in *A. distichus* (Population: $F_{2,32} = 25.30$, $P < 0.001$, SVL: $F_{2,32} = 11.14$, $P = 0.002$) and, to a lesser extent, in *A. cristatellus* (Population: $F_{2,35} = 3.29$, $P = 0.078$, SVL: $F_{2,35} = 5.31$, $P = 0.027$).

Discussion

We found significant differences in sperm morphology and testis size between native and introduced populations of three species of *Anolis* lizards. Sperm morphology is known to vary within and among individuals (Chapter 4) and across lizards species (Uller et al., 2008), but this is the first study to demonstrate significant differences in sperm morphology between native and introduced populations. Additionally, we found that the direction of change in sperm morphology and testis size from native to introduced populations was consistent across all three species, with introduced populations characterized by larger sperm midpieces, shorter sperm tails, and smaller

testis, relative to native conspecifics (Fig. 2). The midpiece, which is the area of the cell containing the mitochondria, and the tail, which influences sperm velocity in other lizards (Blengini et al., 2014), are likely targets of selection because of these links to function and performance (Lüpold et al., 2009; Firman and Simmons, 2010). These consistent changes in sperm morphology and testis size argue against a role of random factors such as genetic drift and founder effects, instead suggesting that either the process of introduction or the local environment in Miami has consistently favored the same adaptive changes or induced the same plastic responses in each of these three species.

Published measurements of sperm morphology in lizards are scattered across the phylogeny and show that head length of lizard sperm ranges from 5.50–23.5 μm (mean = 18.5 μm), midpiece length ranges from 1.82–11.50 μm (mean = 4.39 μm) and tail length ranges from 40.10–85.56 μm (mean = 60.90 μm) (Uller et al., 2010). *Anolis* sperm morphology falls near these interspecific means for the group as a whole, with the exception of sperm tail length, which is longer in anoles than in most other lizard lineages. In particular, *A. distichus* has the longest sperm tail and the longest total sperm length reported for any lizard. Though we know little about the relationships between sperm morphology and function in *Anolis* lizards, sperm velocity increases with sperm tail length in *Tupinambis* lizards (Blengini et al., 2014).

Variation in the local environment can affect the expression of sexually selected traits via both phenotypic plasticity (Griffith et al., 1999; Harris and Moore, 2004; Karubian et al., 2011; Somjee et al., 2015) and genetic adaptation (Boughman, 2001; Hettyey and Roberts, 2005). As an example of the former, native *Anolis sagrei* males

vary in sperm count and sperm morphology as a function of their body condition, and this variance can also be induced by dietary manipulation (Chapter 4), suggesting that population differences in prey availability or local environmental quality could generate intraspecific variation in sperm phenotypes. However, we found no differences in body condition between native and introduced populations of *A. cristatellus* and *A. distichus*, nor did we find significant correlations between sperm morphology and body condition in any population but the native population of *A. sagrei*. This suggests that, although sperm morphology may condition-dependent in some contexts, it is unlikely that plasticity is driving the differences between populations in this study.

Sperm morphology, velocity, and count are under sexual selection in a variety of species (Hettyey and Roberts, 2005; Manier and Palumbi, 2008; Álvarez et al., 2013), and the strength and direction of this selection can vary among populations due to differences sex ratio (Sasson and Brockmann, 2016), predator abundance (Elgee et al., 2010), or latitude (Pitcher and Stutchbury, 1998; Lüpold et al., 2011). Given that sperm phenotypes are often heritable and evolve rapidly in response to selection in other species (Landry et al., 2003; Pitnick et al., 2009; Hogner et al., 2013), adaptive differences between native and introduced *Anolis* populations could emerge over the relatively short timescales (20–80 generations) since introductions first occurred in southern Florida (Kolbe et al., 2004, 2007a, 2007b). Although such evolutionary change could also result from random sampling due to population bottlenecks and genetic drift (Stewart et al., 2016), these factors would be unlikely to result in parallel responses (i.e., larger midpieces, shorter tails, smaller testes) across species. Therefore, although we do not

know the causes of the observed differences in sperm morphology and testis size between native and introduced populations, our data suggest that these differences are more consistent with adaptive genetic change or phenotypic plasticity than with random divergence due to drift.

The three species of *Anolis* in our study are widely distributed across islands in the Greater Antilles and have been introduced multiple times to the southern United States over the past 80 yrs (Kolbe et al., 2004, 2007a, 2007b). Genetic analysis has established that the introduced population of *A. sagrei* that we sampled in Miami is likely descended from multiple native source populations in Cuba (Kolbe et al., 2007b), the introduced population of *A. cristatellus* is an admixture from San Juan and Aguas Claras, Puerto Rico, and the introduced population of *A. distichus* is an admixture from the Dominican Republic and the Bahamas (Kolbe et al., 2007a). Though our sampling locations for the native populations of *A. cristatellus* and *A. distichus* are close to the source locations for our introduced populations, the introduced populations are admixed and therefore our native populations should not be viewed as true genetic source populations. Likewise, our native population of *A. sagrei* in the Bahamas is genetically distinct from Cuban populations (the major genetic source for the introduced population that we sampled in Miami), which may explain why population differences in sperm morphology were most pronounced in this species (Fig. 2). Despite this caveat, the consistency in the direction of observed changes in sperm morphology and testis size across these three species suggests that some combination of convergent adaptation or phenotypic plasticity may be driving this population-level variation. The extent to which

this convergent adaptation and/or plasticity is due to the process of introduction per se, or simply the local environment in Miami, is currently unknown. Therefore, further studies characterizing differences local environments, assessing phenotypic plasticity, and quantifying selection on sperm morphology are needed to rigorously test these alternatives.

Acknowledgments

We thank M. Johnson, B. Kircher, M. Oberndorf, J. Strecula, J. Murray, B. Andre, M. Jaramillo, M. Webber, A. Zed, F. Deckard, and A. Hanninen for collection assistance. We also thank E. D. Brodie III, and two anonymous reviewers for their helpful comments on this manuscript. This study was conducted under under permits from Estado Libre Asociado de Puerto Rico, Bahamas Ministry of Agriculture, Bahamas Environment Science and Technology Commission, and Miami-Dade County Parks and Recreation, and approved under the University of Virginia IACUC protocol 3896. This work was supported by startup funding from The University of Virginia (to RMC), an E.E. Williams Research Grant from the Herpetologist's League (to AFK), a Theodore Roosevelt Memorial Grant from the American Museum of Natural History (to AFK), and a Doctoral Dissertation Improvement Grant from the National Science Foundation (DEB-1501680 to RMC and AFK). The authors declare that they have no conflict of interest.

Literature Cited

- Álvarez, D., L. Viesca, and A. G. Nicieza. 2013. Sperm competitiveness differs between two frog populations with different breeding systems. *Journal of Zoology* 292:202–205.
- Bartlett, R. D., and P. P. Bartlett. 1999. A field guide to Florida reptiles and amphibians. Gulf Pub. Co., Houston, TX.
- Boughman, J. W. 2001. Divergent sexual selection enhances reproductive isolation in sticklebacks. *Nature* 411:944–948.
- Cope, E. D. 1861. Notes and descriptions of anoles. *Proceedings of the Academy of Natural Sciences of Philadelphia* 13:208–215.
- Davidson, A. M., M. Jennions, and A. B. Nicotra. 2011. Do invasive species show higher phenotypic plasticity than native species and, if so, is it adaptive? A meta-analysis. *Ecology Letters* 14:419–431.
- Duméril, A. M. C., and G. Bibron. 1837. *Erpétologie Générale ou Histoire Naturelle Complete des Reptiles*. Roret, France.
- Elgee, K. E., J. P. Evans, I. W. Ramnarine, S. A. Rush, and T. E. Pitcher. 2010. Geographic variation in sperm traits reflects predation risk and natural rates of multiple paternity in the guppy. *Journal of Evolutionary Biology* 23:1331–1338.
- Griffith, S. C., I. Owens, and T. Burke. 1999. Environmental determination of a sexually selected trait. *Nature* 400:358–360.
- Harris, W. E., and P. J. Moore. 2004. Sperm competition and male ejaculate investment in *Nauphoeta cinerea*: effects of social environment during development. *Journal of*

- Evolutionary Biology 18:474–480.
- Hettyey, A., and J. D. Roberts. 2005. Sperm traits of the quacking frog, *Crinia georgiana*: intra- and interpopulation variation in a species with a high risk of sperm competition. Behavioral Ecology and Sociobiology 59:389–396.
- Higginson, D. M., K. B. Miller, K. A. Segraves, and S. Pitnick. 2012. Female reproductive tract form drives the evolution of complex sperm morphology. Proceedings of the National Academy of Sciences 109:4538–4543.
- Hogner, S., T. Laskemoen, J. T. Lifjeld, V. Pavel, B. Chutný, J. García, M.C. Eybert, E. Matsyna, A. Johnson. 2013. Rapid sperm evolution in the bluethroat (*Luscinia svecica*) subspecies complex. Behavioral Ecology and Sociobiology 67:1205–1217.
- Immler, S. S., S. S. Calhim, and T. R. Birkhead. 2008. Increased postcopulatory sexual selection reduces the intramale variation in sperm design. Evolution 62:1538–1543.
- Immler, S. S., S. R. Pryke, T. R. Birkhead, and S. C. Griffith. 2010. Pronounced within-individual plasticity in sperm morphometry across social environments. Evolution 64:1634–1643.
- Johnson, D. W., K. Monro, and D. J. Marshall. 2012. The maintenance of sperm variability: context-dependent selection on sperm morphology in a broadcast spawning invertebrate. Evolution 67:1383–1395.
- Kahrl, A. F., and R. M. Cox. 2015. Diet affects ejaculate traits in a lizard with condition-dependent fertilization success. Behavioral Ecology 26:1502–1511.
- Kaldun, B., and O. Otti. 2016. Condition-dependent ejaculate production affects male mating behavior in the common bedbug *Cimex lectularius*. Ecology and Evolution

6:2548–2558.

- Karubian, J., W. R. Lindsay, H. Schwabl, and M. S. Webster. 2011. Bill coloration, a flexible signal in a tropical passerine bird, is regulated by social environment and androgens. *Animal Behaviour* 81:795–800.
- Kolbe, J. J., R. E. Glor, L. Rodríguez Schettino, A. C. Lara, A. Larson, and J. B. Losos. 2004. Genetic variation increases during biological invasion by a Cuban lizard. *Nature* 431:177–181.
- Kolbe, J. J., R. E. Glor, L. R. Schettino, A. C. Lara, A. Larson, and J. B. Losos. 2007a. Multiple sources, admixture, and genetic variation in introduced *Anolis* lizard populations. *Conservation Biology* 21:1612–1625.
- Kolbe, J. J., A. Larson, and J. B. Losos. 2007b. Differential admixture shapes morphological variation among invasive populations of the lizard *Anolis sagrei*. *Molecular Ecology*
- Landry, C., L. B. Geyer, Y. Arakaki, T. Uehara, and S. R. Palumbi. 2003. Recent speciation in the Indo-West Pacific: rapid evolution of gamete recognition and sperm morphology in cryptic species of sea urchin. *Proceedings of the Royal Society of London B: Biological Science* 270:1839–1847.
- Laskemoen, T., T. Albrecht, A. Bonisoli-Alquati, J. Cepak, F. de Lope, I. G. Hermosell, L.E. Johannessen, O. Kleven, A. Marzal, T. A. Mousseau, A. P. Møller, R. J. Robertson, G. Rudolfsen, N. Saino, Y. Vortman, J. T. Lifjeld. 2013. Variation in sperm morphometry and sperm competition among barn swallow (*Hirundo rustica*) populations. *Behavioral Ecology and Sociobiology* 67:301–309.

- Lee, J. C. 1985. *Anolis sagrei* in Florida: phenetics of a colonizing species I. Meristic characters. *Copeia* 1985:182–194.
- Lüpold, S., D. F. Westneat, and T. R. Birkhead. 2011. Geographical variation in sperm morphology in the red-winged blackbird (*Agelaius phoeniceus*). *Evolutionary Biology* 25:373–390.
- Manier, M. K., and S. R. Palumbi. 2008. Intraspecific divergence in sperm morphology of the green sea urchin, *Strongylocentrotus droebachiensis*: implications for selection in broadcast spawners. *BMC Evolutionary Biology* 8:283–14.
- Merrells, K. J., H. Blewett, J. A. Jamieson, C. G. Taylor, and M. Suh. 2009. Relationship between abnormal sperm morphology induced by dietary zinc deficiency and lipid composition in testes of growing rats. *British Journal of Nutrition* 102:226–232.
- Novak, S. J. 2007. The role of evolution in the invasion process. *Proceedings of the National Academy of Sciences of the United States of America* 104:3671–3672.
- Pitcher, T. E., and B. J. M. Stutchbury. 1998. Latitudinal variation in testis size in six species of North American songbirds. *Canadian Journal of Zoology* 76:618–622.
- Pitnick, S., G. T. Miller, K. Schneider, and T. A. Markow. 2003. Ejaculate-female coevolution in *Drosophila mojavensis*. *Proceedings of the Royal Society of London B: Biological Science* 270:1507–1512.
- Pitnick, S., D. J. Hosken, and T. R. Birkhead. 2009. Sperm morphological diversity. Pp. 69–149 in T. R. Birkhead, D. J. Hosken, and S. Pitnick (Eds.), *Sperm Biology: An Evolutionary Perspective*. Academic Press, USA.
- Prentis, P. J., J. R. U. Wilson, E. E. Dormontt, D. M. Richardson, and A. J. Lowe. 2008.

- Adaptive evolution in invasive species. *Trends in Plant Science* 13:288–294.
- Rahman, M. M., J. L. Kelley, and J. P. Evans. 2013. Condition-dependent expression of pre- and postcopulatory sexual traits in guppies. *Ecology and Evolution* 3:2197–2213.
- Sasson, D. A., and H. J. Brockmann. 2016. Geographic variation in sperm and ejaculate quantity and quality of horseshoe crabs. *Behavioral Ecology and Sociobiology* 70:1715–1725.
- Schwartz, A., and R. W. Henderson. 1991. *Amphibians and Reptiles of the West Indies: Descriptions, Distributions, and Natural History*. University Press of Florida, USA.
- Simmons, L. W., and A. J. Moore. 2008. Evolutionary quantitative genetics of sperm. Pp. 405–434 in T. R. Birkhead, D. J. Hosken, and S. Pitnick (Eds.), *Sperm Biology: An Evolutionary Perspective*. Academic Press, USA.
- Somjee, U., P. E. Allen, and C. W. Miller. 2015. Different environments lead to a reversal in the expression of weapons and testes in the heliconia bug, *Leptoscelis tricolor* (Hemiptera: Coreidae). *Biological Journal of the Linnean Society* 115:802–809.
- Stewart, K. A., R. Wang, and R. Montgomerie. 2016. Extensive variation in sperm morphology in a frog with no sperm competition. *BMC Evolutionary Biology* 16:1–11.
- Tourmente, M., M. Gomendio, E. R. S. Roldan, L. C. Giojalas, and M. Chiaraviglio. 2009. Sperm competition and reproductive mode influence sperm dimensions and structure among snakes. *Evolution* 63:2513–2524.
- Uller, T., D. Stuart-Fox, and M. Olsson. 2010. Evolution of primary sexual characters in

reptiles. Pp. 425–452 in J. L. Leonard and A. Córdoba-Aguillar (Eds.), *The Evolution of Primary Sexual Characters in Animals*. Oxford University Press, Oxford.

Tables and Figures

Table 1. Samples size, locality information, sperm morphology mean $\pm$ SD, (min–max), and collection date for six populations of three species of *Anolis* lizards. Population abbreviations: N=Native, I=Introduced.

Species	Locality	Pop.	N	Head Length	Midpiece Length	Tail Length	Date Collected	GPS
<i>A. cristatellus</i>	El Yunque, Puerto Rico	N	19	15.11 $\pm$ 0.39 (14.44–15.81)	3.24 $\pm$ 0.08 (3.08–3.42)	82.36 $\pm$ 1.49 (78.8–84.6)	June 2014	N 18° 20' 32.28" W 65° 49' 33.72"
<i>A. cristatellus</i>	Miami, Florida	I	18	15.16 $\pm$ 0.34 (14.66–15.83)	3.26 $\pm$ 0.12 (3.05–3.46)	80.44 $\pm$ 1.14 (78.68–82.37)	May 2014	N 25° 42' 28.51" W 80° 09' 27.75"
<i>A. distichus</i>	Bani, Dominican Republic	N	20	16.37 $\pm$ 0.34 (15.75–16.80)	3.17 $\pm$ 0.15 (2.91–3.43)	96.76 $\pm$ 1.71 (94.69–101.67)	June 2015	N 18° 13' 50.80" W 70° 20' 44.15"
<i>A. distichus</i>	Miami, Florida	I	15	16.26 $\pm$ 0.72 (14.75–17.55)	3.38 $\pm$ 0.15 (3.16–3.72)	94.50 $\pm$ 2.47 (91.45–99.26)	May 2014	N 25° 42' 28.51" W 80° 09' 27.75"
<i>A. sagrei</i>	Great Exuma, The Bahamas	N	20	14.11 $\pm$ 0.38 (13.39–14.89)	2.46 $\pm$ 0.09 (2.24–2.65)	80.88 $\pm$ 2.39 (78.06–86.58)	May 2013	N 23° 30' 23.17" W 75° 45' 57.53"
<i>A. sagrei</i>	Miami, Florida	I	19	13.50 $\pm$ 0.16 (13.32–13.97)	2.62 $\pm$ 0.11 (2.44–2.76)	73.24 $\pm$ 0.99 (71.18–74.98)	May 2014	N 25° 42' 28.51" W 80° 09' 27.75"

Table 2. Results from a generalized linear model testing for effects of species, population (native or introduced) and their interaction on sperm morphology in three species of *Anolis* lizard.

Effect	df	Head Length		Midpiece Length		Tail Length	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Species	2,110	344.13	<0.0001	470.33	<0.0001	1078.46	<0.0001
Population	1,110	7.72	0.0065	30.88	<0.0001	140.55	<0.0001
Species * Population	2,110	6.34	0.0025	6.62	0.0020	32.69	<0.0001

Table 3. REML variance component estimates partitioning the variation in sperm morphology between native and introduced populations of *Anolis* lizard. Estimates for sperm morphology were made with all six populations, while testis size estimates (here measured as residual testis size from a regression with body mass) were calculated from only the populations of *A. cristatellus* and *A. distichus*.

Phenotype	Species	Population	Individual
Sperm head length	0.873	0.032	0.094
Sperm midpiece length	0.872	0.056	0.072
Sperm tail length	0.862	0.107	0.030
Testis size	0.112	0.270	0.617

Figure 1. Species range maps and the locations of our four sampling sites. See Table 1 for detailed locality coordinates.

Figure 2. *Anolis* spermatozoa (A). Population means $\pm$ SE calculated from individual mean values (across 15 cells per male) for length of the sperm head, midpiece and tail in native (black symbols) and introduced (white symbols) populations of three species of *Anolis* lizards. Significant differences between populations ($P < 0.05$) are noted with an asterisk.

CHAPTER 4:

Diet affects ejaculate traits in a lizard with condition-dependent fertilization success⁴

⁴ As published: Kahrl, A.F., and R.M. Cox. 2015. Diet affects ejaculate traits in a lizard with condition-dependent fertilization success. *Behavioral Ecology*, 26(6): 1502-1511.

Abstract

Sexually selected traits are often driven to costly extremes by persistent directional selection. Energy acquisition and allocation can therefore influence variation in traits subject to both precopulatory and postcopulatory sexual selection, though the later have received much less attention. We tested the condition dependence of sperm morphology, sperm count, and fertilization success in a promiscuous lizard (*Anolis sagrei*) by (1) collecting sperm samples from wild males that varied naturally in body condition, (2) experimentally altering the body condition of captive males through dietary restriction, and (3) analyzing genetic paternity data from competitive mating trials between captive males that differed in body condition. In both wild and captive males, the length of the sperm midpiece decreased with body condition. Experimental food restriction decreased sperm production, decreased length of the sperm head, increased length of the sperm midpiece, and increased variance in sperm morphology within individuals. When restricted to a single copulation, males on high-intake diets exhibited a slight but non-significant fertilization advantage. Reanalysis of a previous experiment in which high- and low-condition males were sequentially allowed to copulate *ad libitum* for one week revealed a significant fertilization bias in favor of high-condition males. When controlling for mean treatment effects on the proportion of offspring sired and on sperm phenotypes, multiple regression revealed negative correlations between fertilization success and sperm head length, midpiece length, and sperm count. Collectively, our results suggest that condition-dependent fertilization success in *A. sagrei* may be partially mediated by underlying condition dependence of sperm morphology and sperm count.

Introduction

Sexually selected traits often exhibit condition dependence in their expression because chronic directional selection due to female choice or male competition can drive them to expensive and exaggerated states, the costs of which can only be borne by individuals in good condition (Andersson 1986; Cotton et al. 2004). Condition dependence is therefore predicted to maintain both phenotypic and genetic variation in sexually selected traits even in the face of strong selection, and is consequently thought to be of general evolutionary significance (Falconer and Mackay 1981; Rowe and Houle 1996). Though most studies of condition dependence have focused on elaborate traits subject to precopulatory sexual selection (e.g., ornaments, weapons), traits experiencing postcopulatory sexual selection (e.g., ejaculate size and quality) can also be costly to produce, and equally important for male fitness (Dewsbury 1982; Perry and Rowe 2010). Consequently, an understanding of the condition dependence of fertilization success and of features of the ejaculate that contribute to this component of fitness is necessary to fully understand how variation in male condition influences the dynamics of sexual selection (Cotton et al. 2004).

Whereas individual sperm cells are relatively inexpensive, ejaculates are often costly to produce (Dewsbury 1982; Van Voorhies 1992; Olsson et al. 1997). Hence, their quality and composition can change based on diet and nutritional state (Gage and Cook 1994; Merrells et al. 2009; Perry and Rowe 2010; Rahman et al. 2013; Tigreros 2013). Likewise, male fitness is often influenced by sperm count (Laskemoen et al. 2010; Boschetto et al. 2011), sperm morphology (LaMunyon and Ward 1998; Simmons and

Kotiaho 2002; Fitzpatrick et al. 2012; Johnson et al. 2013; Bakker et al. 2014), and sperm quality (Gage and Cook 1994; Malo et al. 2005a; Malo et al. 2005b; Devigili et al. 2012), particularly in promiscuous species that experience strong postcopulatory sexual selection. Though the condition dependence of both sperm morphology and fertilization success have been examined in several species, they have rarely been assessed simultaneously within a species (Amitin and Pitnick 2007). This comparison is critical to our understanding of the evolution of condition-dependent traits because it can help identify the causes and consequences of intraspecific variation in sexually selected traits.

Fertilization success of males has been shown to change with body condition or diet in several species (Bonduriansky and Rowe 2005; Tigreros 2013; Zikovitz and Agrawal 2013). For species that experience high levels of sperm competition, increased sperm production can be critical for male fitness (Møller 1989; Møller and Briskie 1995; Boschetto et al. 2011; Kelly and Jennions 2011). However, to prevent functional and competitive sperm from being diluted by faulty or low-quality cells, increased sperm production must be matched by the maintenance of sperm quality. Aspects of sperm morphology that increase sperm performance (e.g., velocity, longevity), such as head size (Malo et al. 2006; Pitcher et al. 2009) midpiece length (Lüpold et al. 2009a; Firman and Simmons 2010) and tail length (Malo et al. 2006; Lüpold et al. 2009a; Mossman et al. 2009; Helfenstein et al. 2010) may be related to sperm quality. The quality of an ejaculate may also be reflected in morphological variance among cells, and increased selection on sperm can lead to reduced variation in sperm morphology (Calhim et al. 2007; Immler et al. 2008; Kleven et al. 2008; Fitzpatrick and Baer 2011). Though sperm morphology is

linked to fertilization success in several species (Simmons and Kotiaho 2002; Laskemoen et al. 2010; Fitzpatrick et al. 2012; Johnson et al. 2013; Bakker et al. 2014), a link between condition dependence of sperm morphology and its effects on male fitness has only been demonstrated in a few species (LaMunyon and Ward 1998; Amitin and Pitnick 2007; García-González and Simmons 2007). Males that produce large and costly sperm are more successful at fertilization in some species (LaMunyon and Ward 1998), but in other species, effects of diet or condition on fitness occur without associated differences in sperm morphology (Schulte-Hostedde and Millar 2004; Amitin and Pitnick 2007).

The brown anole (*Anolis sagrei*) is a small, promiscuous lizard that likely experiences strong postcopulatory selection due to the high rates of multiple mating and multiple paternity that occur in wild populations (Tokarz 1998; Calsbeek and Bonneaud 2008). Female anoles store sperm for several months after mating, and 80% of females from wild populations produce offspring by more than one sire when using stored sperm (Calsbeek and Bonneaud 2008). We used several complementary approaches to explore the condition dependence of sperm morphology, sperm count, and fertilization success in *A. sagrei*. First, we examined ejaculates of wild males that varied naturally in body condition to test for correlations between body condition and sperm morphology in a natural context. Second, we experimentally altered body condition by manipulating the diets of captive males to directly test for condition dependence of sperm count and sperm morphology. Next, we sequentially mated two males from different diet treatments to the same female, then genotyped the progeny of each female to test for condition-dependent fertilization success in a situation where each male was allowed a single copulation.

Finally, we reanalyzed data from a previous mating experiment to test for condition-dependent fertilization success in pairs of captive males that varied naturally in body condition and were allowed to mate *ad libitum* with the same female. We predicted that (1) sperm morphology (head, midpiece, and tail length) would be correlated with natural variation in body condition and influenced by diet treatment, (2) sperm count (cells per ejaculate) would be influenced by diet and increase with body condition, (3) within-male variance in sperm morphology (head, midpiece, and tail length) would be influenced by diet and decrease with body condition, indicating a more stringent maintenance of sperm quality when sufficient resources are available, and (4) fertilization success would be influenced by diet and biased in favor of high-condition males.

Methods

Natural variation in body condition

To assess variation in sperm morphology as a function of natural variation in body condition, we collected 21 adult *A. sagrei* males from February Point, near Georgetown on Great Exuma, The Bahamas (23°30'N, 75°45'W). We collected males in early June of 2013, during the middle of their prolonged breeding season, which extends from approximately February through September (Tokarz et al. 1998). For each male, we measured snout-vent length (SVL, nearest 1 mm), body mass (nearest 0.1 g), and collected a sperm sample into a glass microcapillary tube by applying pressure to the abdomen, anterior to the cloaca. We fixed sperm cells in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for five minutes, at which point the fixative was

removed and the cells were dried on slides. We stained cells with Sperm Blue™ (Microptic SL, Barcelona, Spain) and then imaged them with an Olympus Magnafire camera (Olympus America, Melville, NY) at 100x magnification using differential interference contrast microscopy. We measured the length of the head, midpiece, and tail for 25 sperm cells per male using ImageJ (NIH, Bethesda, Maryland, USA), then calculated the mean and coefficient of variation (CV) in each measure for each male. We used a resampling procedure to confirm that sampling 25 cells per individual was sufficient to reach asymptotically low levels of variance in our measures of individual means and CVs for each measure of sperm morphology (Supplemental Fig. 1). We did this through simulated resampling (with replacement) of a variable number of cells (range 2-25 cells), with 1000 simulations at each sample size for 34 individual males.

For each individual, we estimated body condition in two ways: (1) as residuals from the regression of $\log_{10}$ mass on $\log_{10}$ SVL (residual index, R_i), and (2) as a scaled mass index (M_i), which was recently proposed as a superior alternative to the residual index (Peig and Green 2009; 2010). We then tested for correlations between R_i or M_i and individual means and CVs for sperm head, midpiece, and tail length using ordinary least-squares regression. These condition indices have the advantage of generating a body condition phenotype for each individual, which facilitates visualization of correlations, though some authors advocate for the use of multiple regression with mass and length as separate independent variables (García-Berthou 2001; Freckleton 2002). Therefore, we also performed multiple regressions with $\log_{10}$ mass and $\log_{10}$ SVL as independent variables in models with individual means or CVs of sperm head, midpiece, and tail as

response variables. In brown anoles, these three approaches (R_i , M_i , multiple regression) tend to generate similar results in subsequent analyses (Cox and Calsbeek 2014). All statistical analyses were performed using JMP (SAS Institute Inc., Cary NC, Version 9).

Diet manipulation

We collected 34 adult *A. sagrei* males and 34 females from the same population on Great Exuma in January 2012 and acclimated them to captivity for one year before initiating diet treatments. Adults were housed individually in small plastic cages at 82°F and 60% relative humidity on a 12L:12D photoperiod. We separated the 34 male *A. sagrei* into two treatment groups placed on different diets: a high-intake diet consisting of five crickets per feeding, or a low-intake diet consisting of one cricket per feeding ($n = 17$ males per treatment). We fed each group three times per week (i.e., 15 or 3 crickets per male, per week). All crickets were approximately the same size (3/8 inch), and were dusted weekly with vitamin and mineral supplements (Repta-Vitamin, Fluker Farms, Port Allen, LA). We maintained males on these diet treatments for five months to induce a change in body condition (Fig. 1) and to ensure that this change in condition had sufficient opportunity to affect spermatogenesis. Reptiles with long or continuous reproductive seasons, such as *A. sagrei*, produce sperm continually throughout the reproductive season, with spermatogenic cycles typically lasting two months (Gribbins 2011). We monitored body condition every two weeks during the diet treatment to ensure that sperm used during competitive matings (see below) were produced when body condition differed between treatment groups (Fig. 1). To statistically confirm that our diet

treatment affected condition, we used repeated-measures ANOVA to test for a time-by-treatment interaction on body condition, using time as a within-subjects effect and diet treatment as an among-subjects effect.

After 16 weeks of dietary manipulation, we collected a sperm sample from each male to assess treatment effects on sperm morphology using the methods described above. To assess sperm count, we applied pressure to the lower abdomen of each male until he stopped releasing sperm. Ejaculates were collected into a microcapillary tube and transferred to 500 μ l of PBS with 4% PFA to fix the cells. We mixed this suspension by tapping and pipetted 10 μ l onto a hemocytometer to determine the total cell count. This measure of sperm count is correlated with estimates obtained by collecting whole ejaculates from the female reproductive tract immediately following mating ($n = 18$ males, $r^2 = 0.49$, $P = 0.001$). Measures of sperm morphology were obtained immediately after competitive mating trials, and measures of sperm count were obtained one week after competitive mating trials (see below).

To test for treatment effects on sperm morphology, we used nested ANOVA with head length, midpiece length, or tail length as the dependent variable, 25 individual cells per each of 34 individual males as observations, and male identity as a random effect nested within diet treatment. To compare effects of natural and experimentally induced variation in body condition on sperm morphology, we tested for correlations between body condition (R_i or M_i) and individual means for head length, midpiece length, and tail length of sperm. As described above, we also used multiple regressions with $\log_{10}$ mass and $\log_{10}$ SVL as covariates and individual means of sperm head, midpiece and tail

length as response variables. To test whether dietary restriction resulted in increased variance in sperm morphology within individuals, we calculated within-individual coefficients of variation (CV) for each sperm morphological component (head length, midpiece length, tail length). We used *t*-tests to determine whether variance differed between treatment groups ($n = 17$ males in each group). We also used a *t*-test to determine whether diet impacted sperm count.

Competitive fertilization trials

To test for condition dependence of fertilization success, we mated males from each group competitively to females so that ejaculates from high- and low-condition males would be in direct competition for fertilization. Pairs of males ($n = 17$), one from each treatment group, were matched for body size (SVL) and for approximately the same relative difference in body condition across pairs. First, one male from a pair was allowed to copulate once with a female. After 1-2 days, that female was allowed to copulate once with the second male in the pair. Both males were then allowed to recover for one week (to prevent sperm depletion), and the procedure was repeated with a second female. The order of the males was reversed with the second female, to account for observed first-male paternity advantage in this species (Duryea et al. 2013). The order of each mating pair was balanced with respect to treatment, so that half of the males in each treatment mated first in the first round of mating, and half mated first in the second round of mating.

We monitored all trials from behind a blind so that we could remove males immediately after mating and prevent multiple copulations. After mating, we housed

females in individual cages with potted plants in which they oviposited (mean $\pm$ SEM = 5.55 ± 0.90 eggs per female, range 0-16) at approximately 10-day intervals over an ensuing three-month period. Female brown anoles can store sperm and produce viable eggs for upwards of four months following mating (Calsbeek and Bonneaud 2008). Eggs were removed from potted plants, placed individually in small containers with a mixture of vermiculite and deionized H₂O (1:1 by weight), and incubated at 28°C and 80% relative humidity for two weeks, until embryo sex could be determined, at which point all embryos were sacrificed for genetic material. We stored embryonic and parental tissue in 95% ethanol at 4°C until DNA extraction.

Paternity analysis

We extracted genomic DNA from parental and embryonic tissue (tail clip, 1 mm) by incubating samples for 180 min at 55°C and denaturing for 10 min at 99°C in 150 μ l of 5% Chelex® resin (Bio-Rad, Inc.) in purified H₂O plus 1 μ l Proteinase K (20 mg/ml, Qiagen, Chatsworth, CA, USA) per sample. Following centrifugation, we collected 30 μ l of supernatant from these extractions to genotype individuals at 10 microsatellite loci: AAGG-38, AAAG-61, AAAG-68, AAAG-70, AAAG-76, AAAG-77, AAAG-91, AAAG-94 (Bardeleben et al. 2004), and ACAR11, ACAR23 (Wordley et al. 2010). We performed polymerase chain reaction (PCR) using Qiagen Multiplex PCR Kits with 1 μ l of template DNA in a total volume of 10 μ l. PCR cycles consisted of one denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 57°C for 90 sec, and extension at 72°C for 90 sec, with a final extension step

at 60°C for 30 sec. We ran all PCR reactions on an Eppendorf Mastercycler (Applied Biosystems, Carlsbad, CA, USA), and performed fragment analysis using a GeneScan LIZ500 size standard on an ABI 3130 Genetic Analyzer (Applied Biosystems). We scored genotypes using GeneMarker v2.2.0 software (SoftGenetics, State College, PA, USA).

We analyzed paternity using a likelihood-based method implemented in the program CERVUS 3.0 (Kalinowski et al. 2007). Initial allele-frequency analysis revealed that one locus was not in Hardy-Weinberg equilibrium (AAAG-68), and that two loci (AAAG-38, AAAG-94) had eight null alleles. These three loci were removed from the analysis, leaving a total of seven informative loci (Supplementary Table 1). To assess the power of our markers, we ran a simulation of parentage analysis for 200 offspring and 34 potential sires using the allele frequencies generated above, while assuming 100% of potential sires sampled, a 5% genotyping error rate, and a minimum number of typed loci set to four. These simulated data were used to determine the likelihood-odds ratio (LOD) scores in the paternity analysis. We analyzed paternity using the “one parent known” option in CERVUS, which allowed us to specify the known mother and the two candidate sires for each offspring.

Condition dependence of fertilization success

We tested for condition-dependent fertilization success using data from the fertilization trials described above, in which condition was experimentally altered via dietary manipulation, and by analyzing data from a previous study in which captive-bred

males varied naturally in condition (Cox et al. 2011). The design of this previous study was similar to that described above, with each female mated sequentially to two males, and each pair of males mated to two females, once as the first and once as the second male to mate. In contrast to the present study, pairs of males in the previous study were matched for age (rather than size), each male was allowed to mate *ad libitum* with each female for one week (rather than being limited to one copulation), and males were classified into “high” and “low” condition groups on the basis of positive or negative residuals from the regression of $\log_{10}$ body mass on $\log_{10}$ SVL (rather than being assigned to diet treatments). We filtered this dataset to include only those pairs that comprised one high- and one low-condition male ($n = 20$ pairs of sires). Only 30 of the 40 females to which these males were mated produced offspring, similar to the present study, in which 22 of 34 females produced offspring ($n = 14$ pairs of sires). Therefore, we tested for condition-dependent fertilization success in two ways for each experiment. First, we considered each female as a unit of observation and compared the proportion of her offspring sired by high- versus low-condition males using paired t -tests (statistically equivalent to testing whether the proportion of offspring sired by either group differs from the null expectation of 0.5). Second, to avoid double-counting male pairs, we conducted analogous t -tests but considered each male pair as the unit of observation, pooling the progeny they sired across both females to calculate their proportional paternity. With each approach, we conducted weighted (by the total number of offspring from which proportional paternity was calculated) and unweighted tests.

To test whether sperm morphology and sperm count were correlated with fertilization success independent of any overall treatment effects on these variables, we standardized the proportion of offspring sired, sperm morphology, and sperm count within each treatment by subtracting the treatment mean from each male's individual value and dividing by the treatment standard deviation. To avoid any potential biases due to first- or second-male mating advantage (Duryea et al. 2013), we conducted three separate analyses with different response variables: (1) the proportion of offspring sired when the male was the first male to mate with a female (P_1), (2) the proportion of offspring sired when the male was the second male to mate with a female (P_2), and (3) the average proportion of paternity that each male achieved across both of his trials, once as the first and once as the second male to mate (P_{avg}). We used these estimates of proportional paternity as response variables in separate multiple regressions that included treatment-standardized measures of each male's average sperm head length, midpiece length, tail length, and sperm count (square-root transformed) as independent variables. We confirmed that these analyses were not strongly influenced by multicollinearity by examining variance inflation factors ($VIF < 1.6$ for all models, Marquardt 1970).

Results

Condition dependence of sperm morphology and sperm count

Diet treatment significantly altered male body condition (R_i), such that males in the high-intake treatment were significantly more massive for a given length than males in the low-intake treatment (treatment: $F_{1,33} = 19.39$, $P < 0.0001$; Fig. 1). This effect of

diet on condition increased over time (time*treatment: $F_{1,33} = 7.44$, $P < 0.0001$; Fig. 1). Diet treatment also affected sperm morphology. Males on a high-intake diet had marginally longer sperm heads and significantly smaller sperm midpieces, relative to low-intake males, while sperm tail length did not differ between treatment groups (Table 1, Fig. 2). Treatment also significantly affected within-male variance in sperm morphology, such that males on the high-intake diet had marginally lower variance in head and tail length and significantly lower variance in midpiece length (Table 1, Fig. 2). Males on the high-intake diet also had higher sperm counts ($6.92 \pm 3.42 \times 10^6$ cells per ejaculate) than males in the low-intake group ($3.82 \pm 3.22 \times 10^6$ cells per ejaculate; $t = 2.526$, $P = 0.017$).

Natural patterns of covariance in body condition and sperm morphology generally corroborate this experimental evidence for condition dependence. In the wild, we detected a weak negative correlation between natural variation in body condition and mean midpiece length, but found no relationship between body condition and mean head length or tail length (Fig. 3). These results were robust to the choice of R_i , M_i , or multiple regression (i.e., correlations with body mass while controlling for SVL) to assess body condition (Supplementary Table 2). Correlations were similar across experimentally induced variation in body condition. We found a significant negative relationship between body condition and mean midpiece length, but no correlations with mean head length or tail length, and these results were again robust to the method used to assess condition (Fig. 3, Supplementary Table 2). Though we found significant differences in the variance in sperm morphology between treatment groups, we found no relationship

between natural variation in body condition and variation in the length of the sperm head ($P > 0.49$ for all three methods), midpiece ($P > 0.31$ for all three methods) or tail ($P > 0.41$ for all three methods).

Condition dependence of fertilization success

Loci used in paternity analysis had an average polymorphism information content of 0.72, with a non-exclusion probability of 0.0009 (Supplemental Table 1). In total, we genotyped 188 offspring, and only three of these could not be reliably assigned to a sire. Of the remaining offspring, 82% ($n = 152$) were assigned with $>95\%$ confidence and 18% ($n = 33$) were assigned with $>80\%$ confidence, assuming a 0.05 error rate.

In our reanalysis of data from a previous study in which captive-bred males varied naturally in body condition (Cox et al. 2011), we found a significant fertilization advantage associated with high condition (Fig. 4A), irrespective of whether we used individual females ($n = 30$; weighted: $t = 2.47$, $P = 0.010$; unweighted: $t = 1.74$; $P = 0.046$) or pairs of males as units of observation ($n = 20$; weighted: $t = 2.73$, $P = 0.007$; unweighted: $t = 1.94$; $P = 0.034$). In the present experiment, we did not detect any overall fertilization advantage associated with the high-intake diet (Fig. 4B), irrespective of whether we used individual females ($n = 22$; weighted: $t = 0.19$, $P = 0.57$; unweighted: $t = 0.13$, $P = 0.45$) or pairs of males as units of observation ($n = 14$; weighted: $t = 0.05$, $P = 0.52$; unweighted: $t = 0.32$; $P = 0.38$). When we conservatively limited male pairs to the subset that produced offspring by each of two females, we observed similar overall patterns of condition dependence in both studies (Fig. 4A-B, $n = 9$ pairs of sires), but

neither trend was significant due to low statistical power (previous study: $t = 1.34$; $P = 0.11$; this study: $t = 0.74$; $P = 0.24$; $df = 8$ for each test).

After standardizing fertilization success and sperm phenotypes to a mean of zero and units of standard deviation within each diet treatment, we found no significant relationships between P_1 or P_2 , and any aspect of sperm morphology or sperm count. However, when we used the average proportion of paternity for each male across both mating trials (P_{avg}) as our response variable, multiple regressions revealed significantly negative partial correlations between P_{avg} and sperm head length, sperm midpiece length, and sperm count (Table 2).

Discussion

Over the past decade, there has been mounting empirical evidence that ejaculate traits are important determinants of male fitness (LaMunyon and Ward 1998; Gage et al. 2004; García-González and Simmons 2005; García-González and Simmons 2007; Boschetto et al. 2011). Sexual selection for ejaculate quantity and quality has presumably driven some aspects of sperm morphology and production to be costly and therefore condition-dependent (Gage and Cook 1994; Simmons and Kotiaho 2002; Malo et al. 2005b; Perry and Rowe 2010; Gasparini et al. 2013; Rahman et al. 2013). Though traits associated with sperm quantity (e.g., sperm count) have obvious potential costs, traits that are indicative of sperm quality (e.g., sperm morphology and performance) may have more subtle energetic costs that are attributable to the maintenance of spermatogenesis (Hill 2011). Our results demonstrate that sperm count and some aspects of sperm

morphology are condition-dependent in *Anolis sagrei*. Males in lower body condition due to dietary restriction produced fewer and more variable sperm, as well as sperm with larger midpieces (Fig. 2). We observed a similar negative relationship between body condition and midpiece length in wild males that varied naturally in body condition (Fig. 3). Males in higher body condition also tended to sire more offspring in competitive mating trials with size- or age-matched males in lower condition (Fig. 4). Though we cannot directly link this apparent condition dependence in fertilization success to underlying condition dependence in sperm morphology or sperm count, the proportion of offspring sired by each male was negatively correlated with sperm head length, midpiece length, and sperm count, even after removing the overall effects of diet treatment on each measure. Below, we discuss these findings as part of the emerging body of literature on the condition dependence of traits subject to postcopulatory sexual selection, and their potential fitness consequences.

Condition dependence of ejaculate traits

We found that sperm morphology, particularly the size of the midpiece, was condition-dependent in both a wild population and in response to experimental diet treatments. We also found that sperm count was condition-dependent in our experimental diet treatments. In promiscuous species, such as *A. sagrei*, males who are able to produce more competitive ejaculates (e.g., higher sperm count, morphologically sound cells, greater velocity, increased cellular longevity), are expected to be more successful in sperm competition. Our findings are generally consistent with other studies that have

measured sperm count (Gage and Cook 1994; Malo et al. 2005b; Gasparini et al. 2013; Rahman et al. 2013; but see Perry and Rowe 2010), and sperm morphology (Simmons and Kotiaho 2002; Bonanno and Schulte-Hostedde 2009; Rahman et al. 2013) in relation to body condition. In general across these studies, males in high body condition produce more sperm than males in low body condition, though relationships between body condition and sperm morphology are more variable across these studies. The differences we observed between treatment groups in sperm morphology and sperm quantity suggest that sperm may be energetically costly to produce in large quantities, in high quality, or with particular morphologies (Perry and Rowe 2010).

In some cases, traits that are condition-dependent are predicted to experience positive selection for size or symmetry and therefore require greater energy expenditure for their production and maintenance (Rowe and Houle 1996). Consistent with this idea, when food quantity or quality is low, total sperm length often decreases (Alavi et al. 2009; Merrells et al. 2009; Rahman et al. 2013) whereas within-individual variation in sperm morphology increases (Hellriegel and Blanckenhorn 2002). However, other studies found no relationship between condition and sperm length (Gage and Cook 1994; Amitin and Pitnick 2007; Gasparini et al. 2013). Unfortunately, the majority of these studies did not separately quantify the length of each sperm component, but looked only at total sperm length. Those studies that have quantified individual morphological components of sperm size have found both context- and nutrient-dependent variation in cellular morphology (Alavi et al. 2009; Merrells et al. 2009; Immler et al. 2010). In the present study, we found no association between body condition and total sperm length, but we

did find a weak effect of diet treatment on the length of the sperm head, and a strong effect of diet treatment on the length of the sperm midpiece, suggesting that males in better condition produce sperm with slightly larger heads, but smaller midpieces.

High-condition males produced sperm with slightly smaller midpieces than those of low-condition males in the wild and in captivity, a result that encompasses both natural and experimentally induced variation in body condition. Although correlations between body condition and midpiece length were generally weak ($0.12 < r^2 < 0.17$) and effects of diet on midpiece size were fairly modest and subject to considerable variation among individuals (Fig. 2), midpiece size has been linked with cellular performance, and may play a critical role in male fitness (Bakker et al. 2014). The midpiece of the sperm cell contains its mitochondria, which are potentially costly to produce in high numbers. In comparative studies of birds, mammals, rodents, fish, and snakes, polygamous species and/or species with larger testes tend to produce sperm with larger midpieces, suggesting that sperm competition may select for large midpiece size (Breed and Taylor 2000; Anderson et al. 2005; Lüpold et al. 2009a,b; Tourmente et al. 2009). Within species, Gouldian finches placed in environments with greater male-male competition produced sperm with larger midpieces, which demonstrates that individual variation in sperm morphology may respond to environmental cues of intrasexual competition (Immler et al. 2010). This association between competitive environment and midpiece size within and among species may occur because midpiece size is often positively related to measures of performance, such as velocity (Firman and Simmons 2010) and longevity (Helfenstein et

al. 2010), suggesting that larger midpieces could improve cellular performance and potentially increase fertilization success.

Despite this general pattern across species, other studies have found negative intraspecific relationships between body condition and midpiece length (Bonanno and Schulte-Hostedde 2009), and between body condition and adenosine triphosphate content of sperm cells (Burness et al. 2008), comparable to our results for *A. sagrei*. Because the midpiece may be one of the most energetically expensive portions of the cell to produce, this pattern could represent a trade-off in investment between midpiece size and sperm number (Parker and Begon 1993). One explanation for the opposing pattern across species and the pattern we observed in our study may be that midpiece size is more important for long-term storage and viability in the female reproductive tract, but may not affect paternity when competition for fertilization occurs over shorter intervals. Species that store sperm for months or years may rely on the mitochondria to increase cellular longevity (Tourmente et al. 2009), while glycolysis in the tail can provide the energy needed for short-term competition. In our study, females produced offspring from stored sperm over a three-month span following mating, whereas in the wild, females mate repeatedly over a six-month reproductive season, potentially minimizing the importance of sperm longevity. Producing more sperm with shorter midpieces could therefore be more beneficial in competition for fertilizations using stored sperm. Though our data cannot directly link sperm morphology with fertilization success, males producing smaller midpieces (as well as smaller sperm heads) sired a larger proportion of offspring

even when controlling for overall treatment effects on proportional paternity and sperm morphology (Table 2).

Condition dependence of fertilization success

When we reanalyzed data from Cox et al. (2011; Fig. 4A), we found that males in high condition produced a greater proportion of offspring (63-68%) when competitively mated against males in low condition in a design that eliminated the potential for precopulatory male-male competition. We found a similar trend in the present study (Fig. 4B), where males on a high-intake diet produced a slightly higher proportion of offspring (53-60%) than males on a low-intake diet, though this trend was not statistically significant. In the previous experiment (Cox et al. 2011), males were allowed unlimited access to females for one week, rather than being limited to a single copulation, as in the present study. This difference could influence relative fertilization success in several ways. First, males in high condition could have mated more frequently than males in low condition, therefore increasing the relative abundance of their sperm in the female reproductive tract beyond what would result from a single mating by each male. Second, even if low-condition males were able to mate as often as high-condition males, their ability to continuously produce ejaculates with high sperm counts could have declined more steeply with successive mating, relative to high-condition males. Lastly, though all males mounted and appeared to copulate with females in the present study, we could not confirm the actual transfer of ejaculates to the female for all trials. Collectively, these factors could explain why we only detected a slight condition dependence of paternity in

the present study, whereas this effect was large and significant in a previous experiment (Cox et al. 2011).

Irrespective of these potential differences between studies, our results collectively indicate that male anoles in high condition tend to sire a higher proportion of offspring than males in low condition, even when direct, precopulatory male-male competition is eliminated. We cannot exclude the possibility that this apparent condition dependence of fertilization success actually reflects cryptic female choice (e.g., based on precopulatory assessment of male phenotypes) or some aspect of male behavior or performance unrelated to properties of the ejaculate per se (e.g., deeper intromission with more efficient sperm transfer). Nonetheless, our simultaneous documentation of condition dependence in sperm morphology and sperm count indirectly supports the hypothesis that properties of the ejaculate may contribute to these observed fitness differences. Moreover, our multiple regression analyses revealed significant correlations between the proportion of offspring sired and head length, midpiece length, and sperm count when proportional paternity was averaged over two matings (P_{avg}), though these same ejaculate phenotypes were unrelated to variance in P_1 or P_2 (Table 2). This difference may arise in part from the small number of offspring from which estimates of proportional paternity were derived (median 12.5, range 1-26 per male pair), such that averaging across multiple mates may provide a better representation of a male's competitive ability.

We emphasize that our evidence for a relationship between sperm phenotypes and fertilization success is subject to many caveats, including the correlative nature of the data, our relatively low sample sizes in terms of both parents and offspring, and the fact

that significant effects are only detectable under certain analytical conditions (e.g., using P_{avg} rather than P_1 or P_2 ; using multiple regression rather than univariate comparisons). Under these conditions, we found that proportional paternity decreased as the average size of the sperm head and midpiece increased, even when controlling for overall treatment effects on proportional paternity and sperm phenotypes. In other species, midpiece size is often correlated with sperm velocity (Anderson and Dixson 2002; Malo et al. 2006; Firman and Simmons 2010), and large head size may contribute to drag on the cell, preventing the cell from moving efficiently (Humphries et al. 2008). However, without a better understanding of the functional significance of sperm morphology in *A. sagrei*, and of the aspects of female reproductive anatomy and physiology that influence sperm performance, any adaptive interpretations are merely speculative. We also note that, after removing overall treatment effects (i.e., when considering only variance within treatments), we observed a significant negative relationship between proportional paternity and sperm count using multiple regressions including all sperm phenotypes, a result that seems difficult to reconcile with any adaptive interpretation.

Quantifying the condition dependence of male phenotypes, as well as the fitness consequences of this condition dependence, may aid in our understanding of how phenotypic and genetic variance is maintained in the face of chronic sexual selection. Sperm morphology is highly variable among species, and often within species, but few studies have identified the processes that generate and maintain this intraspecific variation (Hellriegel and Blanckenhorn 2002; Simmons and Kotiaho 2002; Rahman et al. 2013). We have demonstrated that some aspects of sperm morphology and sperm count

exhibit signatures of condition dependence in wild *A. sagrei* populations, as well as in direct response to dietary restriction in captivity. Moreover, we have shown that high-condition males tend to sire a greater proportion of offspring in competitive mating trials with low-condition males. These data suggest that condition-dependent reproduction may be mediated in part by the condition dependence of sperm quantity and quantity, and our results also provide limited correlative evidence that variation in sperm phenotypes may be associated with variation in fertilization success. The nature of this association, and its relevance for the evolution of sperm and ejaculate traits via sexual selection in *Anolis sagrei* and other organisms, will require a deeper understanding of the factors that influence sperm performance, along with more comprehensive tests for selection on sperm morphology and sperm count.

Acknowledgments

We thank Laura Zemanian, Jennifer Price, and Hannah Donald-Cannon for assistance with animal care and mating trials, and Henry Wilbur for assistance with statistical analyses. We also thank Aaron Reedy, Amanda Hanninen, Christian Cox, Edmund Brodie III, Corlett Wood, Brian Sanderson, Malcolm Augat and Michael Hague for discussion and comments on the manuscript. Finally, we would like to thank Francisco García-González and two anonymous referees for their constructive comments on this manuscript. This study was conducted under protocol from the Animal Care and Use Committee at the University of Virginia (protocol 3896). Animals were acquired under permits from the United States Fish and Wildlife Service, the Bahamas Ministry of

Agriculture, and the Bahamas Environment, Science and Technology (BEST)

Commission. This work was supported by startup funding to RMC from the University of Virginia.

References

- Alavi SMH, Pšenička M, Policar T, Rodina M, Hamáčková J, Kozák P, Linhart O. 2009. Sperm quality in male *Barbus barbus* (L.) fed different diets during the spawning season. *Fish Physiol. Biochem.* 35:683-693.
- Amitin, EG, Pitnick S. 2007. Influence of developmental environment on male- and female-mediated sperm precedence in *Drosophila melanogaster*. *J. Evol. Biol.* 20:381–391.
- Anderson MJ, Dixson AF. 2002. Sperm competition: motility and the midpiece in primates. *Nature.* 416:496
- Anderson MJ, Nyholt J, Dixson AF. 2005. Sperm competition and the evolution of sperm midpiece volume in mammals. *J. Zool. Lond.* 267:135-142.
- Andersson M. 1986. Evolution of condition-dependent sex ornaments and mating preferences: sexual selection based on viability differences. *Evolution.* 40:804–816.
- Bakker T C, Hollmann M, Mehlis M, Zbinden M. 2014. Functional variation of sperm morphology in sticklebacks. *Behav. Ecol. Sociobiol.* 68:617-627.
- Bardeleben C, Palchevskiy V, Calsbeek R, Wayne RK. 2004. Isolation of polymorphic tetranucleotide microsatellite markers for the brown anole (*Anolis sagrei*). *Mol. Ecol. Notes.* 4:176–178.
- Bonduriansky R, Rowe L. 2005. Sexual selection, genetic architecture, and the condition dependence of body shape in the sexually dimorphic fly *Prochyliza xanthostoma* (Piophilidae). *Evolution.* 59:138–151.

- Bonanno VL, Schulte-Hostedde AI. 2009. Sperm competition and ejaculate investment in red squirrels (*Tamiasciurus hudsonicus*). *Behav. Ecol. Sociobiol.* 63:835–846.
- Boschetto C, Gasparini C, Pilastro A. 2011. Sperm number and velocity affect sperm competition success in the guppy (*Poecilia reticulata*). *Behav. Ecol. Sociobiol.* 65:813–821.
- Breed WG, Taylor J. 2000. Body mass, testes mass, and sperm size in murine rodents. *J. Mammal.* 81:758–768.
- Burness G, Schulte-Hostedde AI, Montgomerie R. 2008. Body condition influences sperm energetics in lake whitefish (*Coregonus clupeaformis*). *Can. J. Fish. Aquat. Sci.* 65:615–620.
- Calhim S, Immler S, Birkhead TR. 2007. Postcopulatory sexual selection is associated with reduced variation in sperm morphology. *PLoS ONE* 2:e413.
- Calsbeek R, Bonneaud C. 2008. Postcopulatory fertilization bias as a form of cryptic sexual selection. *Evolution.* 62:1137–1148.
- Cotton S, Fowler K, Pomiankowski A. 2004. Do sexual ornaments demonstrate heightened condition-dependent expression as predicted by the handicap hypothesis? *Proc. R. Soc. Lond. B.* 271:771–783.
- Cox, RM, Calsbeek, R. 2014. Survival of the fattest? Indices of body condition do not predict viability in the brown anole (*Anolis sagrei*). *Funct. Ecol.* 29: 404–413.
- Cox RM, Duryea MC, Najarro M, Calsbeek R. 2011. Paternal condition drives progeny sex-ratio bias in a lizard that lacks parental care. *Evolution.* 65:220–30.

- Devigili A, Kelley JL, Pilastro A, Evans JP. 2012. Expression of pre- and postcopulatory traits under different dietary conditions in guppies. *Behav. Ecol.* 24:740-749.
- Dewsbury D. 1982. Ejaculate cost and male choice. *Am. Nat.* 119:601–610.
- Duryea MC, Kern AD, Cox RM, Calsbeek R. 2013. A novel application of Approximate Bayesian Computation for detecting male reproductive advantages due to mating order. *Behav. Ecol. Sociobiol.* 67:1867–1875.
- Falconer DS, Mackay TFC. 1981. Introduction to quantitative genetics. 4th edition. Essex, England: Pearson.
- Firman RC, Simmons LW. 2010. Sperm midpiece length predicts sperm swimming velocity in house mice. *Biol. Letters.* 6:513-516.
- Fitzpatrick JL, and Baer B. 2011. Polyandry reduces sperm length variation in social insects. *Evolution.* 65:3006–3012.
- Fitzpatrick JL, Simmons LW, Evans JP 2012. Complex patterns of multivariate selection on the ejaculate of a broadcast spawning marine invertebrate. *Evolution.* 66:2451–2460.
- Freckleton R. 2002. On the misuse of residuals in ecology: regression of residuals vs. multiple regression. *J. Anim. Ecol.* 71:542-545.
- Gage M, Cook PA. 1994. Sperm Size or Numbers? Effects of nutritional stress upon eupyrene and apyrene sperm production strategies in the moth *Plodia interpunctella* (Lepidoptera: Pyralidea). *Funct. Ecol.* 8:594–599.
- Gage M, Macfarlane CP, Yeates S, Ward RG, Searle JB, Parker GA. 2004. Spermatozoal traits and sperm competition in Atlantic Salmon. *Curr. Biol.* 14:44–47.

- García-Berthou E. 2001. On the misuse of residuals in ecology: testing regression residuals vs. the analysis of covariance. *J. Anim. Ecol.* 70:708–711.
- García-González F, Simmons LW. 2005. Sperm viability matters in insect sperm competition. *Curr. Biol.* 15:271-275.
- García-González F, Simmons LW. 2007. Shorter sperm confer higher competitive fertilization success. *Evolution.* 61:816–824.
- Gasparini C, Devigili A, Dosselli R, Pilastro A. 2013. Pattern of inbreeding depression, condition dependence, and additive genetic variance in Trinidadian guppy ejaculate traits. *Ecol. Evol.* 3:4940-4953.
- Gribbins KM. 2011. Reptilian spermatogenesis: A histological and ultrastructural perspective. *Spermatogenesis* 1:250–269.
- Helfenstein F, Podelvin M, Richner H. 2010. Sperm morphology, swimming velocity, and longevity in the house sparrow *Passer domesticus*. *Behav. Ecol. Sociobiol.* 64:557-565.
- Hellriegel B, Blanckenhorn WU. 2002. Environmental influences on the gametic investment of yellow dung fly males. *Afr. Zool.* 16:505–522.
- Hill GE. 2011. Condition-dependent traits as signals of the functionality of vital cellular processes. *Ecol. Lett.* 14:625–634.
- Humphries S, Evans JP, Simmons LW. 2008. Sperm competition: linking form to function. *BMC Evol. Biol.* 8:319.
- Immler S, Calhim S, Birkhead TR. 2008. Increased postcopulatory sexual selection reduces the intramale variation in sperm design. *Evolution.* 62:1538–1543.

- Immler S, Pryke SR, Birkhead TR, Griffith SC. 2010. Pronounced within-individual plasticity in sperm morphometry across social environments. *Evolution*. 64:1634–1643.
- Johnson DW, Monro K, Marshall DJ. 2013. The maintenance of sperm variability: context-dependent selection on sperm morphology in a broadcast spawning invertebrate. *Evolution*. 67:1383-1395.
- Kalinowski ST, Taper ML, Marshall TC. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol. Ecol.* 16:1099-1106.
- Kelly CD, Jennions MD. 2011. Sexual selection and sperm quantity: meta-analyses of strategic ejaculation. *Biol. Rev.* 86:863–884.
- Kleven O, Laskemoen T, Fossøy F, Robertson RJ, Lifjeld JT. 2008. Intraspecific variation in sperm length is negatively related to sperm competition in passerine birds. *Evolution*. 62:494–499.
- LaMunyon CW, Ward S. 1998. Larger sperm outcompete smaller sperm in the nematode *Caenorhabditis elegans*. *Proc. R. Soc. Lond. B.* 265:1997-2002.
- Laskemoen T, Kleven O, Fossøy F, Robertson RJ, Rudolfson G, Lifjeld JT. 2010. Sperm quantity and quality effects on fertilization success in a highly promiscuous passerine, the tree swallow *Tachycineta bicolor*. *Behav. Ecol. Sociobiol.* 64:1473–1483
- Lüpold S, Calhim S, Immler S, Birkhead TR. 2009a. Sperm morphology and sperm velocity in passerine birds. *Proc. R. Soc. B.* 276:1175-1181.

- Lüpold S, Linz GM, Birkhead TR. 2009b. Sperm design and variation in the New World blackbirds (Icteridae). *Behav. Ecol. Sociobiol.* 63:899–909.
- Malo AF, Garde JJ, Soler AJ, García AJ, Gomendio M, Roldan ER. 2005a. Male fertility in natural populations of red deer is determined by sperm velocity and the proportion of normal spermatozoa. *Biol. Reprod.* 72:822–829.
- Malo AF, Roldan ERS, Garde J, Soler AJ, Gomendio M. 2005b. Antlers honestly advertise sperm production and quality. *Proc. R. Soc. B.* 272:149–157.
- Malo AF, Gomendio M, Garde J, Lang-Lenton B, Soler AJ, Roldan ERS. 2006. Sperm design and sperm function. *Biol. Letters.* 2:246–249.
- Marquardt D. 1970. Generalized inverses, ridge regression, biased linear estimation, and nonlinear estimation. *Technometrics.* 12:591–612.
- Merrells KJ, Blewett H, Jamieson JA, Taylor CG, Suh M. 2009. Relationship between abnormal sperm morphology induced by dietary zinc deficiency and lipid composition in testes of growing rats. *Brit. J. Nut.* 102:226–232.
- Møller AP. 1989. Ejaculate quality, testes size and sperm production in mammals. *Funct. Ecol.* 3:91–96.
- Møller AP, Briskie JV. 1995. Extra-pair paternity, sperm competition and the evolution of testis size in birds. *Behav. Ecol. Sociobiol.* 36:357–365.
- Mossman J, Slate J, Humphries S, Birkhead T. 2009. Sperm morphology and velocity are genetically codetermined in the zebra finch. *Evolution.* 63:2730–2737.
- Olsson M, Madsen T, Shine R. 1997. Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. *Proc. R. Soc. B.* 264:455–459.

- Parker GA, Begon ME. 1993. Sperm competition games: sperm size and sperm number under adult control. *Proc. Biol. Sci.* 253:245–254.
- Peig J, Green AJ. 2009. New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. *Oikos*. 118:1883–1891.
- Peig J, Green AJ. 2010. The paradigm of body condition: a critical reappraisal of current methods based on mass and length. *Funct. Ecol.* 24:1323–1332.
- Perry JC, and Rowe L. 2010. Condition-dependent ejaculate size and composition in a ladybird beetle. *Proc. R. Soc. B.* 277:3639–3647.
- Pitcher TE, Beausoleil JJ, Abbott JA, Vandereerden JL. 2009. Sperm design and function in the redbreasted dace *Clinostomus elongatus*. *J. Fish Biol.* 75:924–31.
- Rahman MM, Kelley JL, Evans JP. 2013. Condition-dependent expression of pre- and postcopulatory sexual traits in guppies. *Ecol. Evol.* 3:2197–2213.
- Rowe L, Houle D. 1996. The lek paradox and the capture of genetic variance by condition dependent traits. *Proc. R. Soc. Lond. B.* 263:1415–1421.
- Schulte-Hostedde AI, Millar JS. 2004. Intraspecific variation of testis size and sperm length in the yellow-pine chipmunk (*Tamias amoenus*): implications for sperm competition and reproductive success. *Behav. Ecol. Sociobiol.* 55:272–277.
- Simmons LW, Kotiaho J. 2002. Evolution of ejaculates: patterns of phenotypic and genotypic variation and condition dependence in sperm competition traits. *Evolution*. 56:1622–1631.
- Tigeros N. 2013. Linking nutrition and sexual selection across life stages in a model

- butterfly system. *Funct. Ecol.* 27:145–154.
- Tokarz R. 1998. Mating pattern in the lizard *Anolis sagrei*: Implications for mate choice and sperm competition. *Herpetologica*. 54:388–394.
- Tokarz, RR, McMann S, Seitz L, John-Alder HB. 1998. Plasma corticosterone and testosterone levels during the annual reproductive cycle of male brown anoles (*Anolis sagrei*). *Physiol. Zool.* 71:139-146.
- Tourmente M, Gomendio M, Roldan ER, Giojalas LC, Chiaraviglio M. 2009. Sperm competition and reproductive mode influence sperm dimensions and structure among snakes. *Evolution*. 63:2513–24.
- Van Voorhies WA. 1992. Production of sperm reduces nematode lifespan. *Nature*. 360:456-458.
- Wordley C, Slate J, Stapley J. 2010. Mining online genomic resources in *Anolis carolinensis* facilitates rapid and inexpensive development of cross-species microsatellite markers for the *Anolis* lizard genus. *Mol. Ecol. Resour.* 11:126–133.
- Zikovitz AE, Agrawal AF. 2013. The condition dependency of fitness in males and females: the fitness consequences of juvenile diet assessed in environments differing in key adult resources. *Evolution*. 67:2849–2860.

Tables and Figures

Table 1. (A) Results of three nested ANOVAs testing for difference in mean head length, midpiece length, and tail length of sperm between diet treatments, with male identity nested within treatment as a random effect in each model. (B) Results of paired *t*-tests for differences in individual coefficients of variation for sperm morphology between diet treatment groups. Asterisks indicate significant ($P < 0.05$) effects.

A. Nested ANOVAs (means)	df	<i>F</i>	<i>P</i>
Head Length	1, 32.87	3.13	0.086
Midpiece Length	1, 32.87	8.33	0.007*
Tail Length	1, 32.87	0.44	0.513
B. <i>t</i>-tests (coefficients of variation)	df	<i>t</i> ratio	<i>P</i>
Head Length	16	1.91	0.065
Midpiece Length	16	2.67	0.011*
Tail Length	16	1.92	0.068

Table 2. Regression analyses testing for fitness consequences of variation in sperm head length, midpiece length, tail length, and sperm count (square-root transformed) using three measures of fertilization success as dependent variables in separate analyses: P_1 is the proportion of paternity when males were the first to mate with a female, P_2 is the proportion of paternity when males were the second to mate with a female, and P_{avg} is the average proportion of paternity across P_1 and P_2 . Males are only included in the analysis of P_{avg} if both of their mates produced offspring. All dependent and independent variables were first standardized to a mean of zero and units of standard deviation within each diet treatment to eliminate any collinearity due to overall treatment effects. Results are shown separately for a single multiple regression with all four ejaculate phenotypes as separate independent variables and as separate univariate regressions for each ejaculate phenotype.

Model	P_1 (df = 21)			P_2 (df = 20)			P_{avg} (df = 16)		
Effect	<i>r</i>	<i>F</i>	<i>P</i>	<i>r</i>	<i>F</i>	<i>P</i>	<i>r</i>	<i>F</i>	<i>P</i>
Multiple regression									
Head length	-0.064	0.106	0.748	-0.402	3.030	0.099	-0.513	6.958	0.021*
Midpiece length	-0.080	0.469	0.502	-0.197	0.594	0.449	0.134	8.267	0.014*
Tail length	0.248	1.158	0.296	-0.090	0.081	0.777	-0.721	0.892	0.363
Sperm count	0.174	0.190	0.667	-0.358	2.424	0.136	-0.766	5.437	0.026*
Univariate regressions									
Head length	-0.168	0.291	0.594	-0.277	1.115	0.302	0.111	4.580	0.048*
Midpiece length	-0.124	0.293	0.593	-0.147	0.083	0.774	-0.014	1.521	0.235
Tail length	0.218	0.880	0.358	-0.027	0.121	0.730	-0.446	0.414	0.529
Sperm count	0.208	0.102	0.752	-0.141	0.289	0.597	-0.529	0.538	0.474

Figure 1. Experimental timeline plotting mean ($\pm$ SE) body condition for high-intake and low-intake groups from the start of the diet treatment (week 0) to the onset of competitive matings (week 16). Condition is expressed as residuals from separate regressions of $\log_{10}$ body mass on $\log_{10}$ SVL at each time point.

Figure 2. (A) *Anolis sagrei* sperm cell. (B) Individual means ($\pm$ SD) for head length, midpiece length, and tail length of 25 sperm cells per individual for each of 17 males from the low-intake diet treatment and 17 males from the high-intake diet treatment, grouped by treatment and ranked by mean within each group. (C). Treatment means ($\pm$ SE) of individual means in head length, midpiece length, and tail length. (D) Treatment means ($\pm$ SE) of individual coefficients of variation (CV) in head length, midpiece length, and tail length.

Figure 3. (A) Correlations between natural variation in body condition and individual means for head length, midpiece length, and tail length of sperm across 21 wild males from Great Exuma, The Bahamas. (B) Correlations between experimental variation in body condition and individual means for head length, midpiece length, and tail length of sperm across 17 high-intake and 17 low-intake males in captivity. Individual means are derived from 25 cells per male. Body condition is derived from residuals of regressions of $\log_{10}$ body mass on $\log_{10}$ SVL.

Figure 4. Mean ($\pm$ SE) proportion of progeny sired by males that were (A) categorized into high and low condition pairs on the basis of natural variation in body condition (data reanalyzed from Cox et al. 2011), and (B) assigned to high-intake and low-intake diet treatments (this study). For each experiment, condition-dependence was assessed in three ways: (1) using each dam as a unit of observation and estimating the proportion of paternity for each of her two mates ($n = 30$ and 23 dams), (2) using each pair of potential sires as a unit of observation and estimating the proportion of paternity for each male across one dam (if the other dam did not produce offspring) or across both dams ($n = 20$ and 14 pairs of sires), and (3) using each pair of potential sires as a unit of observation but restricting the comparison to the subset of pairs for which both dams produced offspring ($n = 9$ and 9 pairs or sires). See text for details.

Supplementary Table S1. Data from seven microsatellite loci used in paternity assignment. PIC = polymorphism information content, Non-exclusion = non-exclusion probability, H_o = observed heterozygosity, H_e = expected heterozygosity, Null Freq = frequency of null alleles.

Locus	No. of Alleles	PIC	Non-exclusion	H_o	H_e	Null Freq
AAAG-61	10	0.756	0.410	0.783	0.787	-0.0024
AAAG-70	9	0.836	0.295	0.820	0.855	0.0206
AAAG-76	6	0.709	0.482	0.675	0.754	0.0557
AAAG-77	15	0.909	0.172	0.853	0.917	0.0338
AAAG-94	11	0.601	0.569	0.627	0.623	-0.0086
ACAR-11	19	0.888	0.206	0.888	0.899	0.0047
ACAR-23	7	0.380	0.776	0.409	0.413	0.0078
Mean	11	0.726		0.722	0.749	
Total			0.0009			

Supplementary Table S2. Comparison of three methods used to assess relationships between body condition and sperm morphology. Values of r are correlation coefficients from univariate regressions of sperm morphology on the residual index (R_i) or the Scaled Mass Index (M_i) of body condition, or partial correlation coefficients for the relationships between sperm morphology and $\log_{10}$ body mass in multiple regressions including $\log_{10}$ snout-vent length (SVL). Data are shown separately for natural variation in a wild population and diet-induced variation in a lab experiment.

Study	Head length		Midpiece length		Tail length	
	r	P	r	P	r	P
Wild (natural variation)						
Residual Index (R_i)	0.135	0.954	-0.406	0.075	-0.055	0.818
Scaled Mass Index (M_i)	0.023	0.920	-0.401	0.079	-0.057	0.810
Mass (multiple regression)	0.014	0.954	-0.418	0.075	-0.055	0.823
Lab (diet manipulation)						
Residual Index (R_i)	0.183	0.301	-0.352	0.040*	0.259	0.138
Scaled Mass Index (M_i)	0.199	0.258	-0.338	0.050*	0.271	0.121
Mass (multiple regression)	0.192	0.283	-0.353	0.043*	0.264	0.138

Supplemental Figure 1. We used repeated resampling to validate the use of 25 cells to estimate an individual's mean and coefficient of variation (CV) for each sperm morphological component. We conducted 1000 iterations of resampling per each sample size of 2 to 25 cells sampled for each of 34 individual males, generating a measure of variance in our estimate of the mean and CV for each sperm component in each individual male. Graphs illustrate the mean ($\pm$ SD) across 34 males for individual estimates of variance in the mean and CV for each sperm component at each sample size. At 25 cells, the variance in each summary statistic has reached an asymptotically low level.

CHAPTER 5:

Sperm midpiece length and sperm velocity are positively correlated in the brown anole lizard⁵

⁵ Formatted as a coauthored manuscript: Kahrl, A. F., Kustra, M. C., Reedy, A. M., and R. M. Cox

Abstract

Sperm competition results in selection on a variety of ejaculate traits, including traits associated with sperm performance or sperm velocity. Selection on sperm morphology is thought to be mediated through selection for sperm function. Several interspecific analyses have demonstrated associations between sperm morphology and velocity, but within species, the strength and direction of patterns are mixed. We tested for an association between sperm velocity and morphology by measuring the mean sperm velocity and mean sperm head, midpiece, and tail lengths of 15 cells per male in a wild population of *Anolis sagrei*. We found that across individuals, sperm velocity has a weak positive association with mean sperm midpiece length. Surprisingly, the sperm head and tail length were not correlated with sperm velocity, potentially due to high intra-male variation in both velocity and morphology. This relationship provides a potential functional explanation for the rapid evolution of sperm midpiece length relative to other sperm traits in anoles.

Introduction

Describing the relationship between sperm form and function is critical to interpreting patterns of evolution among species and to understand processes that generate variation in sperm morphology within species. In general, faster sperm improve male fertilization success by enhancing their competitive ability for both storage in the female reproductive tract and arrival time at the site of fertilization (Gage et al. 2004; Malo 2005; Casselman et al. 2006; Boschetto et al. 2011; Bennison et al. 2014). In many species sperm length is also positively correlated with fertilization success (LaMunyon and Samuel 1999; Malo 2005; Bennison et al. 2014), which is not surprising as sperm morphology is associated with sperm performance. Specifically, in comparative analyses among species, sperm midpiece and tail length are positively correlated with sperm velocity (Gomendio and Roldan 1991; Lüpold et al. 2009; Fitzpatrick et al. 2009; Gasparini et al. 2010). These studies provide a link between morphology, performance, and fitness, which helps explain the evolution of sperm morphology among species.

Within species, the strength of the correlation between sperm velocity and morphology varies across species, likely due to high intra-male variation in both sperm morphology and velocity (Humphries et al. 2008; Fitzpatrick et al. 2010). Many intraspecific studies are conducted in external fertilizing species, which makes it difficult to draw general conclusions about sperm functional morphology for internally fertilizing species from these analyses (summarized in Humphries et al. 2008). Because the magnitude of the relationship between sperm velocity and morphology is variable among species, and very few species have been described on an intraspecific level, it is

necessary to characterize this functional relationship within a group to interpret patterns of evolution and selection on sperm morphology.

Anolis lizards have high rates of multiple mating and multiple paternity, with females storing sperm for months at a time, thereby creating the opportunity for postcopulatory selection. In *Anolis* lizards, the sperm midpiece is condition-dependent and evolves more quickly than the rest of the cell (Chapters 2, 4), which may be due to selection on sperm function. Other parts of the cell may also influence sperm velocity, as the sperm head is predicted to reduce velocity by increasing drag, while the sperm tail may increase velocity as it physically propels the cell (Humphries et al. 2008). Our goal was to test for associations between sperm velocity and sperm morphology in a wild population of brown anoles. We measured sperm morphology and sperm velocity from 15 cells per male for 107 males in a wild population of *A. sagrei* in Florida, and tested for a correlation between curvilinear velocity and sperm morphology. We also used variance partitioning to determine how much of the total variation in sperm morphology and sperm velocity was due to within- and among-male variation. Given that sperm morphology and sperm count are condition-dependent in this species, we also tested for associations between body condition and sperm velocity across males.

Methods

We collected 107 adult male lizards in May 2015 from an island population of *Anolis sagrei* in the Guana Tolomato Matanzas National Estuarine Research Reserve in Palm Coast Florida (29°37'59.15" N, 81°12'45.64" W). We measured the mass (nearest

0.01g) and snout-vent length (SVL, nearest 1mm) of every male and then held the males overnight to allow them to replenish their sperm stores. We then collected a sperm sample from each male by manually depressing their lower abdomens and collecting the sperm from their cloacae using glass capillary tubes. We quickly added these samples into room temperature (27°C) Dulbecco's Modified Eagle Medium (Gibco, Thermo Fisher Scientific, Waltham, MA) and immediately added 100 µl of this suspension to a covered well slide. For each male, we took a one-minute video of their sperm at 25 frames per second and 40x magnification using an AmScope digital camera (AmScope, Irvine, CA) using the software ToupView (ToupTek Photonics, Zhejiang, P.R. China). We then tracked 15 cells from each video for at least 1.6 seconds (minimum = 40 frames, mean = 54.8 frames) to measure their velocity using the Manual Tracking plugin in Image J (NIH, Bethesda, MD). We selected these cells by starting in the upper left quadrant of the first frame of the video and tracking every motile cell in that area. We then moved to the upper right quadrant, then the lower right quadrant and finally the lower left quadrant of the video frame until we had measured the tracks of 15 cells. We did not track any cell that was not moving, or was stuck to the slide or to another cell. This tracking method calculates the distance and velocity for each frame of a single sperm path. The distribution of sperm velocity for each sperm track was right-skewed, therefore, we calculated the median curvilinear velocity of each sperm cell. From these measurements, we then calculated the mean velocity for each male.

From the same sperm suspension, we fixed the remaining cells using 4% paraformaldehyde to create slides for measuring sperm morphology. These cells were

then stained with Sperm Blue™ (Microptic SL, Barcelona, Spain) and imaged using 100x DIC microscopy and the software Magnafire camera (Olympus America, Melville, NY). We measured the sperm head, midpiece, and tail length for 15 cells per male in ImageJ (NIH, Bethesda, MD), and calculated the mean length of each part of the sperm cell for each male.

To test for a relationship between sperm morphology and sperm velocity, we used multiple regressions with individual sperm head, midpiece and tail length as predictors. We then pooled all sperm morphology and velocity measurements for all individuals and calculated the restricted maximum likelihood (REML) variance component estimates to partition the total variance in sperm morphology and velocity into within- and among-male variation. Finally, to test for condition-dependence of sperm velocity, we first calculated body condition using the residuals from a regression of $\log_{10}$ mass on $\log_{10}$ SVL. We then used an ordinary least squares regression to test for an association between body condition and sperm velocity.

Results

We found that males with longer midpieces produced sperm with higher sperm velocity ($r = 0.203$, $t = 2.20$, $P = 0.030$), but we found no relationship between sperm head ($r = 0.046$, $t = 0.51$, $P = 0.611$) or tail length ($r = 0.028$, $t = 0.28$, $P = 0.778$) and sperm velocity (Figure 1). When we partitioned the total variance in sperm morphology, we found that the total phenotypic variance in head size was distributed almost equally within and among males, however, within-male variation was much higher than among

male variation for the sperm midpiece, tail, and for sperm velocity (Table 1). Finally, we found no evidence for condition dependence of sperm velocity ($r = 0.102$, $t = 0.98$, $P = 0.327$).

Discussion

We found weak, but significant, positive correlations between mean sperm midpiece length and sperm velocity in a wild population of brown anoles. We found no relationship between sperm head or tail length and sperm velocity. This is surprising considering that the sperm tail provides the propulsion for the cell, and sperm head size can increase the amount of drag the cell experiences (Humphries et al. 2008). We also found high within-male variation in both sperm morphology and velocity, which may explain why the relationship between sperm midpiece and velocity is weak (or undetectable for the sperm head and tail). Finally, we found no correlation between condition and sperm velocity in this population.

In comparative surveys across other species, there are frequently positive associations between sperm morphology and sperm velocity (Gomendio and Roldan 1991; Lüpold et al. 2009; Fitzpatrick et al. 2009, Firman and Simmons 2010). However, intraspecific studies frequently fail to find correlations between sperm velocity and morphology (Humphries et al. 2008), likely because of high intra-male variation in both sperm morphology and velocity (Fitzpatrick et al. 2010). Those studies that have found a significant correlation between morphology and velocity often report that sperm velocity is positive correlated with both tail and midpiece length (Firman and Simmons 2010), and

negatively correlated with head length (Humphries et al. 2008, Ramon et al. 2013). The sperm midpiece contains mitochondria and the tail both propels the cell and has glycolytic activity in its fibrous sheath (Eddy et al. 2003; Miki et al. 2004), so their link with sperm velocity is understandable. Our data corroborate these previous studies, as intra-male variation in both sperm morphology and sperm velocity was high.

Very little is known about the functional morphology of sperm in squamates. In fact, there are only two studies that have tested for an association between sperm morphology and velocity in lizards (Blengini et al. 2014). In contrast to our findings, sperm with a longer tails and shorter midpieces have a higher sperm velocity in *Tupinambis* lizards (Blengini et al. 2014). The difference in the relationship between morphology and velocity in these two species of lizards highlights the need to conduct intraspecific tests for the functional morphology of sperm, as the patterns may be species-specific. Though it is unclear why these differences occur, it may be that the relative lengths of each part of the sperm cause the differences in the functional morphology in these species. The sperm midpiece of *Tupinambis* lizards is approximately two times longer than the *Anolis sagrei* midpiece, and their tail length is about 3/4 the length of the *A. sagrei* tail.

The link between sperm morphology and sperm velocity in *A. sagrei* can provide context for the variation in sperm morphology within and among species. Previous studies demonstrated that the sperm midpiece is condition-dependent (Chapter 4), and evolves faster than other parts of the cell among species of anoles (Chapter 2). The variation in sperm morphology across species of anoles may be the result of selection on

sperm velocity within species. We found that the midpiece is correlated with velocity, which may explain why the rate of evolution of the sperm midpiece is faster than the rest of the cell. However, the fitness consequences of sperm velocity remain untested in lizards. In the brown anole sperm midpiece length and sperm count are both condition-dependent (Chapter 4), however, we found no relationship between condition and sperm velocity in this study. Sperm movement, sperm production, and cellular mitochondria all require energy, but it is unclear how energy is allocated to each trait and how those traits relate to body condition and sperm performance. The energetic cost of sperm velocity is unclear in other species as well, where many species exhibit condition dependence of some sperm traits, such as morphology or count, but not sperm velocity (Urbach et al. 2007; Burness et al. 2008; Devigili et al. 2013; Rahman et al. 2013). The interactions or tradeoffs between these traits may be complex and require further experiments to disentangle. In this species males in poor condition do not have reduced sperm velocity suggesting that maintaining energy available for sperm velocity may be under strong selection.

Acknowledgments

Thanks to Dan Warner, Lauren Finks, Cara Giordano, Cheyanne Sams, and Alana Castro-Gilliard who helped collect the lizards. We thank Guana Tolomato Matanzas National Estuarine Research Reserve for permission to conduct research in their reserve. This work was supported by a National Science Foundation Career award to R.M.C. (DEB-1453089), and a Doctoral Dissertation Improvement Grant from the National Science

Foundation (DEB-1501680 to AFK and RMC). University of Virginia's Animal Care and Use Committee approved all procedures (protocol 3896).

References

- Bennison, C., N. Hemmings, J. Slate, and T. Birkhead. 2014. Long sperm fertilize more eggs in a bird. *Proc. Biol. Sci. Lond. B.* 282:20141897.
- Boschetto, C., C. Gasparini, and A. Pilastro. 2011. Sperm number and velocity affect sperm competition success in the guppy (*Poecilia reticulata*). *Behav. Ecol. Sociobiol.* 65:813–821.
- Burness, G., A. I. Schulte-Hostedde, and R. Montgomerie. 2008. Body condition influences sperm energetics in lake whitefish (*Coregonus clupeaformis*). *Can. J. Fish. Aquat. Sci.* 65:615–620.
- Casselman, S. J., A. I. Schulte-Hostedde, and R. Montgomerie. 2006. Sperm quality influences male fertilization success in walleye (*Sander vitreus*). *Can. J. Fish. Aquat. Sci.* 63:2119–2125.
- Devigili, A., J. L. Kelley, A. Pilastro, and J. P. Evans. 2013. Expression of pre- and postcopulatory traits under different dietary conditions in guppies. *Behav. Ecol.* 24:740–749.
- Eddy, E. M., K. Toshimori, and D. A. O'Brien. 2003. Fibrous sheath of mammalian spermatozoa. *Microsc. Res. Tech.* 61:103–115.
- Firman, R. C., and L. W. Simmons. 2010. Experimental evolution of sperm quality via postcopulatory sexual selection in house mice. *Evolution* 64:1245–1256.
- Fitzpatrick, J. L., F. Garcia-Gonzalez, and J. P. Evans. 2010. Linking sperm length and velocity: the importance of intramale variation. *Biol. Lett.* 6:797–799.
- Fitzpatrick, J. L., R. Montgomerie, J. K. Desjardins, K. A. Stiver, N. Kolm, and S.

- Balshine. 2009. Female promiscuity promotes the evolution of faster sperm in cichlid fishes. *Proc. Natl. Acad. Sci. U.S.A.* 106:1128–1132.
- 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. *Curr. Biol.* 14:44–47.
- Gasparini, C., L. W. Simmons, M. Beveridge, and J. P. Evans. 2010. Sperm swimming velocity predicts competitive fertilization success in the Green Swordtail *Xiphophorus helleri*. *PLoS ONE* 5:e12146.
- Gomendio, M., and E. R. S. Roldan. 1991. Sperm competition influences sperm size in mammals. *Proc. Biol. Sci. Lond. B.* 243:181–185. The Royal Society.
- Humphries, S., J. P. Evans, and L. W. Simmons. 2008. Sperm competition: linking form to function. *BMC Evol. Biol.* 8:319.
- Kahrl, A. F., and R. M. Cox. 2015. Diet affects ejaculate traits in a lizard with condition-dependent fertilization success. *Behav. Ecol.* 26:1502–1511.
- LaMunyon, C. W., and W. Samuel. 1999. Evolution of sperm size in nematodes: sperm competition favours larger sperm. *Proc. R. Soc. Lond. B.* 266:263–267.
- Lüpold, S., S. Calhim, S. Immler, and T. R. Birkhead. 2009. Sperm morphology and sperm velocity in passerine birds. *Proc. R. Soc. Lond. B.* 276:1175–1181.
- Malo, A. F. 2005. Male fertility in natural populations of red deer is determined by sperm velocity and the proportion of normal spermatozoa. *Biol. Reprod.* 72:822–829.
- Miki, K., W. Qu, E. H. Goulding, W. D. Willis, D. O. Bunch, L. F. Strader, S. D. Perreault, E. M. Eddy, and D. A. O'Brien. 2004. Glyceraldehyde 3-phosphate

- dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proc. Natl. Acad. Sci. U.S.A.* 101:16501–16506.
- Rahman, M. M., J. L. Kelley, and J. P. Evans. 2013. Condition-dependent expression of pre- and postcopulatory sexual traits in guppies. *Ecol. Evol.* 3:2197–2213.
- Ramon, M., A. J. Soler, J. A. Ortiz, O. Garcia-Alvarez, A. Maroto-Morales, E. R. S. Roldan, and J. J. Garde. 2013. Sperm population structure and male fertility: an intraspecific study of sperm design and velocity in red deer. *Biol. Reprod.* 89:110–110.
- Simmons, L. W., and J. L. Fitzpatrick. 2012. Sperm wars and the evolution of male fertility. *Reproduction* 144:519–534.
- Urbach, D., D. Bittner, T. L. Lenz, D. Bernet, T. Wahli, and C. Wedekind. 2007. Sperm velocity in an Alpine whitefish: effects of age, size, condition, fluctuating asymmetry and gonad abnormalities. *J. Fish Biol.* 71:672–683.

Tables and Figures

Table 1. REML variance component estimates partitioning the variation in sperm morphology and sperm velocity into within and among-male variation. Estimates were calculated with all cells from all males.

Sperm Trait	Within-male	Among-male
Head Length	0.472	0.528
Midpiece Length	0.641	0.359
Tail Length	0.628	0.372
Curvilinear Velocity	0.707	0.293

Figure 1. The relationship between mean sperm head (A), midpiece (B), and tail (C) length and mean sperm velocity for 107 male *Anolis sagrei*.